Testicular structure following interaction of alcohol with antiretroviral therapy: Role of Virgin coconut oil extract in an experimental animal model

By

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Preface

The study described in this thesis was carried out in the Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from March 2015 to November 2017, under the supervision of Prof Azu O.O and Dr Naidu E.C.S for the award of Doctor of Philosophy Degree in Clinical Anatomy.

Declaration

I, Mr. OGEDENGBE, Oluwatosin Olalekan declare as follows:

1. That the work described in this thesis, to my knowledge, has not been submitted to UKZN or any other tertiary institution for the purpose of obtaining an academic qualification, whether by myself or any other party.
2. That my contributions to the project were as follows:
    - I was involved in the the design and submission of the proposal for ethics approval by the University Animal Research Ethics Committee.
    - I was wholly responsible for carrying out the experiments and data and sample collection.
    - I performed all sample analyses, collate and did all the data analyses.
    - I was responsible for the writing of all the manuscripts and the thesis.

3. This thesis does not contain other person's written, data, pictures or other information unless specifically acknowledged as being sourced from other persons or researchers. Where other written sources have been quoted then:
    - Their words have been re-written but the general information attributed to them has been referenced
    - Where their exact word have been used, then it has been properly referenced in the reference section.

10th March, 2018

Signed

Date

Dedication

To my Parents- Mr. Johnson and Mrs. Janet Opeyemi Ogedengbe, I could never have done this without your faith, support and constant encouragement. Thank you for earning an honest living for us and for teaching me to believe in my God, myself and my dreams. To my siblings Temitope, Folasade and Mary - Thank you all for your continuous effort, giving and permanent support to pursue this noble course.

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Table of Contents

List of figures

Page

List of tables

Page

Abbreviations

ABC	ATP-binding cassette
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ARVs	Antiretrovirals
ATP	Adenosine triphosphate
BCRP	Breast cancer resistant protein
BW	Body weight
cART	Combination antiretroviral therapy
CAT	Catalase
CCR5	Chemokine receptor
CD4	Cluster of differentiation 4
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration of United States of America
FIs	Fusion inhibitors
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GSH	Reduced glutathione
GST	Glutathione-S-transferase
H&E	Haematoxylin and Eosin
HAART	Highly active antiretroviral therapy
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HPG	Hypothalamic pituitary gonadal
IB	Inhibin
INSTIs	Integrase strand transfer inhibitors
IVF	In vitro fertilization
LCFA	Long chain fatty acid
LDL	Low density lipoprotein
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
MCFA	Medium chain fatty acid
MDA	Malondialdehyde
MGT	Male genital tract
MRP	Multidrug resistance associated protein

MRP	Multidrug resistance protein
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
OTC	Over the Counter
PAS	Periodic acid Schiff
PBS	Phosphate buffered solution
P-gp	Permeability glycoprotein
PIs	Protease Inhibitors
PLWHA	People living with HIV and AIDS
RCO	Refined coconut oil
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SLC	Solute carrier
SOD	Superoxide dismutase
TAG	Triacylglycerol
TBARS	Thiobarbituric acid
TW	Testicular weight
UNAIDS	Joint United nation program on HIV and AIDS
VCO	Virgin coconut oil
WHO	World Health Organization

Glossary

Heavy episodic drinking: A high prevalence with consumption of at least 60 g or more of alcoholic drinks on at least a single occasion within a 30-day period.

Binge Drinking: A pattern of drinking that brings blood alcohol concentration (BAC) levels to 0.08 g/dL. This typically occurs after 4 drinks for women and 5 drinks for men—in about 2 hours.

Low-risk drinking: for women, it is defined as no more than 3 drinks on any single day and no more than 7 drinks per week. For men, it is defined as no more than 4 drinks on any single day and no more than 14 drinks per week

Heavy Alcohol Use: this refers to binge drinking on 5 or more days in the past month.

Complementary therapy: this refers to any of a range of medical therapies that fall beyond the scope of conventional medicine but may be used alongside it in the treatment of disease and ill health.
Alternative therapy: generally used instead of conventional medical treatment.

Abstract

The consumption of alcohol by people living with HIV/AIDS is associated with a graver prognosis. Long term use of antiretrovirals has known health challenges that may be aggravated by concomitant alcohol use. This study investigated Virgin coconut oil (VCO) as an adjuvant to the deleterious effects of combining highly active antiretroviral therapy (HAART) and alcohol on the cyto-architecture and functions of the testis in an experimental animal model. The study was conducted in two phases – phase one involving HAART and VCO administration and phase two involving ethanol, HAART and VCO co-administration. In phase 1, twenty adult male Sprague Dawley rats weighing between 153g to 169g were divided into four groups with five animals per group and forty adult male Sprague-Dawley rats weighing between 165g to 176g, were used in phase 2 and divided into eight groups with five animals per group. The animals were subjected to various treatments with HAART, Ethanol and VCO according to the protocol. Blood was collected for hormonal (LH, FSH, Testosterone) and antioxidant marker (MDA and GSH) assays. Epididymal seminal fluid was analysed for sperm concentration, motility and morphology. The testes were examined for histopathological and histochemical changes using H&E, PAS and Gordon and Sweet's silver stains. Testicular ultrastructure was examined using transmission electron microscopy. Morphometric measurements were done on the seminiferous tubules examining seminiferous tubular diameter, epithelial and basement membrane thickness, Leydig cell diameter and nuclear volume. Stereological studies were carried out examining volume densities and absolute volumes of the germinal epithelium, lumen and the intersitium.

Results of the semen parameters showed a significant decline in sperm counts ($P<0.01$) and motility ($P<0.05$) in animals treated with HAART alone, ethanol alone or with HAART + Ethanol when compared to the control animals. The groups receiving adjuvant VCO + ethanol also had significantly increased sperm counts ($P<0.05$) and sperm motility ($P<0.01$) than ethanol alone. Likewise the group receiving VCO + ethanol + HAART showed significantly increased sperm counts ($P<0.05$) and sperm motility ($P<0.01$) than ethanol + HAART alone.

Hormonal assay indicated a significant increase in testosterone levels relative to the control animals ($P<0.01$). There was a significant increase in FSH levels of VCO + ethanol + HAART relative to the ethanol + HAART treated animals ($P<0.01$). Testicular GSH level was significantly decreased ($P<0.05$) in the ethanol alone treated group. A significant increase ($P<0.01$) was also observed in the VCO + ethanol and VCO + ethanol + HAART. Changes in the LH and MDA levels were not statistically significant among all treated animals.

Histo-morphological studies showed HAART caused some damage to seminiferous tubular architecture with a significant decline in epithelial height closely mirrored by extensive abnormal reticulin framework and presence of many positive PAS cells. Testis ultrastructure of HAART-treated animals also showed significant increase ($p<0.01$) in basement membrane thickness with decrease in Leydig cell nuclear diameter ($p<0.05$) and volume ($p<0.01$) when compared to controls.

Mitochondrial cristae appeared collapsed and Sertoli cells showed cytoplasmic vacuolations. In addition, the use of ethanol alone and ethanol + HAART showed extensive degeneration in the seminiferous tubular epithelium, disorganized basement membrane and widened, hypocellular interstitium. Adjuvant treatment of VCO with HAART, ethanol, or ethanol + HAART showed improved testicular morphology, reversed HAART and ethanol histopathology as well as improved HAART-induced testicular ultrastructural alterations. In conclusion, the use of Virgin coconut oil was found to mitigate the deleterious effects of ethanol and HAART thereby significantly preserving and promoting testicular function and fertility.
Keywords: Alcohol, Virgin coconut oil, HAART, Ultrastructure, Testis

CHAPTER ONE

INTRODUCTION

1.0 Background

HIV/AIDS epidemic is one of the largest global health challenges of current times. To date HIV has infected over 70 million people and claimed more than 35 million lives since its inception (UNAIDS, 2017). According to UNAIDS (2017) an estimated 36.7 million people are currently living the virus.

About 1.8 million people were newly HIV infected cases with 1.0 million AIDS related deaths reported in 2016. Globally of the PLWHAs, 51% are females. The Eastern and Southern Africa region accounts for only 5% of the world's population, however it remains home to half of the global population living with HIV. The high rate of poverty, lack of assets and skills have forced people in this region to engage in risky behavior such as commercial sex for survival (Mbirimtengerenji, 2007). In addition, the lack of HIV awareness, decreased access to curative health services, bad governance amongst several others, serve as the driving force behind the high HIV infection rates in these regions (Temah, 2009).

Amidst several countries within this region, South Africa is ranked as the country with the biggest HIV epidemic with about 7.1 million people living with the virus as at 2016. Despite great efforts in tackling the spread of HIV/AIDS in South Africa, a total of 270,000 new HIV infections with 110,000 AIDS related death was reported at the end of 2016 (UNAIDS, 2017).

The global expansion of access to highly active antiretroviral therapy (HAART) in the management of HIV/AIDS in Sub-Saharan Africa have significantly improved the quality of lives of people living with HIV/AIDS (PLWHAs) within this region (Bijker et al., 2017, Taieb et al., 2017). The effective and efficient roll-out of HAART in this region where the vast majority of HIV-infected individuals live, have contributed positively to impacting AIDS patient survival, with a sharp decline in morbidity and a reduction in new HIV cases (Bendavid et al., 2012, Madhombiro et al., 2017). However, despite the success of HAART in improving the quality of lives of PLWHAs, its use has been associated with a plethora of adverse events, some of which include lipodystrophy, insulin resistance, lactic acidosis, hepatic, renal and cardiovascular abnormalities (Montessori et al., 2004, Hawkins, 2010, Nagiah et al., 2015a).

Clinical studies and animal experiments have indicated that the long term use of HAART can negatively impact the male reproductive system (Azu et al., 2014, Pathak et al., 2015). These range from several potentially fatal conditions such as mitochondrial dysfunction and oxidative stress damage to several lesser detrimental effects (Pavili et al., 2010b, Ahmad et al., 2011a).

Depression is another major challenge affecting the HIV-infected population with dire consequences on their quality of life (Ngum et al., 2017). Studies have shown a high prevalence of depression amongst PLWHA with rates that are three time higher than that of the general population (Schneider et al., 2014, Kumar et al., 2015). Depression among HIV-patients has been reported to be associated with poor adherence to HAART with acceleration of HIV disease progression (Marinho et al., 2016). The consistent nature of depressive disorders seen in PLWHAs has triggered the high rate of alcohol consumption among these patients (Palfai et al., 2014). According to reports, the rate of alcohol consumption among the HIV-population is approximately twice as high than the general population (Schneider et al., 2014, Kumar et al., 2015). The habitual or chronic use of alcohol is paralleled with several comorbidities which include testicular toxicity (La Vignera et al., 2013a). Adverse effects arising from testicular toxicity involve deterioration of semen quality and sperm functional parameters, sexual hormonal impairment and overall organ damage resulting in decreased male fertility (Ramlau-Hansen et al., 2010b, Rai and Rai, 2016b).

Furthermore, the consumption of alcohol among several HIV-infected patients undergoing ARV treatments have shown significant increase globally (Roux et al., 2008, Soboka et al., 2014a, Kekwaletswe and Morojele, 2014a). The frequency of use amongst these patients is also known to pose serious health risks relating to drug interactions, compliance, efficacy and toxicity with the possibility of accelerated disease progression (Kumar et al., 2012a). The resulting negative outcome arising from the concomitant use of alcohol in antiretroviral therapy justifies the need to seek alternative remedies to palliate these ravages especially amongst HIV patients on antiretroviral therapy who are dependent on alcohol.

However in recent times, the use of herbal or traditional remedies as complementary adjuvant therapy has become increasingly popular all across the globe. They are widely acknowledge to be affordable, easily accessible and less liable to cause toxic effects. In addition, herbal medicine provides a better alternative to synthetic pharmaceuticals (Gilani, 2017). Amongst several herbs of interest, attention was focused on medicinal plants with pharmacological properties. *Cocos nucifera* (Coconut) falls into this category of plants owing to its wide range health benefits, some of which include anti-inflammatory, antioxidant, antitumor, antimicrobial properties amongst many others (Lima et al., 2015).

1.0 Anatomy of the rat testis

The testicular size of rats in relation to their body weight vary between animals. However within mammals, the size increases when the mating habits are more polygamous than monogamous (Kenagy and Trombulak, 1986). The larger testicular size in these cases can be attributed to high copulatory frequency and sperm production and competition among sperm of different males for fertilization of the same female (Kenagy and Trombulak, 1986, Gomendio and Roldan, 1991). With rats the testes are larger

due to their polygamous mating habits. The testes are contained in two separate membranes called scrotal sacs. In a young rat they descend between 4-6 weeks of age. Throughout the rat's life the testes are able to move up into the rat's abdominal cavity as a result of the rat having an open inguinal canal. Spermatogenesis (formation of sperm) occurs in the epithelial lining of the seminiferous tubes found within the testes. Another role of the testes involves the secretion of steroidal hormones such as testosterone, estrone, and progesterone. In rats, the testis are comparable to those in humans, further details are described below.

1.1 Anatomy of the human testes

The testes are the primary reproductive organs or gonads in the male. They are ovoid reproductive and endocrine organs responsible for sperm production and testosterone and are suspended in the scrotum by scrotal tissues including the non-striated dartos muscle and the spermatic cords. The average dimensions of the testis are: 4-5 cm in length, 2.5 cm in breadth and 3 cm in anteroposterior diameter. Their weight varies from 10.5-14g. The left testis usually lies at a lower level than the right testis within the scrotum. Each testis lies obliquely within the scrotum, its upper pole tilted anterolaterally and the lower posteromedially. The anterior aspect is convex, the posterior nearly straight, with the spermatic cord attached to it. Anterior, medial and lateral surfaces and both poles are convex, smooth and covered by the visceral layer of the serosal tunica vaginalis, which separates them from the parietal layer and the scrotal tissues external to this. Between these two layers there is always a very fine film of fluid. This fluid layer can increase on occasions, creating a hydrocele. The posterior aspect is only partly covered by tunica serosa; the epididymis adjoins its lateral part.

The testis is invested by three coats, which are, from outside inwards, the tunica vaginalis, tunica albuginea and tunica vasculosa. Each testis is separated from its fellow by a fibrous median raphe, which is deficient superiorly.

- The *tunica vaginalis* is a serous pouch comprises of two layers of serous membranes that cover the Tunica vaginalis albuginea – a layer of fibrous substance that enwraps the testes. It extends 1 cm into the spermatic cord
- The *tunica albuginea* is a dense, bluish-white covering for the testis. It is composed mainly of interlacing bundles of collagen fibers, and is covered externally by the visceral layer of the tunica vaginalis, except at the epididymal head and tail and the posterior aspect of the testis, where vessels and nerves enter.
- The *tunica vasculosa* contains a plexus of blood vessels and delicate loose connective tissue, and extends over the internal aspect of the tunica albuginea, covering the septa and therefore all the testicular lobules (Gray et al., 2005).

Septa from the mediastinum extend internally to partition the testis into 250 lobules. These differ in size, and are largest and longest in the center. Each lobule contains one to four convoluted seminiferous tubules, which are much-coiled loops whose free ends both open into channels (the rete testis) within the mediastinum. The loose connective tissue between seminiferous tubules contains several layers of contractile peritubular myoid cells, and clusters of steroid-producing interstitial (Leydig) cells (Gray et al., 2005). There are about 400-600 seminiferous tubules in each testis and the length of each is 70-80 cm. Their diameter varies from 0.12-0.3 mm. They are pale in early life, but in old age they contain much fat and are deep yellow. Each tubule is surrounded by a basal lamina, on which rests a complex, stratified seminiferous epithelium containing spermatogenic cells and supportive Sertoli cells (Junqueira and Carneiro, 2005). When active, the spermatogenic cells include basally situated spermatogonia and their progeny in the adluminal compartment, spermatocytes, spermatids and mature spermatozoa. Among the spermatids may be residual bodies, which are spherical structures derived from surplus spermatid cytoplasm shed during maturation and phagocytosed by Sertoli cells (Gray et al., 2005).

1.1.1 Overview of spermatogenesis

Spermatogenesis is the process involving the gradual transformation of germ cells into spermatozoa. The site of spermatogenesis are the seminiferous tubules. The two types of cells involved in spermatogenesis are the germ cells (which develop to form sperms) and the Sertoli cells (which nourish and support the germ cells throughout the developmental process) (Clermont, 1963). In humans, the production of sperms originate from the primordial germ cells which differentiate to form Spermatogonia (the stem cells for all spermatozoa) (Gilbert, 2000, Griswold, 2016). Several types of spermatogonia are recognized on the basis of cell and nuclear dimensions, distribution of nuclear chromatin (dark, condensed or pale, euchromatic) and histochemical and ultrastructural data.

The three basic groups of spermatogonia are;

- dark type A
- pale type A
- type B

The dark type A cells divide mitotically to maintain the population of spermatogonia which, before puberty, is small but increases under androgenic stimulation. Some divisions give rise to pale type A cells which also divide mitotically but remain linked in clusters by fine cytoplasmic bridges. These are the precursors of type B cells, which are committed to the spermatogenic sequence. At about the time type B cells enter a final round of DNA synthesis, without undergoing cytokinesis, they leave the basal compartment and cross the blood-testis barrier to enter meiotic prophase as pre-leptotene spermatocytes. These coordinated processes are under the control of Sertoli cells (Gray et al., 2005). They undergo leptotene, zygotene, pachetene and diakinetic steps in the prophase of the first meiotic division. Then the

first maturation division proceeds through metaphase, anaphase, and telophase giving rise to secondary spermatocytes. After a short interphase the secondary spermatocytes go through the second maturation division which yields spermatids (Clermont, 1966). In man, some spermatocytes degenerate during the two maturation divisions, since it is found that, for each primary spermatocyte, only 2.5 instead of the theoretical 4 spermatids are produced (Clermont, 1966).

The spermatids formed remained connected to one another by their cytoplasmic bridges. The spermatids that are connected in this manner have haploid nuclei, but are functionally diploid, since a gene product made in one cell can readily diffuse into the cytoplasm of its neighbors. From the initial cell divisions of type A spermatogonium to spermatids, the cells move farther away from the basement membrane of the seminiferous tubules and move closer to the lumen. The spermatid are however located at the border of the lumen, and here they lose their cytoplasmic connections and differentiate into sperm cells. In humans, the progression from spermatogonial stem cell to mature sperm takes 64 days (Gilbert, 2000).

The haploid spermatid is a round, unflagellated cell that looks nothing like the mature vertebrate sperm. However these spermatids undergo sperm maturation to form the sperm cells by a process known as spermiogenesis or spermateliosis. For fertilization to occur, the sperm has to meet and bind with the egg, and spermiogenesis prepares the sperm for these functions of motility and interaction. The first steps involve the construction of the acrosomal vesicle from the Golgi apparatus. The acrosome forms a cap that covers the sperm nucleus. As the acrosomal cap is formed, the nucleus rotates so that the cap will be facing the basal membrane of the seminiferous tubule. This rotation is necessary because the flagellum is beginning to form from the centriole on the other side of the nucleus, and this flagellum will extend into the lumen. During the last stage of spermiogenesis, the nucleus flattens and condenses, the remaining cytoplasm (the “cytoplasmic droplet”) is jettisoned, and the mitochondria form a ring around the base of the flagellum. The resulting sperm then enter the lumen of the tubule (Gilbert, 2000).

The process of spermatogenesis in humans, rodents and most other mammals are remarkably alike, though the duration is shorter in mice and rats (Clermont, 1963, Pryor et al., 2000). The formation of spermatozoa and maturation of sperm in adult men take approximately 64 and 5–10 days, respectively. The total duration of these two processes in mice and rats take 34.5 and 48–52 days, respectively (Clouthier et al., 1996, Bruckner et al., 2009). In addition, human spermatozoa show obvious differences in the 3-dimensional (3D) organization of the seminiferous epithelium and are lower in quality compared to rodents and most other mammals (Amann, 2008). Men produce fewer spermatozoa per gram of testis. Furthermore, there are more abnormal forms, lower motility, and fewer motile sperm in human semen (Pryor et al., 2000, Bruckner et al., 2009).

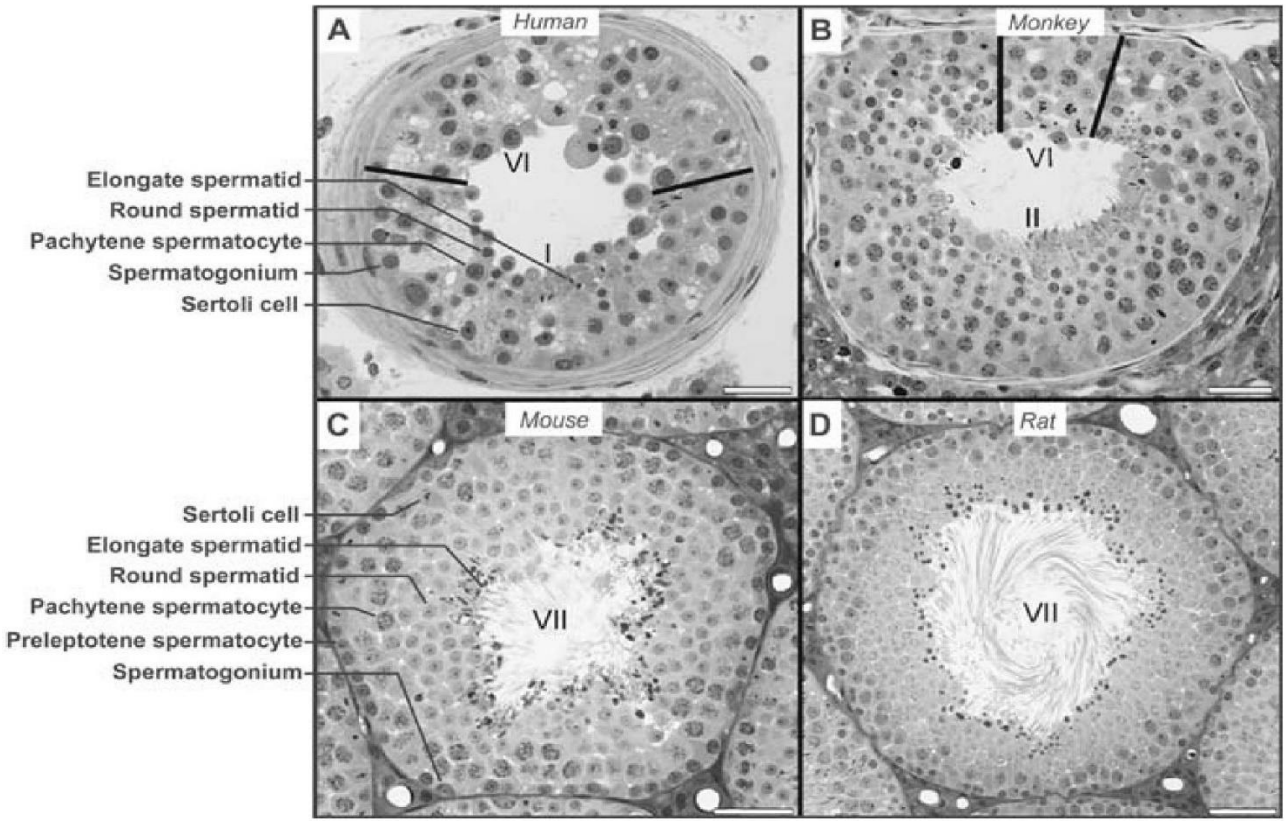


Figure 1.1 Seminiferous tubule cross-sections in different mammalian species. In the human (A), monkey (B), mouse (C), rat (D). Adapted from (Hess and de Franca, 2009).

1.1.4 The cycle and waves of the seminiferous epithelium

The complex biological process of spermatogenesis responsible for the production of male haploid germ cells from diploid spermatogonial cells has been categorized morphologically into cellular associations, also known as 'stages' and 'phases' of spermatogenesis (Clermont, 1963). The cells do not migrate laterally along the length of the seminiferous tubule; however, an unusual successive order of the stages is observed, whereby sequential stages occur with repetition along the length of the tubules, in a 'wave' of the seminiferous epithelium. Cellular associations progress through precisely timed and highly organized cycles which are essential for continuous sperm production and depends on numerous factors involving intrinsic (Sertoli and germ cells), extrinsic (androgens, retinoic acids) and the variability of species-specificity (Hess and de Franca, 2009). Spermatogenic classification into cellular association allows description and quantitation of the seminiferous epithelium and reveals the cycle of the seminiferous epithelium. In humans, the spermatogenic cycle is divided into six stages or cell associations and the duration of spermatogenesis does not change (Clermont, 1963). This is in contrast to mice and rats with 12 and 14 stages respectively (Leblond and Clermont, 1952, Oakberg, 1956). Testicular descent in human is concluded before the differentiation of pale type A spermatogonia to type B sermatogonia. Whereas in

rats and mice, spermatogenesis is initiated shortly after birth and before testes are positioned in the scrotum. The interval from initial commitment of spermatogonia through formation of preleptotene spermatocytes is accelerated for the first 10–12 days after birth, and the process then slows to adult timing (Van Haaster and de Rooij, 1993). The temporary acceleration of spermatogenesis might be due to elevated temperature in non-scrotal testes (Amann, 2008).

1.1.5 Assessment of semen quality

Table 1.1: Cut-off reference values for semen characteristics as published in consecutive WHO manuals

Semen characteristics	WHO 1980	WHO 1987	WHO 1992	WHO 1999	WHO 2010[α]
Volume (mL)	ND	≥ 2	≥ 2	≥ 2	1.5
Sperm count (10^6/mL)	20-200	≥ 20	≥ 20	≥ 20	15
Total sperm count (10^6)	ND	≥ 40	≥ 40	≥ 40	39
Total motility (% motile)	≥ 60	≥ 50	≥ 50	≥ 50	40
Progressive motility[β]	≥ 2[γ]	≥ 25%	≥ 25% (grade a)	≥ 25% (grade a)	32% (a + b)
Vitality (% alive)	ND	≥ 50	≥ 75	≥ 75	58
Morphology (% normal forms)	80.5	≥ 50	≥ 30[δ]	14[ε]	4[Θ]
Leukocyte count (10^6/mL)	< 4.7	< 1.0	< 1.0	< 1.0	< 1.0

[α]Lower reference limits generated from the lower fifth centile value;

[β]Grade a = rapid progressive motility (> 25μm/s); grade b = slow/sluggish progressive motility (5-25μm/s); Normal = 50% motility (grades a +b) or 25% progressive motility (grade a) within 60 min of ejaculation; [γ]Forward progression (scale 0-3);

[δ]Arbitrary value;

[ε]Value not defined but strict criterion is suggested;

[Θ]Strict (Tygerberg) criterion; ND = not defined.

Reference - World Health Organization (Esteves, 2014).

1.16 The male reproductive hormones

The two principal functions of the testis is for the secretion of testosterone (a hormone which control male sexual characteristics and behaviour) and production of sperms. Meanwhile, the amount of testosterone

production and secretion is regulated by the hypothalamus (located at the base of the brain) and the anterior pituitary glands (located at the base of the brain) (Ilacqua et al., 2016). The hypothalamus, anterior pituitary, and the testis constitutes the hypothalamic-pituitary-gonadal (HPG) axis.

Furthermore, the hypothalamus produces luteinizing hormone releasing hormone (LHRH), which is released in pulses into a system of blood vessels that connect the hypothalamus and the pituitary gland. In response to the LHRH signal, the pituitary gland produces two protein hormones called gonadotropins (Emanuele and Emanuele, 2001). These two gonadotropin hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH) are then released into the body's general circulation and act primarily at the level of the gonads (Rehfeld et al., 2017). The production of testosterone is males is stimulated by the LH from specialized cells called the Leydig cells (located in the connective tissues surrounding the seminiferous tubules), whereas FSH acts on the Sertoli cells (embedded in the inner walls of the seminiferous tubules) of the testes to stimulate sperm production (spermatogenesis) (Emanuele and Emanuele, 1998).

Aside from testosterone role in reproduction, it helps in promoting male secondary sexual characteristics such as increasing bone and muscle mass, sexual drive (i.e., libido), growth of facial and body hair, and deepening of the voice during puberty (Dohle et al., 2016). The anterior pituitary becomes less responsive to stimulation by GnRH as testosterone levels in the blood increases. The outcome of this leads to a reduced LH and FSH secretion. Reduced LH production will in turn result in a lowered testosterone levels. This process is called a negative feedback mechanism. On the contrary, a decrease in blood testosterone levels will stimulate anterior pituitary’s responsiveness to GnRH increases. This will result in more secretion of LH and FSH, with an overall increase in testosterone production by the Leydig cells (Emanuele and Emanuele, 1998, Emanuele and Emanuele, 2001).

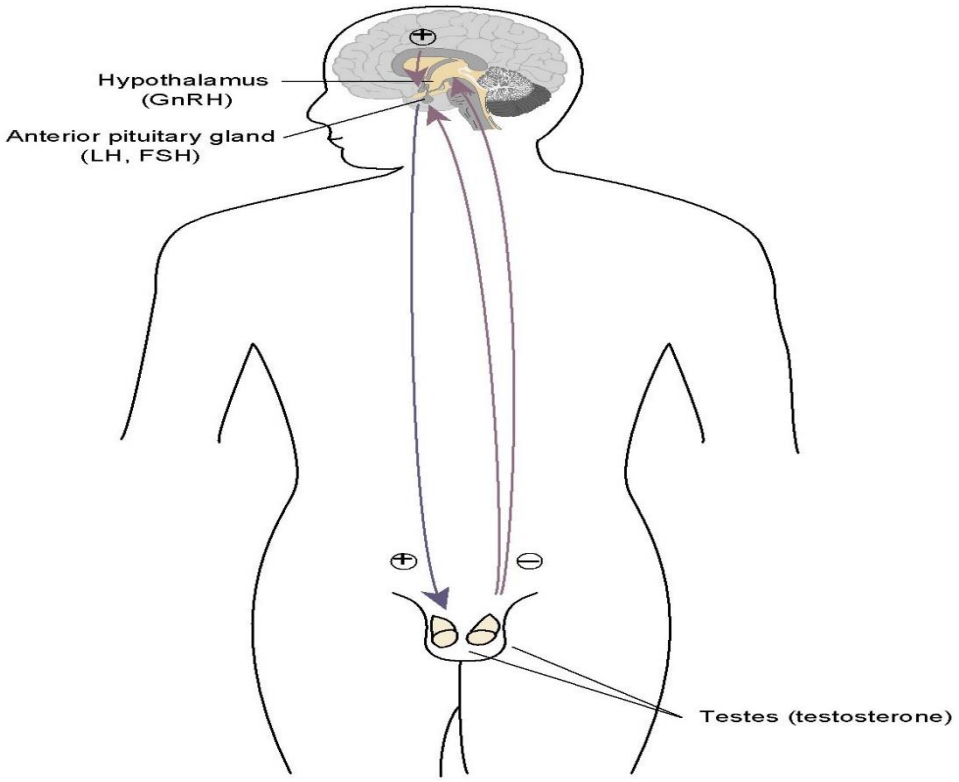


Figure 1.2: Components of the hypothalamic-pituitary-gonadal axis. The hypothalamus releases gonadotropin-releasing hormone (GnRH) to the anterior pituitary gland, which in turn releases luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. In males, LH stimulates testosterone production and FSH is important to sperm maturation. Testosterone circulates in the blood back to the hypothalamic pituitary unit and regulates the further production and secretion of GnRH, LH and FSH (Adapted from Emanuele and Emanuele (1998). NOTE: + = stimulatory effect; = inhibitory effect.

1.2 HIV and AIDS

HIV can be categorized as a lentivirus (subgroup of retrovirus) containing RNA, it is the causative agent responsible for AIDS (Sridevi et al., 2016, Wolfson et al., 2017). It carries single-stranded RNA is its genetic material rather than the double-stranded DNA human cells carry. Retroviruses also have the enzyme reverse transcriptase, which allow it to copy RNA into DNA and use that a DNA "copy" to infect human, or host cells. HIV attacks the immune system by destroying CD4 positive ($CD4^+$) helper T-cells, which is a type of white blood cell responsible for militating against infections, diseases and other complications in the body (Doitsh and Greene, 2016). When HIV infects a cell, HIV binds to the host cell leading to a fusion of the cell and the viral membrane. Then the viral RNA is converted into DNA and the virus uses the host cell's machinery to replicate itself during a process called reverse transcription (WHO, 2009a). Newly formed virus then break from the host, destroying the cell in the process, and move on to

infect other cells (Wilen et al., 2012). The mutation rate of HIV is very high (approximately 10^{-4} to 10^{-5} mutation per nucleotide and cycle of replication) with an intense frequency of recombination (Menéndez-Arias, 2013), There are two main forms of HIV: HIV1 and HIV2. The HIV-1 is the primary cause of AIDS worldwide. The HIV-2 is found predominantly in West Africa and its vertical transmission develops more slowly and it is much less virulent (usually not resulting in full blown AIDS, but still fatal) (Sanders et al., 2007). Following acute primary HIV infection, one may remain free of HIV-related illnesses, often for years, despite ongoing replication of HIV in the lymphoid organs and relentless destruction of the immune system. However, during the period, the immune system remains sufficiently competent to provide immune surveillance and to prevent most infections. AIDS is the final stage of HIV infection. A person infected with HIV is diagnosed with AIDS when the infected person is vulnerable to a variety of infections called "opportunistic" infections) and has a dangerously low number of CD4+ T cells (less than 200 cells per cubic millimeter of blood). The effect of HIV on the immune system is monitored by measuring the CD4 (helper) lymphocyte count in the blood. A normal CD4 count (between approximately 600 and 1,200 cells/μL) indicates that the immune system has not undergone sufficient damage to put the individual at risk for opportunistic illness. However, those with CD4 counts above 350-500 cells/μL are at elevated risk for a number of conditions that were not previously recognized as related to HIV infection. These include cardiovascular disease, kidney and liver disease, malignancies, and neurocognitive decline. As a result, experts have increasingly recommended initiation of treatment at higher CD4 counts. CD4 counts <500 cells/μL indicate that impairment of immune function is present, and are an indication for ART. CD4 counts <200 cells/μL indicate imminent risk of serious opportunistic" infections or other complications of HIV disease, and prompt treatment is recommended (WHO, 2009a).

1.2.1 Mode of Transmission of HIV

The spread of HIV from person-to-person can only be carried out through certain body fluids of an infected individuals. The three main routes of spreading this virus are: sexual exposure with infected individual, contact with infected blood, and mother-to-child transmission (Corstjens et al., 2016, Thomas et al., 2017)

To date, the major route of the HIV spread globally is through unprotected sexual acts, either ‘homosexual’ or ‘heterosexual’ with infected person. This is carried out through penetrative penile-vaginal intercourse, penetrative penile-anal intercourse and oral-genital contact (Swan and Evans, 2017, Thomas et al., 2017).

The risk of a male contracting HIV from an infected female during genital sex is low owing to the fact that the penis is harder to infect than the vaginal. The only route of infection in a male is the urethra opening or an open sore on the penis compared to several openings on the mucous membrane lining of a

female vagina (Hladik and Hope, 2009). HIV transmission via heterosexual contact is reportedly high in females compared to males (Ramjee and Daniels, 2013, Amin, 2015).

The penetrative penile-anal intercourse is considered as the riskiest form of sex in transmitting or contracting HIV (Baggaley et al., 2010, Swan and Evans, 2017). This is because the membrane lining the anal to rectal area is more delicate and easily broken when compared to that of the vagina making it easier for HIV to gain access into the lymphatics during sexual intercourse (Duby et al., 2016, Thomas et al., 2017). It risk of HIV transmission during homosexual (men and men) and heterosexual (men and women) intercourse is about 10-20 fold greater in unprotected penetrative penile-anal intercourse than unprotected penile-vaginal intercourse and therefore plays a significant role in HIV epidemic (McGowan, 2013, Kelly-Hanku et al., 2014).

The role of oral-genital sex as a route of transmission of HIV is poorly studied in populations other than homosexuals. It is because individuals who engage in oral genital sex rarely do so to the exclusion of other forms of sexual contact. It is difficult to attribute transmission of HIV to oral sex and not to other types of sexual exposures. The possibility of transmitting HIV from the vagina to the mouth seems possible, although it is not documented. Similarly, the feasibility of transmitting of HIV from the mouth to the genitals is unclear. One can speculate about plausible roots of transmission in any type of oral-genital contact. HIV bearing lymphocytes present in semen could contact damaged mucosa in the mouth and allow the entry of HIV into tissue. Likewise, traces of menstrual blood or vaginal discharge containing HIV could serve as a conduit of infection from the genitals to the mouth. In case the virus is shed in the saliva, it is not infective as the dose of virus needed to transmit the infection is very low. Saliva also contains an enzyme that inhibits the virus. Hence, it does not easily transmit the infection (Thomas et al., 2017).

HIV can also be transmitted from an infected mother to her baby while the baby is still in the woman's uterus or, more commonly, during childbirth. The virus can also be transmitted through the mother's breast milk during breast-feeding. Mother-to-child transmission accounts for 90 percent of all cases of AIDS in children. Mother-to-child transmission is particularly prevalent in Africa (Ciaranello et al., 2012).

HIV testing in individuals can be diagnosed only through laboratory tests on various body fluids such as blood, plasma, semen or vaginal fluid among others (WHO, 2013). Antibodies are detectable four to six weeks after exposure to HIV infection and employed by confirmatory tests such as ELISA (enzyme-linked immunosorbent assay), IFA (immuno-florescent assay) Western Blot.

1.2.2 Epidemiology of HIV/AIDS

In 2016, it was estimated that a total number of 36.7 million people worldwide are living HIV virus with more than 35 million deaths from AIDS related illnesses since its inception (UNAIDS, 2017). Roughly 1.8 million were new infections, whereas about 1 million people died of AIDS- related deaths in 2016. Among PLWHA in 2016, 34.5 million are adults, 17.8 million are women (+15 years), and 2.1 million are children (< 15 years). However in 2016, AIDS-related illnesses constitute the major cause of deaths globally among women of 15-49 years of age (WHO, 2017). Based on regional statistics, the Eastern and Southern Africa with approximately 6.2% of the global population has the highest hit of HIV in the world. This region has at least 19 million people are infected with the virus, accounting for over 50% of HIV/AIDS infected patients worldwide (UNAIDS, 2016, UNAIDS, 2017).

Table 1.2: Regional HIV and AIDS statistics and features in 2016

Regions	Adults and children living with HIV	Adults and children newly infected with HIV	Adults and child deaths due to AIDS
Eastern and Southern Africa	19.4 million	790,000	420,000
Western and Central Africa	6.1 million	370,000	310,000
Asia and the Pacific	5.1 million	270,000	170,000
Western and central Europe and North America	2.1 million	73,000	18,000
Latin America	1,8 million	97,000	36,000
Eastern Europe and central Asia	1.6 million	190,000	40,000
Caribbean	310,000	18,000	9,400
Middle East and North Africa	230,000	18,000	11,000
Total	36.7 million	1.8 million	1.0 million

Source: The Joint United Nations Programmed on HIV/AIDS (UNAIDS, 2017)

Next in order of prevalence is the Western and Central Africa, alongside the Asia and the pacific regions accounting for 16.6% and 13.8% of global HIV respectively (Table 1). Regions like Western and Central

Europe and North America, Latin America, Eastern and central Asia have less prevalent global HIV rates of about 5.7%, 4.9% and 4.4% respectively (UNAIDS, 2017). This explains why high HIV rates is confined to the poorest regions of the world, as seen in the sub-Saharan Africa – (Eastern, Southern, Western and Central Africa) (Mbirimtengerenji, 2007).

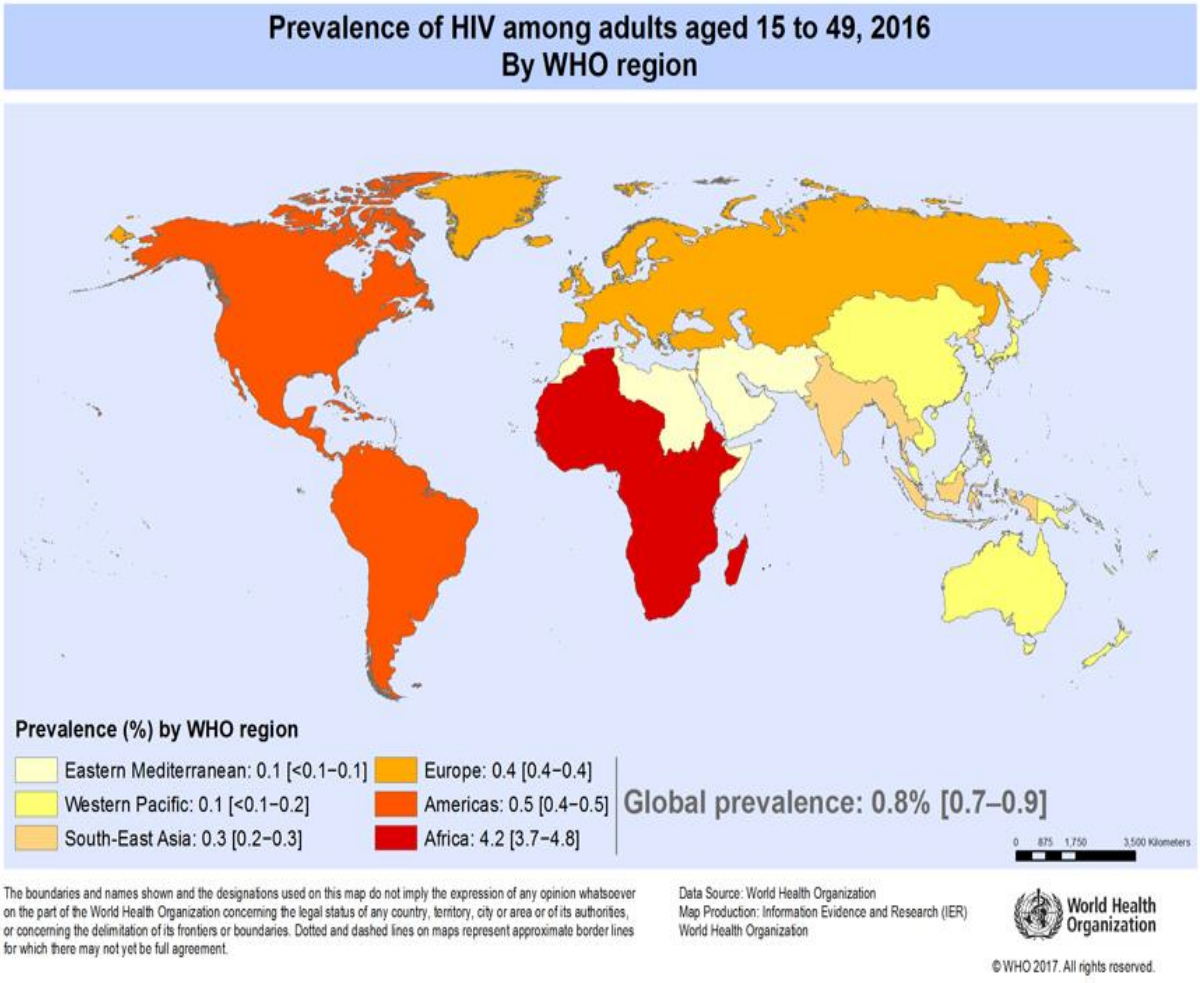


Figure 1.3: Global prevalence of HIV by regions (WHO 2017)

1.2.3 HIV/AIDS prevalence in Africa

HIV/AIDS is a major public health challenge in recent times which have resulted into decreased life expectancy on the African continent (WHO, 2017). Africa is a home of 15.2% of the world's population, with approximately 25.5 million HIV-infected individuals living in the Sub-Sahara Africa alone, which accounts for about 69% of the global HIV (UNAIDS, 2017). The spread of HIV/AIDS affect every country, however nine (9) countries in the Sub-Sahara Africa experience the most severe HIV prevalence

in the world. These countries include: Botswana, Lesotho, Malawi, Mozambique, Namibia, South Africa, Swaziland, Zambia and Zimbabwe. Swaziland has the highest HIV prevalence rate of 28.8% in the world, followed by Botswana (22.2) and Lesotho (22.7%) respectively (figure 1.4). The other six (6) countries showed at least 10% adult HIV prevalence with South Africa (19.2%) having the world's largest epidemic in terms of raw HIV case number of 7.1 million HIV infected individuals. In South Africa, the KwaZulu-Natal province has the highest HIV/AIDS population prevalence of 24.7% in the country (Hunt et al., 2017). The impact of HIV/AIDS on Africa development and economy has been reported to have dire consequences. Labor productivity and supply has been adversely affected owing to increasing mortality and morbidity rate, which in turn results in loss of skilled personnel in key sectors (Dixon et al., 2002). In addition, HIV/AIDS have led to a rise in number of children orphaned by AIDS, as well as threatening the survival of millions in Africa (Foster and Williamson, 2000).

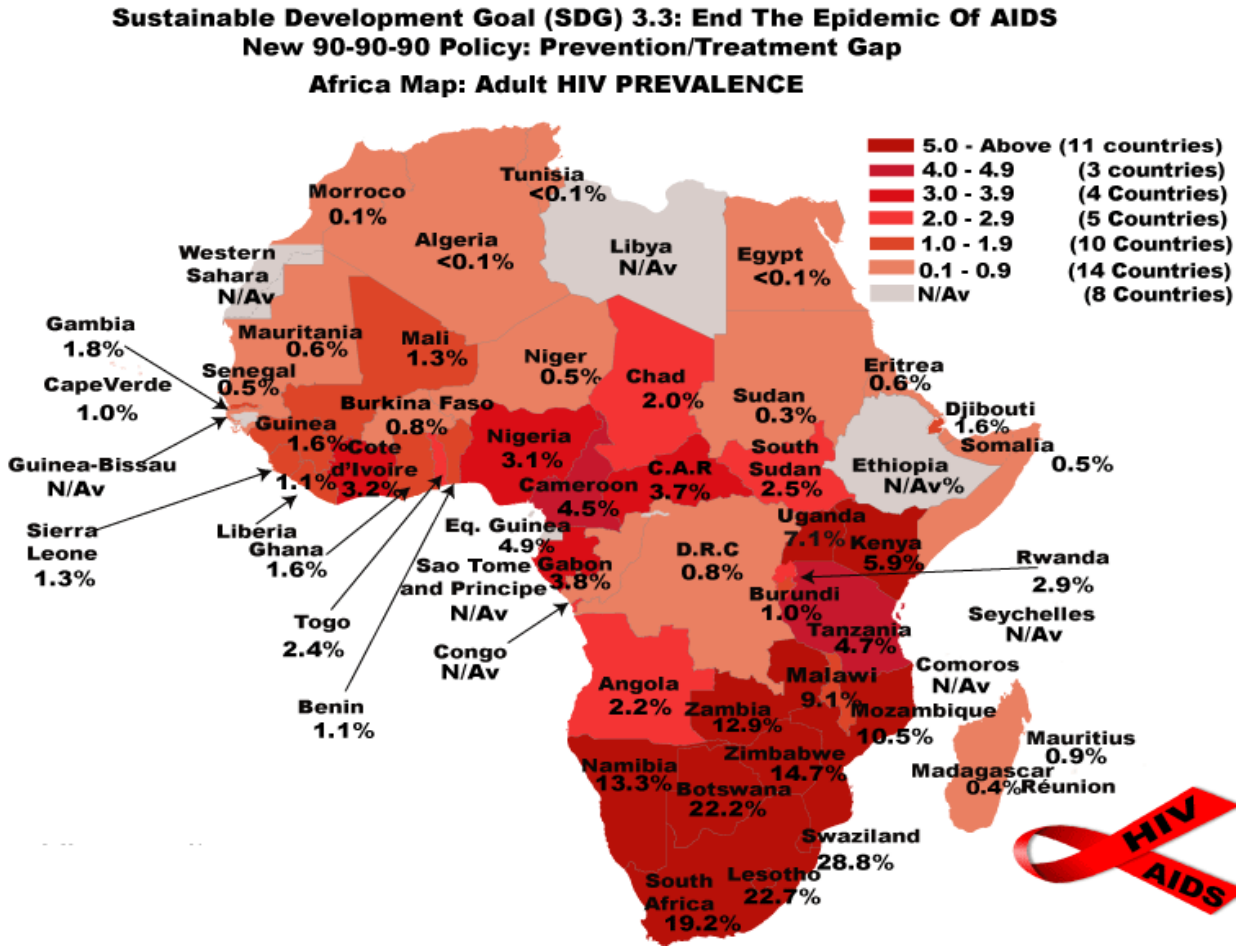


Fig 1.4: HIV adult prevalence in Africa (UNAIDS, 2017)

1.2.4 Treatment of HIV/AIDS

Although there is currently no cure for HIV, however there are treatment options available for improving the quality of life of PLWHAs. The management of HIV/AIDS involves the initiation of anti-HIV drugs called antiretroviral therapy (ART). The primary goal of ART is to decrease patient's total burden of HIV, restore and preserve immunological functions, prevents opportunistic infection that often leads to death, and to prevent HIV transmission (Council, 2011). Recommendation of ART to HIV-positive individuals should be carried out regardless of CD4 T lymphocyte cell counts. The use of ART should be continued indefinitely for the improvement of immunological functions as well as maintenance of viral suppression. HIV patients should be enlightened on the benefit of ART, as well as addressing adherence for maximum optimization of these regimen (Council, 2011, Mugoh et al., 2016). The treatment of HIV with anti-HIV medications is recommended for all infected individual, however certain conditions necessitate the urgent use of this therapy. These include: pregnancy, acute opportunistic infections (OIs) and malignancy, lower CD4 counts, acute or early infections, HIV/hepatitis B virus co-infection, and HIV/hepatitis C virus co-infection (Council, 2011).

ART consists of drugs from six (6) classes. These include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CCR5 antagonists) (Tabe et al., 2015). These drugs are approved by the United States of America Food and Drug Administration (FDA). The table below (Table 1.2) shows the various classes of antiretroviral drugs approved by the FDA.

Table 1.3: Classification of antiretroviral drugs

Mechanism of action	Generic Name (Other names and acronyms)	Brand Name	FDA Approval Date	Manufacturer name
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)				
Inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation.	Abacavir-ABC	Ziagen	December 17, 1998	GlaxoSmithKline
	Didanosine (ddI, EC)	Videx	October 9, 1991	Bristol Myers-Squibb
		Videx EC (enteric-coated)	October 31, 2000	Bristol Myers-Squibb
	Emtricitabine-FTC	Emtriva	July 2, 2003	Gilead Sciences
	Lamivudine-3TC	Epivir	November 17, 1995	GlaxoSmithKline
	Stavudine (d4T)	Zerit	June 24, 1994	Bristol Myers-Squibb
	Tenofovir disoproxil fumarate-TDF	Viread	October 26, 2001	Gilead Sciences
	Zidovudine-ZDV	Retrovir	March 19, 1987	GlaxoSmithKline
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)				
Inhibit reverse transcriptase directly by binding to the enzyme and	Delavirdine-DLV	Rescriptor	April 4, 1997	Pfizer
	Efavirenz-EFV	Sustiva	September 17, 1998	Bristol Myers-Squibb
	Etravirine-ETR	Intelence	January 18, 2008	Tibotec Therapeutics

interfering with its function.	Nevirapine-NVP	Viramune (Immediate Release)	June 21, 1996	Boehringer Ingelheim
		Viramune XR (Extended Release)	March 25, 2011	Boehringer Ingelheim
	Rilpivirine-RPV	Edurant	May 20, 2011	Tibotec Therapeutics
Protease Inhibitors (PIs)				
Target viral assembly by inhibiting protease enzyme used by HIV to cleave nascent proteins for final assembly of new virons.	Atazanavir-ATV	Reyataz	June 20, 2003	Bristol-Myers Squibb
	Darunavir-DRV	Prezista	June 23, 2006	Tibotec, Inc.
	Fosamprenavir-FPV	Lexiva	October 20, 2003	GlaxoSmithKline
	Indinavir-IDV	Crixivan	March 13, 1996	Merck
	Nelfinavir-NFV	Viracept	March 14, 1997	AgouronPharmaceuticals
	Ritonavir-RTV	Norvir	March 1, 1996	Abbott Laboratories
	Saquinavir-SQV	Invirase	December 6, 1995	Hoffmann-La Roche
	Tipranavir-TPV	Aptivus	June 22, 2005	Boehringer Ingelheim
Fusion or entry Inhibitors				
Prevent HIV from binding to or entering human immune cells.	Enfuvirtide-T20	Fuzeon	March 13,2003	Hoffmann-La Roche & Trimeris
	Maraviroc-MVC	Selzentry	August 6, 2007	Pfizer
Integrase Inhibitors				
Inhibit integrase enzyme needed by HIV to insert its genetic material into human cells.	Dolutegravir-DTG	Tivicay	August 13, 2013	GlaxoSmithKline
	Elvitegravir-EVG	Vitekta	September 24, 2014	Gilead Sciences
	Raltegravir-RAL	Isentress	October 12, 2007	Merck & Co., Inc.

CCR5 antagonist				
Binds to human chemokine receptor CCR5 present on the membrane of CD4 cells (T-cells), thereby blocking the HIV-1 gp120 from associating with the receptor necessary for CCR5-tropic HIV-1 to enter cells	Maraviroc/MVC	Selzentry	August 6, 2007	Pfizer

Source: *https://aidsinfo.nih.gov/education.../fact.../fda-approved-hiv-medicines*
(Last updated 4/28/2015; last reviewed 4/28/2015).

Highly active antiretroviral therapy

The use of multi-drug antiretroviral treatments as well as fixed dose combination has been very effective in the inhibition of viral replication and restoration of immunological functions in PLWHAs, as earlier results with single-drug therapies had been discouraging (Saag and Kilby, 1999). HAART which constitutes two nucleoside reverse transcriptase inhibitor (NRTIs) in combination with a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor (PI) or an integrase strand transfer inhibitor (INSTIs) as approved by the FDA, serves as the standard treatment for HIV infection (WHO, 2009b, Arenas-Pinto et al., 2015a) (Table 1.3). Recent improvement on HAART had led to patients switching from three separate antiretroviral (ARV) drugs to the new, single, fixed-dose combination (FDC) tablet, as single pill is easier to take.

The advent of HAART and FDC recommendations has ultimately led to significant improvement regarding decreased viral loads, increased CD4 counts, improved T- cell counts with life expectancies much closer to general mortality have been reported in HIV patients who received them (Conklin and Pineda, 2017). In addition, there is improvement in patients' overall quality of life, aside from longevity (Arenas-Pinto et al., 2015a).

Table 1.4: Possible combination antiretroviral therapy- FDA approved

Drug Class	Generic Name (Other names and acronyms)	Brand Name	FDA Approval Date	Manufacturers name
Multi class combination products	Abacavir and Lamivudine (ABC/3TC)	Epzicom	August 2, 2004	GlaxoSmithKline
	Abacavir, Dolutegravir, and Lamivudine (ABC/DTG/3TC)	Triumeq	August 22, 2014	ViiV Health care and Shionogi & Co Ltd.
	Abacavir, Lamivudine, and Zidovudine (ABC/3TC/ZDV)	Trizivir	November 14, 2000	GlaxoSmithKline
	Atazanavir and Cobicistat (ATV/COBI)	Evotaz	January 29, 2015	Bristol-Myers Squibb
	Darunavir and Cobicistat (DRV / COBI)	Prezcobix	January 29, 2015	Jassen Therapeutics
	Efavirenz, Emtricitabine, and Tenofovir disoproxil fumarate (EFV/FTC/TDF)	Atripla	July 12, 2006	Bristol-Myers Squibb and Gilead Sciences
	Elvitegravir, Cobicistat, Emtricitabine, and Tenofovir disoproxil fumarate (EVG/COBI/FTC/TDF)	Stribild	August 27, 2012	Gilead Sciences
	Emtricitabine, Rilpivirine, and Tenofovir disoproxil fumarate (FTC/RPV/TDF)	Complera	August 10, 2011	Gilead Sciences
	Emtricitabine and Tenofovir disoproxil fumarate (FTC/TDF)	Truvada	August 2, 2004	Gilead Sciences, Inc.
	Lamivudine and zidovudine (3TC/ZDV)	Combivir	September 27, 1997	GlaxoSmithKline
	Lopinavir and Ritonavir (LPV/RTV)	Kaletra	September 15, 2000	Abbott Laboratories

Source: *https://aidsinfo.nih.gov/education.../fact.../fda-approved-hiv-medicines*

(Last updated 4/28/2015; last reviewed 4/28/2015).

1.2.6 Adverse effects of HAART

The advent of HAART have seen remarkable progress over the years against HIV/AIDS in increasing life expectancy, with a dramatic drop in overall morbidity and mortality (Arenas-Pinto et al., 2015a, Chang et al., 2015a). Despite these achievements, HAART has been unable to fully restore health in HIV- patients owing to several adverse effects incurred from its chronic use (Nagiah et al., 2015a). Enthusiasm generated by HAART has been diminished by the severe consequences mediated by hypersensitivity reactions, mitochondrial dysfunction, disturbances of lipid and glucose metabolism, and direct cell stress (Núnez, 2006). HAART adverse effects may be short term such as: headache, rash, dizziness, nausea and vomiting; others are long term and can be life threatening, e.g. hepatic, renal and cardiovascular abnormalities (Montessori et al., 2004, Hawkins, 2010). Many possible reasons for switching or discontinued therapy which could limit adherence and increase detectable viral loads amongst HIV patients were attributed to these adverse events (Azia et al., 2016).

In addition, HIV-patients on HAART are susceptible to an increased risk of several non-AIDS complications known as comorbid conditions (Collaboration, 2008, Gazzola et al., 2010). The long-term treatment with HAART is associated with several comorbid conditions including metabolic abnormalities such as; abnormal carbohydrate metabolism, fat redistribution syndrome (lipodystrophy), hypertension, and dyslipidemia (Pirrone et al., 2013, Bryant et al., 2016).

1.2.7 HAART- related testicular damage

Antiretroviral drugs are known to negatively impact testicular functions via altered mitochondrial DNA and oxidative stress mechanisms leading to sexual dysfunction (Day and Lewis, 2004b, Lambert-Niclot et al., 2011). Clinical studies have shed light on the effects of ARVs on sperm quality with a majority of them indicating that ARVs can adversely affect seminal parameters. Decrease in sperm mitochondrial DNA (mtDNA), and sperm mtDNA deletions with severe impact on fertility via impairment of the acrosome reaction were observed in HIV positive men undergoing treatments with antiretroviral therapy (White et al., 2001, Pavili et al., 2010b, Ahmad et al., 2011a). Additional findings have shown a positive correlation between ARVs and low sperm counts with a decrease in progressive motility and semen volume (Dulioust et al., 2002, van Leeuwen et al., 2008, Nicopoullos et al., 2011).

Furthermore, animal studies have buttressed the negative influence of ARVs on sperm functional parameters causing lowered sperm quality. This manifested as induction of abnormalities in the genetic makeup (Kaushik et al., 2014, Adaramoye et al., 2015), structural abnormalities to testicular tissues (Chris-Ozoko et al., 2013), atrophy of the seminiferous tubules, decrease epithelial height, depletion of the Sertoli cells and spermatogenic arrest (Azu et al., 2014). Based on these findings, it is evidently clear that ARVs can be toxic to the mitochondria.

It is well established that mitochondria energy metabolism is critical to the efficient functionality of sperm cells which can be compromised when exposed to toxicants (Piomboni et al., 2012b, Song et al., 2014a). Hence these drugs target the energy supply and hence compromise fertility of the individual.
Emerging counter results from some other authors reveal no difference in semen parameters that may affect fertility following antiretroviral therapy (Politch et al., 1994, Robbins et al., 2001). While acknowledging these discrepancies, it is noteworthy to point out that much of the observations and reports have been based on different sample populations or experimental cohorts and under diverse aetio-pathological underpinnings that might have influenced the outcomes. However, in a previous review (Azu, 2012), it was noted that the association of HAART and sexual dysfunction in seropositive HIV patients remains a subject of intense research amongst researchers. It is reasonable to believe that this argument may still remain unresolved until such time as exhaustive tools are available to assess the male genital tract (MGT) morphologically and correlate changes with other associated parameters of fertility.

1.2.8 The testis, HAART related complications

Studies have shown that metabolic complications arising from ARVs can lead to a marked reduction in fecundity (La Vignera et al., 2009, Oliveira et al., 2012). Abnormal carbohydrate metabolism can precipitates into various pathologies of insulin resistance, glucose intolerance and diabetes mellitus (Brown et al., 2010). However carbohydrate (mainly glucose) supplies the energy required for passive transport of glucose across the blood testes barrier in the process of spermatogenesis (Alves et al., 2013). Disruption in glucose metabolism and/or transport can adversely affect the germinal cells, leading to spermatogenic arrest in the seminiferous tubules (Azu et al., 2014, Rato et al., 2012). With interrupted energy supply (due to altered glucose metabolism), protein synthesis in germinal cells become deranged resulting in lipid accumulation in the cytoplasm of germinal cells and interstitial connective tissue of the testes (Malekinejad et al., 2011, Razi et al., 2012). This will result in fat re-distribution (lipodystrophy) which may include both central fat accumulation (lipohypertrophy) and peripheral fat wasting (lipoatrophy) (Carr et al., 1998). Dyslipidemia, which is also characterized by abnormal lipid and lipoprotein profiles is associated with the use of ARVs (Caron-Debarle et al., 2010). Dyslipidemia patients show elevation of the serum total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride concentrations, and a decrease in the high-density lipoprotein (HDL) cholesterol concentration (Pirrone et al., 2013). However, there is sufficient evidence that testicular lipid accumulation can impair male fertility (Johnson, 1970, Yildiz et al., 2006a).

1.3 Alcohol use in patients with HIV/AIDS

The impact of depression on HIV infected population cannot be underestimated as a large percentage of these patients constantly suffer from symptoms caused by shame, trauma, increased stigmatization and

adverse effects of medications (WHO, 2008). Reports have shown that depression is three times more common among HIV infected individuals when compared to the general population (DeJean et al., 2013, Tesfaw et al., 2016). The high rates of depression seen in these patients has triggered the use of alcohol consumption among these people to alleviate its symptoms (Palfai et al., 2014, Health., 2015). Meanwhile, alcohol briefly produce a pleasant and relaxed state of the mind resulting in a momentarily elevated mood, there after arises its depressant effects which exacerbates its underlying depression. This, in turn, leads to further intake of alcohol, thus perpetuating a vicious cycle (Crocq, 2003, Maheshwari and Sharma, 2016).

According to WHO (2016), South Africa is the fifth largest 'drinking nation' in Africa. South Africa has previously been rated as one of the nations with the most risky drinking patterns in the world (Organization and Unit, 2014). Reported data from the South African national surveys have shown that approximately one-third of the population engage in heavy episodic bouts of drinking (Ramsoomar and Morojele, 2012, Bello et al., 2017) with a total alcohol consumption of 11.2 L of pure alcohol per capita in subjects over 15 years of age. This therefore makes it one of the biggest drinking nations in the world (WHO, 2016). Furthermore, the high consumption of alcohol in South Africa is closely related to the high risk of contracting HIV (Schneider et al., 2014, Probst et al., 2017). This corresponds to similar patterns globally (Organization and Unit, 2014, Young et al., 2017). This may contribute to an understanding of why South Africa has the highest HIV prevalence in the world (Kalichman et al., 2007).

Heavy consumption of alcohol is a common phenomenon among HIV-infected individuals (Hahn and Samet, 2010). The prevalence of heavy and mild-to-moderate drinking rates is approximately 2–2.5 times higher respectively in HIV individuals than the general population (Schneider et al., 2014, Kumar et al., 2015). The habitual or chronic use of alcohol is capable of causing major health problems and destruction to nearly every organ and system in the body including testicular damage (La Vignera et al., 2013a, Caputo et al., 2016).

1.4 Alcohol associated testicular damage

The heavy or chronic consumption of alcohol has been known to cause decreased fertility in men. Excessive alcohol consumption has been shown to drastically reduce testosterone production and even shrink the testicles responsible for the production of this hormone (Adler, 1992). Clinical and experimental animal studies have established decrease in LH levels resulting from alcohol impairment in both LH production and LH secretion, with an overall decrease in testosterone production (Frias et al., 2002, Grover et al., 2014, Oremosu and Akang, 2015). Since testosterone is directly involved in almost all parts of the male reproductive process, the reduction in testosterone caused by alcohol can cause a range of additional effects, including reduced fertility and impotence, or the inability to get an erection. In addition, alcohol permeates directly into the testes bringing about a decrease in spermatogenesis, lowering

the synthesis of testosterone, increasing metabolic clearance of testosterone and resulting in increased estrogen levels (Emanuele et al., 1999, Muthusami and Chinnaswamy, 2005). The primary role of the Sertoli cells are to nourish the developing cells throughout the stages of spermatogenesis for the production of matured sperms. Thus, healthy Sertoli cells function is essential for sperm cell viability and development (Jensen et al., 1997a). Sertoli cell requires both FSH and testosterone stimulation for the maintenance of spermatogenesis (Pappano and Wier, 2012). However, alcohol reduces the production of all of these hormones, which leads to the deterioration of the Sertoli cells (Emanuele and Emanuele, 1998, Emanuele and Emanuele, 2001). This results in human spermatozoa exhibiting decreased sperm motility, severe morphological abnormalities, increased DNA fragmentation and can overtime progress to complete loss of sperm production (Muthusami and Chinnaswamy, 2005, Sermondade et al., 2010b).

More evidences from clinical studies have also indicated that long-term consumption of alcohol alters sperm parameters and potentiates testicular pathology (Vicari et al., 2002, Ramlau-Hansen et al., 2010b, Varshini et al., 2012).

1.4 Testicular interactions of alcohol and antiretroviral

The consumption of alcohol has shown significant usage in HIV-infected individuals who are on antiretroviral drugs (Roux et al., 2008, Soboka et al., 2014a, Kekwaletswe and Morojele, 2014a). Numerous drugs are metabolized by the cytochrome P450 (CYP) pathway. CYP3A4 (a major isoform of the CYPs family) have proven involvement in the metabolism of ARVs and alcohol, thus several ARV drugs can either induce or inhibit CYP3A4 (Walubo, 2007, McCance-Katz et al., 2013). Alcohol can affect the pharmacokinetics and pharmacodynamics of several ARVs via enzymatic induction which is associated with chronic alcohol use, and enzymatic inhibition due to competition of ethanol for various cytochrome P450 (CYP) isozymes, associated with acute ethanol use (Hahn and Samet, 2010, Kumar et al., 2012a). Interaction of alcohol with CYP3A4 inducing ARVs may therefore result in increased metabolism with subsequent decreased plasma concentration, reduced antiretroviral efficacy and acceleration of disease progression (Kumar et al., 2015). Inhibition of CYP3A4 will also increase drug bioavailability which may place patients at significant risk for toxicities (Kumar et al., 2012a).

Studies have reported alcohol exacerbating the side effects of certain ARVs with emphases on the toxicities resulting from their drug–dietary interactions (Cooper and Cameron, 2005a, Braithwaite and Bryant, 2010a). As mentioned earlier, antiretroviral drugs negatively impact testicular functions via altered mitochondrial DNA and oxidative stress mechanisms (Pavili et al., 2010b). The outcomes of these have a negative impact on semen parameters and subverts the architecture of the testicular anatomy with consequent loss of germ cells leading to sexual dysfunction (Lambert-Niclot et al., 2011, Azu et al., 2014). Interaction with alcohol can also significantly affect seminal fluid concentration of ARVs (Ghosn et al., 2004, Van Leeuwen et al., 2007). Habitual consumption of alcohol decrease the male sexual

hormone levels, causes testicular hypofunction with potential for lowered fertility (Vicari et al., 2002, Rai and Rai, 2016b). Concomitant use of ARVs appears to act synergistically to exacerbate this toxicity thus diminishing male fertility.

In addition, alcohol and ARVs have pronounced effects on membrane-associated drug transporters (Weiss et al., 2008, Ingólfsson and Andersen, 2011b). Alcohol alters the properties of the lipid bilayer by changing membrane permeability and protein distribution (Gurtovenko and Anwar, 2009a, Ingólfsson and Andersen, 2011b). The change in membrane properties comes with resultant alteration of protein functions (Eckenhoff, 2001, Patra et al., 2006). Since drug transporters critical to pharmacokinetics are integral membrane proteins, there is a high probability that alcohol will alter accessibility to the protein-binding sites of ARVs substrates (Dallas et al., 2006). The observable of alcohol tends to diminish the activity of both the efflux and influx transporters.

1.5 Herbal remedies as complementary and adjuvant therapy

The use of herbal or traditional remedies as complementary and alternative therapies have become a major component of health care and has been widely embraced across the globe so much so that the validity of the term "complementary" has been questioned (Bielory, 2001, Braun et al., 2010a). Complementary therapy refer to any of a range of medical therapies that fall beyond the scope of conventional medicine but may be used alongside it in the treatment of disease and ill health. An alternative therapy is generally used instead of conventional medical treatment.

The practice of complementary and alternative therapies is growing at a significant pace and its use has been promoted on the African continent. An estimated 80% of Africans rely on this holistic form for therapy in the management of diseases and ailments, which conventional therapies often fail to address (Parekh and Jing, 2011, Romm, 2017).

The use of plant-based adjuvants have been supported by several studies, as it involves the use of plant's seeds, berries, roots, leaves, flowers, bark or whole herbs for medicinal purposes, with these herbs containing several active ingredients that may work together to produce beneficial effects (Ruban and Rodioniva, 2012). Various medicinal herbs are however used to treat several disease conditions such as asthma, diabetes, cancer amongst several others. Also many available conventional drugs originate from plant sources with a majority of them being effective, resulting to the engagement of several drug companies in large scale pharmacological screening of herbs (Vickers and Zollman, 1999).

The heavy financial burden tied to sustaining adherence to HAART among PLWHAs, coupled with its rising toxicities has resulted to a design of several studies accommodating a holistic management approach that includes the use of herbal remedies (Shah, 2007a). Thus, while the use of medicinal herbs (a principal component of complementary and alternative therapies) predates the emergence of HAART, there has been a widespread increase in the consumption of herbal products as adjuvant in management of

HIV (Peltzer et al., 2011). Among several plants of interest, attention was focused on medicinal plants with pharmacological properties. *Cocos nucifera* (Coconut) however fall into this category of plants owing to its wide range health benefits, some of which include its anti-inflammatory, antioxidant, antitumor, antimicrobial properties amongst several others (Lima et al., 2015).

1.5.1 Coconut (*Cocos nucifera*)

The coconut tree (*Cocos nucifera*) is a member of the family Arecaceae (palm family) and the only species of the genus Cocos (Nayar, 2016). It is the most extensively cultivated palm in the tropical and subtropical regions of the world and has been the most useful tree to humans because it meets almost all basic needs of mankind (Raffauf, 1985, Chan and Elevitch, 2006). It is often called 'Kalpavirksha' or 'Tree of Life' owing to its wide range of benefits (DebMandal and Mandal, 2011). Coconut is not distributed or cultivated in South Africa. Rather most of it are imported from Sri Lanka, Philippines and Mozambique (Teulat et al., 2000).

Cocos nucifera is a large palm, growing up to 30 m (98 ft) tall, with pinnate leaves 4–6 m (13–20 ft) long, and pinnae 60–90 cm long; old leaves break away cleanly, leaving the trunk smooth (Lima 2015).

There are mainly two distinct groups of coconut i.e. tall and the dwarf. The tall varieties grow slow and bear fruits 6 to 10 years after planting. Its copra, oil and fiber are of good quality. This type is comparatively hardy, and lives up to a ripe age of 80 to 120 years. As male flowers mature earlier than the female flowers, this type is highly cross-pollinated. Nuts mature within a period of 12 months after pollination. The dwarf varieties are fast growing and bear early i.e. takes 4 to 5 years (DebMandal and Mandal, 2011). Due to overlapping of male and female phases, the dwarf varieties are self-pollinated. The nuts are yellow, red, green and orange colored. These are less hardy and require favorable climatic conditions and soil type for better yield (Chan and Elevitch, 2006).

Botanically, the coconut fruit is a fibrous drupe, not a true nut. It consists of, from the outside in, a thin hard skin (exocarp), a thicker layer of fibrous mesocarp (husk), the hard endocarp (shell), the white endosperm (kernel/seed), and a large cavity filled with liquid ("water"). When immature, the exocarp is usually green. The shell has three germination pores (micropyles) or "eyes" that are clearly visible on its outside surface once the husk is removed. A full-sized coconut weighs about 1.44 kg (3.2 lb) (Lima et al., 2015).

Figure 1.5: Photograph showing Coconut tree with fruits. Adapted from (Fife, 2005)

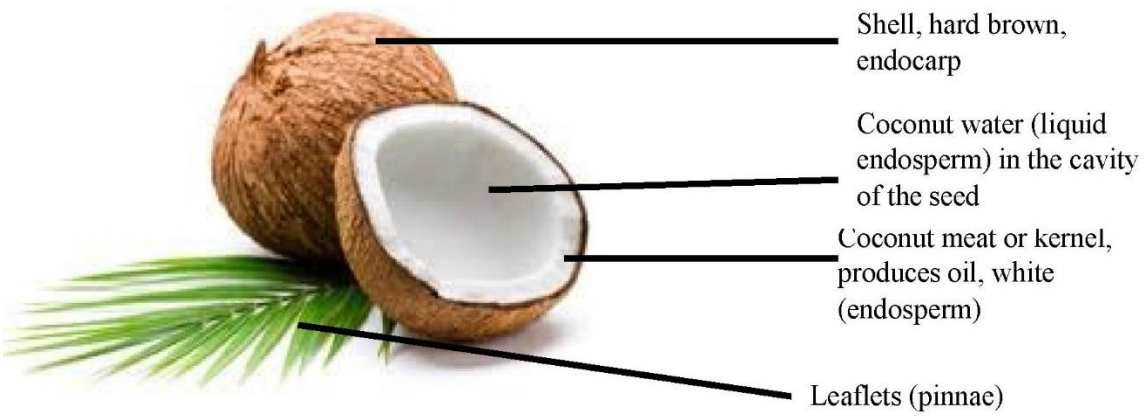


Figure 1.6 Photograph showing Coconut shell, meat and leaflets. Adapted from (Fife, 2013b)

Coconut can produce a variety of edible products. The most common being the meat, water, milk, cream and the oil. The coconut meat is the white edible part of the Kernel. Coconut oil is extracted from the fresh and dried coconut meat (Fife, 2005). Additionally, it has also found wide applications in traditional medicines and as carrier oil in pharmaceutical industries besides being widely used in pharmaceuticals, cosmetics and baking industries. Moreover, it is one of the main ingredients in soap making and infant formulae (Krishna et al., 2010). There are two different types of coconut oil available for edible purposes: refined coconut oil and the unrefined or virgin coconut oil

Refined coconut oil (RCO)

This is made exclusively from dried coconut meat or kernel. The meat contains about 50% moisture and it has to be dried to a moisture content of 6–8% before oil extraction. This can be achieved by drying it under the sun, with direct heat or through the use of hot air. The dried coconut meat is known as copra and has an oil content of 64%. Traditionally, coconut oil is extracted from the copra by crushing in an expeller, followed by solvent extraction to recover the residual oil from the cake (Canapi, 2005). The crude oil is then refined by physical or chemical refining to remove impurities, making it suitable for human consumption and prolonging its shelf-life. Refined coconut oil is sometimes partially hydrogenated, which produces trans-fats (Krishna et al., 2010). Trans-fats are produced when hydrogen is added to liquid plant-derived oils, which makes them more solid, and they can raise your low-density lipoprotein levels. High LDL cholesterol can increase your risk of developing type-2 diabetes, heart disease or risk of having a stroke (Mozaffarian et al., 2006).

Virgin coconut oil (VCO)

Unlike RCO which is extracted from the dried meat (i.e. copra) of the coconut, VCO is obtained from the fresh meat/kernel of mature coconuts by mechanical or natural means. The production of VCO first involves the extraction of coconut milk from the grated coconut meat. This is followed by the separation of VCO from water and other residues of coconut milk either through cold extraction (i.e. without the use of heat), or hot extraction (i.e. with the use of heat) (Srivastava et al., 2016). Other extraction techniques used include chilling, freezing and thawing; fermentation; as well as enzymatic (Marina et al., 2009b). Regardless of the extraction techniques used, coconut milk is always the starting ingredient. As such, this process is often referred to as the "wet" process, in contrast to the dry process of RCO extraction (Nevin and Rajamohan, 2006, Amri, 2011). To be considered "virgin", the extracted oil is not being chemically refined, bleached, deodorized, as in the production of RCO (Amri, 2011, Srivastava et al., 2016).

VCO can be differentiated from RCO from its physical appearance. RCO has a distinct yellow colour. VCO produced by cold extraction is almost colourless, whereas VCO produced by hot extraction has a light yellow colour (Srivastava et al., 2016). RCO has no perceptible aroma while VCO has a strong, sweet, and nutty aroma often associated with roasted coconut or cooked coconut. In terms of flavour, VCO has a detectable sweet taste and nutty flavour contrasting the absence of flavour in RCO. In terms of fatty acid composition, there is no difference between VCO and RCO. The chemical properties in both oils are essentially the same, since they both come from the same source (coconut meat) (Carandang, 2008, Marina et al., 2009a). However, VCO is claimed to be superior or have more health benefits compared to RCO. This is due to its higher content of phenolic compounds which is approximately three times more in VCO compared to RCO, with enhanced antioxidant activity which doubles of RCO (Srivastava et al., 2016).

1.5.2 Medicinal uses of Coconut oil

Coconut oil is highly valued across the globe owing to its versatility of use which provide a wide range of health benefits (Lima et al., 2015). It is believed that coconut oil plays an instrumental role in reducing the viral susceptibility of HIV patients (Fife, 2013a). A preliminary study on the effects of coconut oil on HIV/AIDS gave very encouraging results with subjects' viral load dramatically reduced and immune system enhanced as reflected in the CD4/ CD8 count (Dayrit, 2000, Hardon et al., 2008).

It is also used in traditional medicine to treat a variety of general ailments such as, impotency and infertility, menstrual cycle disorders, asthma and gonorrhea, fever, malaria and venereal diseases (Al-Adhroey et al., 2011, Lima et al., 2015). Several other studies have reported its potency in the treatment of diabetics, cardiovascular disease conditions, cancer, weight loss, bone loss, skin problems and hair loss amongst several others (Carandang, 2008, Ganguly, 2013).

1.5.3 Composition of Coconut oil

The Crude or unrefined Coconut oil is majorly a triglyceride or contains mainly of triacylglycerol (TAG) (about 95%). It also contains minor proportions of mono- and di-glycerides. Other minor constituents include free acids, phospholipids, free and/or acylated sterols, tocols and hydrocarbons such as alkanes, squalene and carotenes (Amri, 2011, Gunstone, 2013). About 0.5% of crude coconut oil is not saponified by caustic treatment. This unsaponifiable matter consists mainly of tocols, sterols, squalene, color pigments, carbohydrates and odour compounds (lactones) (Canapi, 2005). Most of the unsaponifiables are removed during the refining, bleaching and deodorising of crude coconut oil. The crude oil also contains protein, crude fibre and trace amounts of metals such as iron, copper and lead (Amri, 2011).

Unrefined coconut oil melts at 24-25°C and smokes at 177°C, while refined coconut oil has a higher smoke point of 232°C. Coconut oil is a highly saturated oil (about 92%). Its high saturated fat content makes the crude oil very stable against oxidation. The slow oxidation of coconut oil causes its resistance to rancidity making the oil last up to two years (Young, 1983, Laureles et al., 2002, Henna Lu and Tan, 2009). Coconut oil is considered as one of the most stable oils. However, refined oil has less oxidative stability compared to crude oil due to the loss of natural antioxidants during the refining process. Of its total, 62% fatty acid composition of coconut oil are medium-chain fatty acids (MCFA) with a chain length of 6 to 12 carbon atoms. This makes the coconut oil the richest source of MCFA among vegetable oils and gives it a uniqueness among other dietary fats since the majority of fats in the human diet are composed almost entirely of long chain fatty acids (LCFA). Despite being highly saturated, the oil has a relatively low melting point since it contains mainly short- and medium-chain fatty acids (Bhatnagar et al., 2009, Krishna et al., 2010).

Fatty acid composition

The major saturated fatty acids are lauric and myristic acids respectively. The high saturated fattyacid content causes the oil to have a sharp melting property (Refer to table 1.5). A sharp melting point is an advantage when formulating food products that are expected to melt quickly in the mouth. Other fats tends to exhibits a gradual softening with an increase in temperature (Gunstone, 2011).

Table 1.5: Fatty acid composition of coconut oil

Name of fatty acid	Percentage	Type of fat
Lauric acid	45% to 52%	Saturated fat
Myristic acid	16% to 21%	Saturated fat
Caprylic acid	5% to 10%	Saturated fat
Capric acid	4% to 8%	Saturated fat
Caproic acid	0.5% to 1%	Saturated fat
Palmitic acid	7% to 10%	Saturated fat
Oleic acid	5% to 8%	Monounsaturated fat
Palmitoleic acid	in traces	Saturated fat
Linoleic acid	1% to 3%	Polyunsaturated fat
Linolenic acid	up to 0.2%	Unsaturated fat
Stearic acid	2% to 4%	Saturated fat

Source: (Amri, 2011).

Phenolic compounds of virgin coconut oil

The level of phenolic compounds found in VCO is around 400 to 800 μg/g. This level is comparable to the phenolic compounds found in virgin olive oil (Lockyer and Stanner, 2016), which is known to be responsible for many health promoting benefits of virgin coconut oil (Marina et al., 2009b). Some of the phenolic acids identified in VCO include gallic, fellic, catechin, chlorigenic, epicatechin, homovallin, vallin, rutin, p-coumaric, sinapic, and quercetin acids (Srivastava et al., 2016). Studies have suggested

that the contribution of antioxidant activity in VCO could be due to phenolic compounds. Natural phenolic compounds from plant-sources also play an important role in cancer prevention and treatment (Huang et al., 2009). Both hot extraction and cold extraction VCO are healthy versions of coconut oil than RCO (Srivastava et al., 2016). Chemical analysis have shown that the phenolic compounds in VCO triples that of RCO while the antioxidant activity in VCO is more than double the level of activity in RCO (Srivastava et al., 2016). VCO produced under hot extraction is superior to VCO produced under cold extraction due to higher amount of phenolic compounds and antioxidant activity recovered in VCO extracted under hot condition when compared with VCO extracted under cold condition (Seneviratne et al., 2009, Srivastava et al., 2016). The high temperature used in the hot extraction of coconut oil favoured the incorporation of more thermally stable phenolic antioxidants into coconut oil. It was hypothesized that the concentration of the phenolic substances increased when the water in the emulsion evaporated during the hot extraction of the oil (Marina et al., 2009b). However, according to the Codex standard (Codex, 2003), application of heat is permitted in producing virgin oil, but there is no definite temperature set as to how high the application of heat should be limited (Marina et al., 2009b).

Tocols in virgin coconut oil

The tocols (tocopherols and tocotrienols) are natural antioxidants present in vegetable oils and fats. Coconut oil generally contains low level of tocols, as expected for a highly saturated oil. The major tocol component is α-tocotrienol, ranging from 0–44 mg/kg, while the minor tocols are α-, β- and γ-tocopherols, ranging from 0–17 mg/kg, 0–11 mg/kg and 0–14 mg/kg respectively (Firestone, 2013). In addition to these minor tocols, Alimentarius (2009) includes γ-tocotrienol at 0–1 mg/kg. Tocols contribute to the oxidative stability of the oil. In comparison, animal fats have lower oxidative stability than vegetable oils since they contain only trace amount of tocols. Refining of crude coconut oil leads to a reduction of tocol content and consequently reduces the oxidative stability of the oil. Thus very low amount of the tocopherol obtained in all RCO samples in comparison to VCO samples (Srivastava et al., 2016). Addition of 50–200 ppm α-tocopherol does not increase the stability. It seems that the natural level of tocopherol in the oil is the optimum concentration for the oil stability. The oxidative stability can, however, be improved by adding citric acid to the refined oil as a chelating agent for trace metals (Gordon and Rahman, 1991). Recent studies have also shown that cold extracted VCO contains more tocopherol than the hot extracted VCO (Srivastava et al., 2016).

Sterols in virgin coconut oil

Sterols in vegetable oils are known as phytosterols, as opposed to zoosterols in animal fats. Phytosterols are the major part of the unsaponifiable fraction. They are beneficial to human health (Gunstone, 2013) because of their ability to reduce blood cholesterol levels. Phytosterols also help to reduce symptoms of an enlarge prostate, improve control of blood sugar among diabetics, reduce inflammation among patients

with autoimmune diseases such as rheumatoid arthritis and lupus (Carandang, 2008).The major sterols are campesterol, stigmasterol, β-sitosterol and Δ5-avenasterol, with mean values of 8.7%, 12.5%, 46.7% and 26.6% of total sterols respectively. The total sterol content is 807 mg/kg (Rossell, 2001, Alimentarius, 2009).

Other minor components in virgin coconut oil

Hydrocarbon and lactones are among the other minor components present in Coconut oil. The presence of a high level of hydrocarbon makes the oil unfit for human consumption. Contamination with petroleum products may occur during transport or through handling of the oil. Naturally, Coconut oil contains 7.7 mg/kg of hydrocarbon (*n*-alkanes), while crude petroleum and diesel oil contain 72,200 and 148,562 mg/kg respectively (Moffat et al., 1995). The levels in the mineral oils are of a different order of magnitude. Squalene and polycyclic aromatic hydrocarbon content in crude Coconut are 0.002% (20 ppm) and 0.30% (3000 ppm) respectively (De Greyt and Kellens, 2000, Gunstone, 2013). The pleasant odour and taste of coconut oil are largely due to δ- and γ-lactones, which are present in trace quantities (Young, 1983). γ-Valerolactone is considered to be responsible for the characteristic taste of coconut oil (Amri, 2011).

1.5.4 Pharmacological activity of virgin coconut oil

Antioxidant activity of virgin coconut oil

The antioxidant activity of VCO is due to the high composition of polyphenol compounds in the oil (Colo and Fife, 2003, Marina et al., 2009b, Srivastava et al., 2016). Marina et al. (2009a) estimated the total phenolic content of VCO to be in the range of 7.78–29.18 mg GAE/100 g oil, which is significantly higher than the refined, bleached, and deodorized coconut oil. The major polyphenols in VCO are ferulic acid and p-coumaric acid (Marina et al., 2009a). Seneviratne and Sudarshana Dissanayake (2008) also detected the presence of ferulic acid, p-coumaricacid, and caffeic acid in the commercial and traditional VCO. Polyphenols are stronger as antioxidants than vitamins C and E in vitro on the molar basis (Rice-Evans et al., 1997). The antioxidant properties of ferulic acid has been firmly established. Ferulic acid belongs to phenoxy carboxylic acid family (Graf, 1992). Toda et al. (1991) have proven that ferulic acid has the ability to scavenge the superoxide radical and suppress the lipid peroxidation induced by superoxide anion. The effects of ferulic acid and superoxide dismutase as antioxidants were equal in magnitude, and this characteristic made it superior to caffeic acid and p-coumaricacid as an antioxidant (Toda et al., 1991). In addition, the effect of ferulic acid as inhibitor of lipid peroxidation was similar to the effect of α-tocopherol (Toda et al., 1991). Castelluccio et al. (1996) reported that ferulic acid was more potent as an antioxidant against LDL oxidation than ascorbic acid. It seems that VCO derives most of its effects from the free-radical scavenging and antioxidant properties of ferulic acid. The antioxidant power of ferulic acid is due to its ability to effectively end the terminal radical chain reactions, since any

free radical colliding with ferulic acid molecule can easily extract a hydrogen atom from the phenolic hydroxyl group to form a phenoxy radical which is considered a highly stable compound (Graf, 1992). This phenoxy radical is unable to initiate or propagate the reactive chain reaction. This stability belongs to easy formation and lack of reactivity of phenoxy radical. Moreover, there is extended conjugation in the unsaturated side chain of phenoxy radical, and the unpaired electron may not be attached to oxygen atom, but it can move throughout the entire molecule (Graf, 1992). Sassa et al. (2003) reported that ferulic acid enhanced bone remodeling process by stimulating osteoblasts to compensate for the bone loss by osteoclasts. Ferulic acid also raised serum level of estrogen and progesterone in postmenopausal osteoporotic rat model. Zych et al. (2009) showed that caffeic acid and p-coumaricacid increase serum estrogen levels in estrogen deficiency rat model. The authors suggested that phenolic acids such as caffeic acid may affect the metabolic pathway which regulates the extra-ovarian estrogen release (Zych et al., 2009). (Folwarczna et al., 2009) investigated the effects of phenolic acids on bone loss in postmenopausal osteoporotic rat model, and they reported that p-coumaric acid had a positive effect on the bone mass/body mass ratio and bone mineral mass/body-mass in bone.

VCO alleviates hyperglycemia and improves glucose tolerance probably by its antioxidant effect which consequently leads to improvement of insulin secretion (Iranloye et al., 2013). Virgin coconut oil also attenuates acute chemotherapy hepatotoxicity induced by anticancer drug methotrexate via inhibition of oxidative stress in rats (Famurewa et al., 2017). It effectively lowered alcohol-induced oxidative stress by reducing testicular malondialdehyde levels and ameliorated the deleterious effect of alcohol on serum testosterone level, but showed no effect on serum FSH and LH levels (Dosumu et al., 2010).

Increases digestion and absorption of food

Most of the fatty acids in the diet are long-chain fatty acids (LCFA) whereas coconut oil is predominantly rich in medium-chain fatty acids (MCFA). They are low molecular weight compounds and are highly soluble in biological fluids. These properties make them unique. Medium chain fatty acids are metabolized differently compared to LCFA (Fushiki et al., 1995). They are absorbed directly into the portal circulation without re-esterification in intestinal cells (Ferreira et al., 2014). The MCFA are partly independent of the carnitine transport mechanism into the mitochondria of the liver and are rapidly oxidized for the production of energy (Rubin et al., 2000).

In contrast, the long-chain fatty acid (LCFA) commonly found in most diets are incorporated into chylomicrons after being absorbed in the intestine where they are subjected to re-esterification and then reach the bloodstream via the lymphatic system (Ferreira et al., 2014). Most LCFA are stored in the adipose tissue (Rego Costa et al., 2012). As a result, coconut oil is used in special food preparations for those who suffer digestive disorders and have trouble in digesting fats (Hoagland and Snider, 1943). Medium-chain fatty acids also improve the absorption of many other nutrients. The absorption of

minerals (particularly calcium and magnesium), B vitamins, fat soluble vitamins (A, D, E, K and beta-carotene) and also amino acids has been found to increase when infants are fed a diet containing MCFA. In addition, coconut oil can be digested by the salivary lipase, getting absorbed very fast to give energy like carbohydrates (Hegde, 2006) . All other fats need the pancreatic lipase for digestion that the infants do not have (Armand et al., 1996).

Cardiovascular activity of virgin coconut oil

Cardiovascular diseases (CVD) are one of the most common diseases in different parts of the world especially in developing countries (Yusuf et al., 2001). Earlier it was believed that as coconut oil contains a high amount of saturated fats, it initiates a rise in blood cholesterol levels and promotes heart disease (Kannel and Dawber, 1972, Ulbricht and Southgate, 1991). Later, Kurup and Rajmohan (1994) conducted a study on 64 volunteers and found no statistically significant alteration in the serum total cholesterol or LDL cholesterol from baseline values. Kaunitz and Dayrit (1992) reviewed epidemiological and experimental data regarding coconut-eating people and noted that the population studies show that dietary coconut oil does not lead to high serum cholesterol or to high coronary heart disease mortality or morbidity. The research over four decades concerning the benefits of coconut oil in controlling heart disease is quite clear; coconut oil has been shown to be beneficial (Babu et al., 2014, Schilling, 2016). The saturated fats in most hydrogenated oils are of a far worse kind. These are long chain triglycerides that the body cannot break down as efficiently as medium chains fatty acids. This means that they build up as fatty deposits around heart and arteries increasing the risk of coronary heart disease (Schilling, 2016). Replacing these oils with coconut oil therefore actually decreases the risk of heart disease (Tsuji et al., 2001, Nagaraju and Belur, 2008). Finally researches concluded that natural coconut oil significantly increases HDL (good cholesterol) which promotes healthy heart but the use of hydrogenated coconut oil may increase LDL (low density lipids, 'bad cholesterol') (Harris et al., 1993, Dayrit, 2003).

Neuroprotective activity of virgin coconut oil

The brain is the functionally and metabolically active organ of the body (Fernstrom, 2000). When the blood glucose levels fall, the brain requires an alternative source of fuel instead of glucose for its function. Ketones are high-energy fuel produced in the liver, specifically to nourish the brain (Fife, 2013b). A common feature found in Alzheimer's disease and many other neurological disorders is chronic inflammation which interferes with normal glucose metabolism (De Felice and Ferreira, 2014). This defect in energy conversion starves the brain cells causing them to degenerate and die. Therefore, if enough ketones are available on a continual basis, they could satisfy the brain's energy needs. However, ketones are only produced when food, particularly carbohydrate, consumption is very low. When coconut oil is consumed, a portion of the MCFA is automatically converted into ketones, which in turn, enhance the functioning of the brain (Fife, 2013b). Case histories of Alzheimer's patients receiving coconut oil

have demonstrated that it is possible not only to stop the progression of the disease, but also to bring about significant improvement (Swaminathan and Jicha, 2014, Fernando et al., 2015).

Immunological activity of virgin coconut oil

Maintaining a healthy diet, results in a well-balanced immune system (Hughes, 2001). Including unsaturated oils in the diet disturbs the smooth functioning of the immune system (Lunn and Theobald, 2006). Here, antioxidants are frequently used up to remove free radicals produced by these oils which ultimately slowdown the immune system. In the case of natural coconut oil, saturated fatty acids are stable and do not oxidize easily, which in turn, reduce the use of antioxidants. Caprylic and Capric fatty acids in coconut oil, when broken down, are converted into specific antibodies that enhance the body‘s defenses against a range of diseases, both bacterial and viral in nature. Having the right antibodies to fight specific bacteria is central to body‘s well-being.

Hence, including coconut oil in the diet is an easy way to ensure good health. Consumption of coconut oil enhances the metabolic rate, which in turn, accelerates healing processes, cell regeneration and smooth functioning of the immune system (Fife, 2000). When applied to infected areas, coconut oil forms a chemical layer that protects the infected body part from external dust, air, fungi, bacteria and viruses. Coconut oil is highly effective on bruises because it speeds up the healing process of damaged tissues. Coconut oil has demonstrated anti-inflammatory, analgesic and fever reducing properties. This plays a good role in removal of toxins from cells. It is thought to curb inflammation of cells by improving cellular function. The cells become more efficient in removing toxins. According to Fife (2013b), Coconut Cures lists fifteen toxins neutralized in part or whole by coconut oil. These include aflatoxin, *E. coli* endotoxin, and MSG. Thus in many different aspects coconut oil supports the immune system and is an ideal food for immune suppressed individuals.

Antimicrobial activity of virgin coconut oil

Major saturated fatty acids in coconut oil include 48% lauric acid (an 12 chain saturated fat), 7% capric acid (an 10 chain saturated fat), 8% caprylic acid (an 8 chain saturated fat) and 5% caproic acid (an 6 chain saturated fat) which enhances the antimicrobial properties of coconut oil. Several reports state that these saturated fatty acids and their monoglycerides kill or inactivate microorganisms by lysing the phospholipid bilayer of plasma membrane, interfering in signal transductions, and reduces virus reproduction. (Kabara, 1978, Hierholzer and Kabara, 1982). Monolaurin, the monoglyceride of lauric acid, possesses antiviral and antibacterial properties. They kill the enveloped bacteria and viruses by solubilizing their phospholipid bilayers. Evidences suggest that MCFA are effective in destroying bacteria like *Staphylococcus, Helicobacter Pylori, Chalamydia trachomatis, Streptococcus, Neisseria* etc., fungi like Candida and yeast, protozoans like giardia, viruses including herpes virus, influenza, Epstein-Barr virus, hepatitis C virus, human immunodeficiency virus(HIV), and others (Isaacs et al., 1992).

Antidiabetic potential of virgin coconut oil

Coconut oil provides a good protection against insulin resistance, a major cause of diabetics (Adekola et al., 2017). Medium chain fatty acids in coconut oil put less of a demand on the enzyme production of the pancreas. This lessens the stress on the pancreas during meal time when insulin is produced most heavily, thus allowing the organ to function efficiently (Nagaraj et al., 2016). MCFAs in coconut oil have a greater ability compared to other oils in developing binding affinity between insulin and cells which improves the secretion of insulin, which in turn, controls blood sugar (Yost et al., 1998). It also helps in effective utilization of blood glucose.

Anticancer activity of virgin coconut oil

Coconut oil resists cancer and tumors to a greater extent compared to other unsaturated oils (Hayatullina et al., 2012). The protective effect of coconut oil against chemically induced colon and breast cancer is more profound than that of other oils (Law et al., 2014). They inhibit the carcinogenic agents of colon and mammary glands. Lim-Sylianco (1987) published a 50-year literature review showing the anti-cancer effects of coconut oil. Growing evidences show that cancer is a metabolic disease characterized by cellular mitochondrial respiratory insufficiency. Cancer cells can only survive and thrive off glucose and amino acid fermentation. A ketogenic cleanse has been proposed as a means of staving off cancer cell development (Seyfried, 2012). The ideal fuel source for the ketogenic cleanse is coconut oil and virgin coconut oil with its powerful immune boosting properties. The metabolism of fatty acids in coconut oil results in the liberation of ketones that normal cells can utilize but cancer cells cannot. These ketone bodies provide a great anti-inflammatory fuel source for the body that also starves the cancer cells from their nutrient demands.

Weight loss capacity of virgin coconut oil

Coconut oil is a low calorie fat and helps control body weight. In addition, coconut oil stimulates metabolism to get itself metabolized fast to supply quick energy unlike other fats. This also helps control body weight. Most of the coconut oil is medium chain fat, it gets absorbed and metabolized so fast that it rarely gets transported to fat depots like other fats, altering the lipoprotein fractions of blood– another great boon. Changing the food fat to coconut oil could help reduce weight in obese individuals (Assunçao et al., 2009). Dietary coconut oil also increases conjugated linoleic acid-induced body fat loss in mice independent of essential fatty acid deficiency (Hargrave et al., 2005).

1.6 Problem statement

The prevalence of alcohol consumption globally amongst people on antiretroviral (ARV) treatments remains disturbingly high (40-63%) (Miguez et al., 2003, Kekwaletswe and Morojele, 2014a). Studies across Sub-Saharan Africa have confirmed high consumption of alcohol amongst people on ARVs (Kekwaletswe and Morojele, 2014a, Soboka et al., 2014a). Globally, South Africa has the largest number

of people on antiretroviral treatment (UNAIDS, 2017), and a significant percentage (>40%) of these people have been reported to indulge in excessive alcohol consumption (Kekwaletswe and Morojele, 2014). Fertility and desire to procreate is becoming increasingly important amongst PLWHAs who once took the diagnosis to be a death sentence. HAART has transformed HIV from a terminal to a manageable chronic condition with approximately normal life expectancies. However, the effects of long term HAART on male fertility is recognized as a barrier to PLWHAs realizing their goals to raise normal healthy families.

We postulate that prolonged concomitant intake of HAART and alcohol - both of which have known testicular toxicity, is much more deleterious to the male reproductive system based on synergistic toxicity. As oxidative stress mechanism mediates most pathophysiological processes, finding readily available and effective antioxidants is a high priority in addressing this issue.

The use of traditional remedies in the form of complementary and alternative therapies has been widely embraced across Africa and many developed countries in the world (Braun et al., 2010a). Herbal medicines and products seem to provide a better alternative to synthetic pharmaceuticals (Shah, 2007a). They are generally less liable to cause acute toxic effects, locally accessible and cost effective (Jaarin et al., 2014a). The herbal adjuvant virgin coconut oil, has been reported to have a high antioxidant potential (Nevin and Rajamohan, 2006) which could ameliorate the amplified toxicity of HAART and alcohol on the testis.

To date, there has been no report either in humans or in animal experimental models to demonstrate the possible effect of virgin coconut oil on the concomitant use of HAART and alcohol on the testis.

1.7 Justification for the study

As antiretroviral therapy becomes increasingly accessible, the estimated life expectancy of PLWHAs within the reproductive age has increased dramatically with significantly improved quality of life leading to an increase in reproductive desire among this population (May et al., 2011, Azu, 2012). The long term use of HAART has detrimental effects on the male reproductive system, which could in turn result in male infertility (Pavili et al., 2010b, Ahmad et al., 2011a, Jegede et al., 2017). The risks involved in drug toxicities continue to attract attention with animal models playing a major role in the evaluation of therapeutic efficacy and safety (Mak et al., 2014). This is so even-though animal models may not always predict human responses to drug toxicities, as they are limited in their ability to mimic the extremely complex process involved in human pharmacokinetics and pharmacodynamics of xenobiotics (Perel et al., 2007, Mak et al., 2014). Although 85% of early clinical trials for novel drugs fail despite successful pre-clinical animal testing, with only half surviving to phase III with consequent clinical approval (Arrowsmith, 2011, Ledford, 2011), it is still crucial to conduct extensive preclinical drug-testing with animal models to examine the preliminary efficacy, toxicity and pharmacokinetics before human trials

(Mak et al., 2014). In this study, the rat serve as a suitable animal model for investigating the mammalian testes based on its similar structure and response to key physiological stimuli. They are also cost effective and easier to handle (Vandamme, 2014, Goutianos et al., 2015).

Currently the most specialized form of treatment of infertility is through the assisted reproductive techniques such as *in vitro* fertilization (IVF), however its accessibility and affordability is a major challenge in many developing countries across the globe (Akande, 2008, Inhorn and Patrizio, 2015). Hence the need to ensure maximizing normal fertility with this study focusing on the male.

The use of HAART cocktails predisposes PLWHAs to several potentially serious conditions resulting in organ damage. With the testis commonly being a target organ due to high rates of cell division, hypofunction is expected to result in lowered male fertility (Vernazza, 2008, Azu et al., 2014, Kaushik et al., 2014). The associated toxicities of HAART necessitate the need for safe and less costly adjuvants that can mitigate these effects. Plant based extracts have the potential to fill this need. An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used rural herbal remedies (Dasture, 2002, Singh, 2015). There is increasing demands for the use of medicinal plants on the basis of easy accessibility, affordability, and reduced risk of toxic effects. Medicinal plants provide better alternatives to synthetic pharmaceuticals (Gilani., 2017) they contain biologically active components such as phenolic compounds. Anecodatal evidence has thus far shown VCO to be efficacious against a variety of diseases and as an immune boosters for PLWHAs. However, the scientific validation of these claims remains to be verified.

1.8 Research questions

1. Does VCO possesses antioxidant properties?
2. Can the known toxicities of HAART or alcohol on semen quality, testicular histo-morphology and morphometry and testicular ultrastructural changes be mitigated by the plant based adjuvant VCO?
3. Does ethanol potentiate HAART toxicity in the testis?
4. What are the effects of VCO on the testes following use of alcohol with HAART?
5. Does VCO affect male fertility potential as evidence by reproductive hormonal and biochemical changes in the context of ethanol with HAART use?

1.9 Aims

This study aims to investigate the possible ameliorative adjuvant potential of VCO on the testis of Sprague-Dawley rats following the co-administration of alcohol with HAART.

1.10 Objectives

1. Evaluate the effects of VCO on the testicular histo-morphology using special histochemical staining techniques.
2. Investigate the effects of VCO on sperm quality (sperm counts, sperm motility and morphology) following the co-administration of alcohol and HAART by direct observation using high power light microscopy.
3. Using stereology to objectively quantify the effects of alcohol and HAART co-treatment on testicular structure following adjuvant treatment with VCO by determining the following parameters:
    - Seminiferous tubular diameter and area
    - Germinal epithelial thickness
    - Volume density and absolute volume of the germinal epithelium, lumen and interstitium
4. To investigate using Transmission Electron microscopy (TEM) the testicular ultrastructure, tubular basement membrane, Leydig cell diameter and nuclear volume in the protocol.
5. To measure the concentration of reproductive hormones and biochemical markers using ELISA kits and standard laboratory analytical methods
    - Serum testosterone
    - Serum luteinizing hormone
    - Serum follicle stimulating hormone
    - Testicular tissue malondialdehyde
    - Testicular tissue reduced glutathione

Materials and Method

1.10.1 Chemicals/drugs

The drugs Zidovudine, Lamivudine, and Nevirapine (Aspen) were procured from Pharmed, (Durban, South Africa) with Batch numbers A844552, A847223 and A849377 respectively. Absolute Ethanol (99%) was procured from LABOQUIP (Johannesburg, South Africa - Batch 15/082).

1.10.2 Collection of plant material

The solid endosperm of mature coconuts were commercially purchased from Kies Supermarket, a local store in Reservoir Hills Street, Durban between September to October 2015, and June to July 2016. The identification was authenticated through the WARD Herbarium at the School of Life Sciences on the Westville Campus, University of KwaZulu-Natal, Durban, South Africa.

1.10.3 Extraction of virgin coconut oil

The wet extraction method described by Nevin and Rajamohan (2006) was used for VCO extraction. Solid mature coconuts were crushed and made into viscous slurry. 500 mls water was added to thin down slurry and squeezed through a cheese cloth to obtain the coconut milk. The coconut milk produced was left for 24 hours to aid gravitational separation in accordance with Onsaard et al. (2005b), (Nour et al., 2009b). Three phases resulted; a lower aqueous phase, a middle emulsion phase, and an upper oily phase. The upper oily phase was then removed and heated at 60-70^{0}C for 10-15 minutes to remove visible water and as much moisture as possible. The resultant pure VCO was then filtered through a fine metallic sieve to remove any residual fine particulates and stored in plain bottles for 2 weeks at room temperature ready for use.

1.10.4 Animals

A total of sixty animals was used for the experiment. The animals were bred and maintained at the Animal House of the Biomedical Resources Unit, University of KwaZulu-Natal. All procedures involving the animals was 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 (Council, 2010). The protocol for the study was approved by Animal Ethics Committee (protocol reference number: **AREC/087/015D**). The rats had unrestricted access to food (standard rat pellets) and water. All the rats were housed in plastic cages (3 rats/cage) having dimensions of 30 cm long, 20 cm wide and 13 cm high) and soft wood shavings employed as bedding in the cages. Rats were maintained under standardized animal house conditions (temperature: 28~31^{o}C; light: approximately 12 hr. natural light per day; humidity: 50~55%).

1.10.5 Experimental design

The study was divided into two segments as Phase 1 and Phase 2. Phase 1 was designed to determine the role of VCO on the testis following treatment with HAART. Phase 2 focuses on the role of VCO on the testicular interactions of alcohol under HAART regime.

1.10.5.1 Phase 1

A total of twenty (20) adult male Sprague Dawley rats weighing between 153g to 169g were used for this phase. The animals were randomly distributed into four (4) groups (A-D) and treated as follows:

- Group A received distilled water as control
- Group B received HAART cocktail (Zidovudine, Lamivudine, and Nevirapine) using human therapeutic equivalent doses (600, 300 and 400 mg/day respectively) which was dissolved in 100 ml of distilled water and adjusted to the equivalent animal dose of 1.89, 0.95 and 1.26 mg/kg body weight respectively (Umar et al., 2008).

- Group C received HAART cocktail and VCO (10 mL/kg).
- Group D received VCO (10 mL/kg)

All treatments were applied daily by oro-gastric gavage and at the end of 8 weeks for phases I & II, all animals were killed 24 hours after the last treatment under halothane® anesthesia.

1.10.5.2 Phase 2

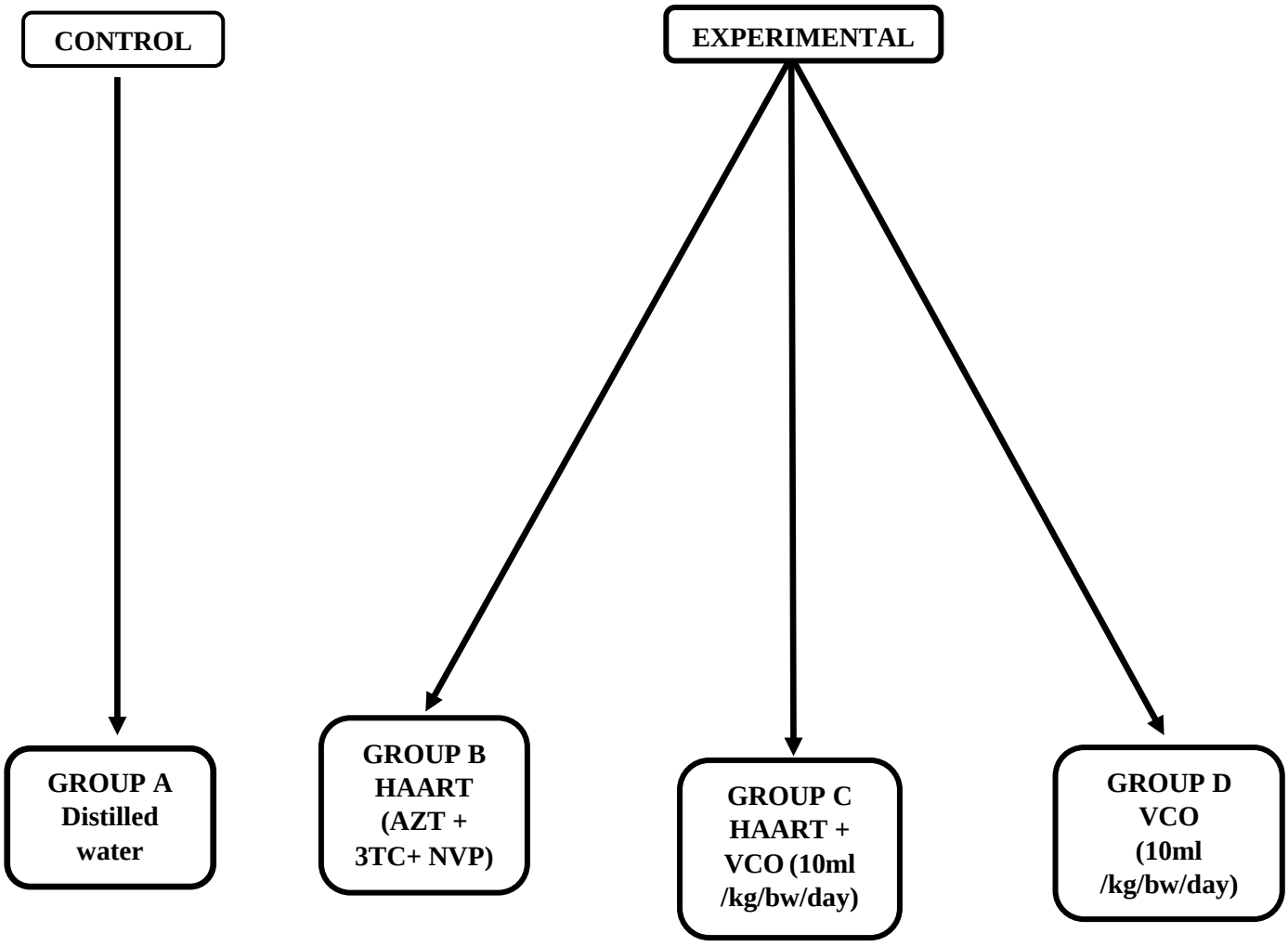
A total number of forty (40) adult male Sprague-Dawley rats weighing between 165g to 176g were used for this phase. The animals were randomly distributed into eight (8) groups (A-H) of five (5) rats per group. The animals were randomly distributed into eight (8) groups (A-H) of five (5) rats per group as indicated below. Animals received treatments 5 days in a week, with 2 days off for the entire 8 weeks study period.

- Group A received only distilled water as control animals
- Group B received only Ethanol (5ml/kg BW of 20% w/v) (Erukainure et al., 2011a)
- Group C received Ethanol (5ml/kg BW of 20% w/v) and HAART cocktail
- Group D received Ethanol (5ml/kg BW of 20% w/v) and HAART cocktail (Reversal group)
- Group E received Ethanol (5ml/kg BW of 20% w/v) and HAART + VCO_1 (2.5 ml/kg body weight)
- Group F received Ethanol (5ml/kg BW of 20% w/v) + HAART cocktail + VCO_2 (5 ml/kg body weight)
- Group G received Ethanol (5ml/kg BW of 20% w/v) + VCO_1 (2.5 ml/kg body weight)
- Group H received Ethanol (5ml/kg BW of 20% w/v) + VCO_2 (5 ml/kg body weight)

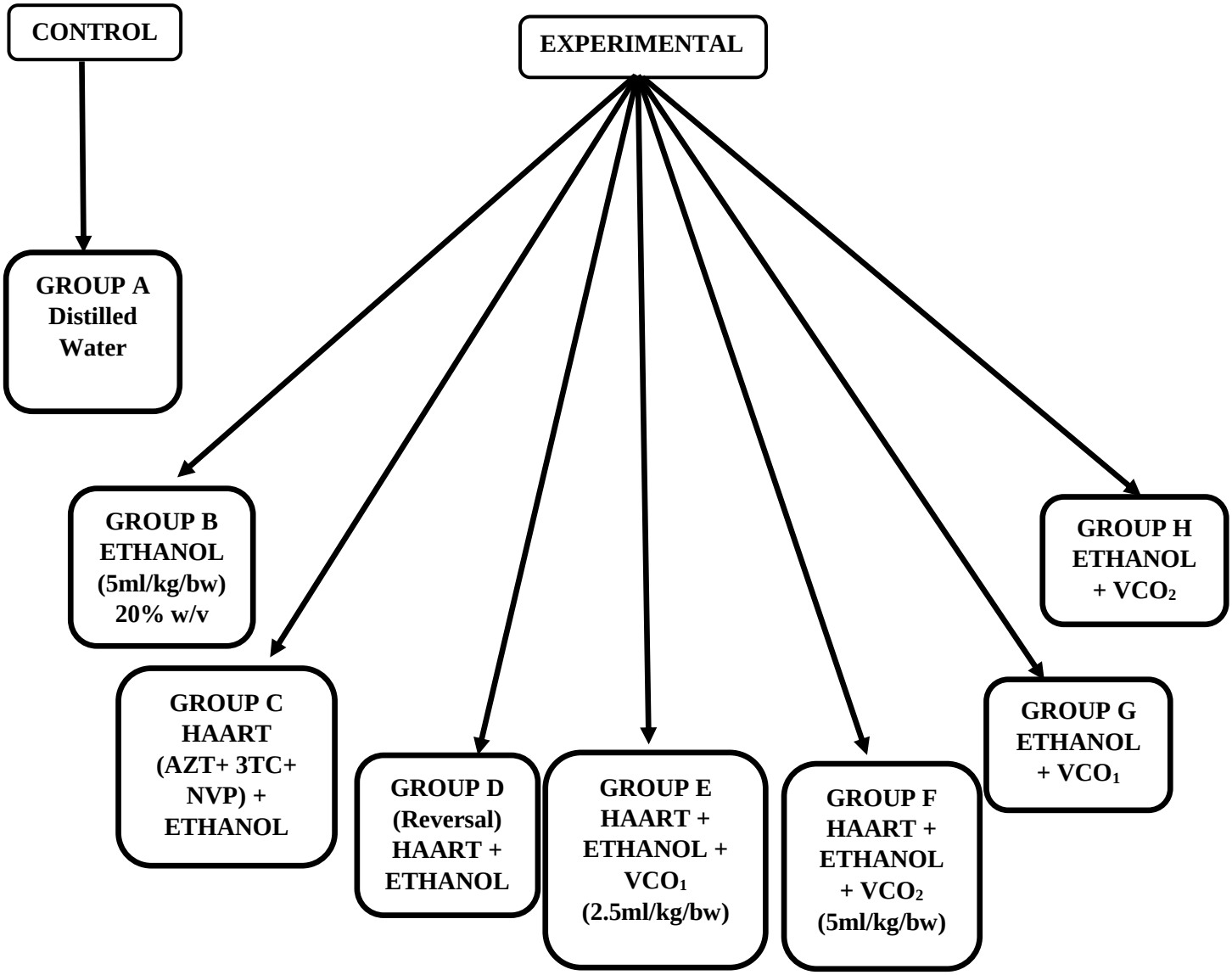
Treatment was administered by oro-gastric gavage. At the end of 56 days the animals were killed 24 hours after the last treatment under halothane® anesthesia except for Group D animals which served as the reversal group. In this group HAART and Ethanol were discontinued to ascertain whether their effects were reversible when withdrawn. These animals were killed on day 84 following withdrawal of all treatment for 4 weeks.

EXPERIMENTAL DESIGN

PHASE 1



PHASE 2


CONTROL
EXPERIMENTAL
GROUP A
Distilled
Water
GROUP B
ETHANOL
(5ml/kg/bw)
20% w/v
GROUP C
HAART
(AZT+ 3TC+
NVP) +
ETHANOL
GROUP D
(Reversal)
HAART +
ETHANOL
GROUP E
HAART +
ETHANOL +
VCO1
(2.5ml/kg/bw)
GROUP F
HAART +
ETHANOL
+ VCO2
(5ml/kg/bw)
GROUP G
ETHANOL
+ VCO1
GROUP H
ETHANOL
+ VCO2

1.10.6 Body and testicular weight

The body weights (BW) of animals were recorded on the first day before treatment (initial) and thereafter on a weekly basis until the day of sacrifice. Testicular weight (TW) was measured by an electronic balance (Mettler Toledo; Microsep (Pty) Ltd, Greifensee, Switzerland). The testes of each rat were measured individually, and the average value obtained for each of the two measurements was regarded as one observation. The values were expressed in grams (g) for TW.

1.10.7 Semen analysis

The caudal epididymis of one testis were excised and minced with an anatomical scissors. A drop of epididymal fluid was placed onto a glass slide, covered with 22×22 *mm* cover slip and immediately examined under a light microscope (Leica DM500, CH-9435 Heerbrugg, Switzerland) (Organization, 1999). The total field was scanned systematically and spermatozoa motility assessed (Rizk and Sallam, 2012) . Motility was graded as progressive, non-progressive and immotile (Vasan, 2011b). The percentage of motile sperm was then estimated and reported to the nearest 5% using a subjective determination of motility (Keel and Webster, 1990).

A portion of the macerated caudal epididymis was diluted with normal saline (1:9) and sperm count determined using Biorad® automated cell counter 1450101TC 20TM with a double slide counting chamber. The dilution was mixed thoroughly and both sides of the counting chamber were scored and the average of the two counts were taken and sperm count expressed in millions/ml (Keel and Webster, 1990).

1.10.8 Collection of blood samples

At the end of the experimental period, all animals were sacrificed by exposure to excess halothane in a gas anaesthetic chamber. Blood samples were collected by cardiac puncture into pre-cooled heparinized tubes and serum bottles, placed on ice for 3 hours 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.

1.10.9 Histopathological examination of the testes

The testes were removed and weighed. The left testis was used for biochemical analyses and the right testis fixed in 10% Neutral buffered formalin and processed for histology. For routine histology, the testis tissues was subject to systematic serial sectioning at 4µm intervals using a microtome (Microm HM 315, Germany) and stained with hematoxylin and eosin (H&E). Examination was done by a histopathologist blinded to the study protocol.

1.10.10 Histochemical examination of the testes

For histochemical studies, testicular tissues were stained with Periodic acid schiff (PAS) technique for the detection of glycogen, neutral polysaccharides and basement membrane; Gordon and Sweet's silver staining method to demonstrate reticular fibers (Bancroft and Gamble, 2008). Examination of the stained tissue sections was done by a histopathologist blinded to the study protocol

1.10.11 Testicular ultrastructure

This procedure was carried out as previously described by Trindade et al. (2013) and Hashish (2015). The testes were removed and sliced into one cubic millimetre segments. The segments were fixed in 2.5% glutaraldehyde and post-fixed in 0.5% osmium tetroxide (OsO_4), washed with 0.1M sodium cacodylate buffer (PH 7.2), dehydrated in graded series of acetone (30%, 50%, 75% and- 100%), infiltrated with propylene oxide-resin and embedded in Spurr resin. Following polymerization, five semi-thin sections (1 µm thick) of each animal were cut using Leica Ultracut R Ultramicrotome (Reichert, Austria) and stained with 1% toluidine blue for light microscopy. Ultrathin sections (50-70nm thick) were cut on the cutting edge of glass knives using Leica EM UC7 Ultramicrotome (Austria), mounted on copper grids and stained with uranyl acetate and lead citrate (Hayat, 1989). The ultrastructural analyses of each section were examined under JEOL JEM-1010 transmission electron microscopy (TEM) at magnification 1000 to 20,000nm in the Microscopic and Microanalysis Unit (MMU) of the University of KwaZulu-Natal, Westville Campus

1.10.12 Morphometric analyses of the testes

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 of the seminiferous tubules (diameter and cross-sectional area) was determined using systematic random sampling (Gundersen and Jensen, 1987) that ensured fair distribution between the polar and equatorial regions of each testis. The 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 was to eliminate longitudinal profiles which might exhibit different degrees of damage along their length and show irregular shrinkage as previously reported (Christensen and Peacock, 1980, Gundersen and Jensen, 1987). The tubules were scanned using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at X 100 magnification using image analyzer Leica (DMLB) and Leica microsystem software. The diameter of the seminiferous tubule was measured across the minor and major axes, and the mean diameter obtained. Cross-sectional area (Ac) of the seminiferous tubules was then calculated from

the formula; $A_C = \pi D^2/4$ (where π is equivalent to 3.142 and D is the mean diameter of the seminiferous tubules).

1.10.13 Stereological analyses of the testes

The procedure was carried out by point counting method in order to calculate the volumes of the germinal epithelium (GE), lumina (L) and testicular interstitium (I). This was done in accordance with the method previously described by Weibel (1979) and Howard and Reed (2004). Four sections per testis and six fields per section were randomly chosen for analysis. Fields were sampled from images captured using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at X 100 magnification using image analyzer Leica (DMLB) and Leica microsystem software.

Volume densities of testicular components (GE, L and I) were determined by randomly superimposing a transparent grid comprising 160 test points per image arranged in a quadratic array (Freitas et al., 2011b). The ratio of the number of point intersections on the grid overlying each tissue component (P_N) to the total number of the points of the grid (P_T) was considered as the volume density (V_V) of the component, with the formula:

$V_V = P_N / P_T$ (Bielli et al., 2001a)

V_V values for GE, L and I were multiplied by 100 and expressed in percentages (%).

The absolute volume (AV) of each testicular components was evaluated based on the previous method of Howard and Reed (2004) with modification. This was obtained by multiplying the corresponding volume densities with the testicular weight (TW). Values were expressed in ml.

1.10.14 Measurement of the testicular tubular basement membrane

Evaluation of the basement membrane thickness was carried out as previously described by Pop et al. (2011) and Shokri et al. (2012). A total of ten seminiferous tubules were randomly selected using the same magnification with 5 random measurements performed on each of the tubules.

1.10.15 Measurement of Leydig cell diameter and nuclear volume

A total of 10 Leydig cell nuclear diameters were measured per animal. The volume of Leydig cell nucleus was calculated using two different methods based the shape of Leydig cell nucleus. The first method was used for the measurement of rounded nuclei using the formula:

$V= 4/3\pi r^3$ (Castro et al., 2002)

The second method was for the measurement of oval nuclei using the revolution of spheroid formula:

$V= P/6\ AP^2$

Where P=short diameter and A=long diameter (Lewiński et al., 1984, El-Sokkary, 2001). Measurements of the basement membrane thickness and Leydig cell nuclei was carried out using iTEM 5.0 Universal TEM Imaging Platform (Olympus Soft Imaging Solutions, Münster, Germany).

1.10.16 Assessment of reproductive hormonal and biochemical parameters

1.10.16.1 Serum analysis of free testosterone, luteinizing and follicle stimulating hormonal levels

Blood was collected from the heart and allowed to clot for 2 hours at room temperature. It was then centrifuged for 15 minutes at (1000xg). The collected supernatant (serum) was kept in the deep freezer (-20^0C) and all hormone measurements done within 6 months. The free testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were analyzed by ELISA method using rat free-testosterone, rat luteinizing hormone and rat follicle stimulating hormone ELISA Kits (Catalog numbers: E-EL-R0389, E-EL-R0026 and E-EL-R0391 respectively - Elabscience Biotechnology, Wu Han, P, R, C., China).

1.10.16.2 Measurement of testicular tissue malondialdehyde level

The testicular tissue was homogenized in 0.2M sodium phosphate buffer (7.8 pH) and then centrifuged for 15 mins at 15000xg. The supernatant was collected and used for the measurement of lipid peroxidation. The procedure was carried out as described by Chenni et al. (2007) with slight modification. This procedure uses the complex formed from the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). Into an assay mixture containing 200µL of 8.1% sodium dodecylsulfate (SDS), 750 µL of 20% acetic acid (pH, 3.5), 2mL of 0.25% TBA and 850µL of distilled water, 200µL of sample of MDA standard series (0, 7.5, 15, 22.5, and 30 µM) was added in a Pyrex screw-capped test tube. The mixture was heated at 95^OC for 60min in a sand bath, cooled down to room temperature and the absorbance read at 532nm using spectrophotometer - Synergy HTX multi-mode reader, VACUTEC, USA. Thiobarbituric acid reactive substances (TBARS) concentrations in the samples were extrapolated from the MDA standard curve.

1.10 16.3 Measurement of testicular tissue glutathione concentration

Glutathione concentration was measured in tissue according to methods modified from Ellman (1959). The sample was first precipitated with 10% TCA and then centrifuged at 2000rpm for 10 min at 25^0C. Reaction mixture contained 100 µL of supernatant, 50 µL of 0.5mM DTNB and 150 µL of 0.2 M sodium phosphate buffer (pH 7.8). After 15 min incubation at 25^0C, the absorbance was measured at 412 nm using a spectrophotometer (Synergy HTX multi-mode reader, VACUTEC, USA) and GSH concentrations extrapolated from a standard GSH curve.

1.10.17 Statistical analysis

The morphometric and stereological data were analyzed using standard parametric tests. The results are expressed as means ± standard error of the mean. These were then subjected to one way analysis of variance (ANOVA) followed by Dunnett's and Tukey's multiple comparison tests performed using

GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). A value of $p<0.05$ was considered significant.

1.11 Publication outcomes

Summary of the manuscripts/Publication

Sn	Title	Journal	Remarks
1	Antiretroviral Therapy and Alcohol Interactions: X-raying Testicular and Seminal Parameters under the HAART Era	European Journal of Drug Metabolism and Pharmacokinetics (https://doi.org/10.1007/s13318-017-0438-6)	Published
2	Coconut Oil Extract Mitigates Testicular Injury Following Adjuvant Treatment with Antiretroviral Drugs	Toxicology Research (http://dx.doi.org/10.5487/TR.2016.32.4.317)	Published
3	Adjuvant Potential of Virgin Coconut Oil Extract on Antiretroviral Therapy-Induced Testicular Toxicity: An Ultrastructural Study	Andrologia Andrologia. 2017 Dec 12. doi: 10.1111/and.12930	Published
4	Virgin Coconut Oil Extract Mitigates Testicular Induced Toxicity of Alcohol Use in Antiretroviral Therapy	Andrology ANDR-2017-0447	Under review

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BRIDGING TEXT

FROM CHAPTER ONE TO TWO

The introduction and literature review from the previous chapter reveal the remarkable contribution of highly active antiretroviral therapy (HAART) towards the management of HIV/AIDs epidemic globally. However the success achieved is not without side effects which in many settings are not monitored. Antiretroviral agents are responsible for a wide range of toxicities, from low-grade intolerance that may be self-limiting, to life-threatening side-effects. The impact of alcohol in the context of HAART is warranted for several reasons as alcohol use is prevalent among HIV-positive individuals. Its frequent use poses serious health risk to the outcomes of HIV-treatment. Thus, the next chapter serves to highlight the current knowledge and pathobiology underlying the pathogenesis of alcohol and antiretrovirals (ARVs) focusing on the testicular and spermatogenic indices as markers of toxicity and adverse interactions as well as other biochemical parameters. We also intend to explore other potential gaps in knowledge that may be needful in addressing the main issues in HIV medicine.

CHAPTER TWO

MANUSCRIPT ONE

Antiretroviral Therapy and Alcohol Interactions: X-raying Testicular and Seminal Parameters under the HAART Era

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REVIEW ARTICLE

Antiretroviral Therapy and Alcohol Interactions: X-raying Testicular and Seminal Parameters Under the HAART Era

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Abstract The prevalence of alcohol use among HIV-infected patients undergoing antiretroviral (ARV) treatments has raised several concerns related to key therapeutic indices. These include drug interactions, compliance, efficacy and toxicity with the possibility of accelerated disease progression. Interaction of ARVs with alcohol can result in therapeutic failures or place patients at significant risk for toxicities. Research findings in this particular area are, however, limited and sometimes conflicting. This review focuses on alcohol and ARV interactions affecting testicular and spermatogenic indices. Antiretroviral drugs are known to negatively impact testicular functions via altered mitochondrial DNA and oxidative stress mechanisms. Interaction with alcohol can significantly affect seminal fluid concentration of ARVs. Habitual consumption of alcohol causes testicular hypofunction with potential for lowered fertility. Concomitant use of ARVs appears to act synergistically to exacerbate this toxicity. Alcohol also induces cytochrome P450 (CYPs) microsomal enzymes, which in turn affect ARVs metabolized by these enzymes. In the presence of ARVs with strong inhibitory activity, increased bioavailability with toxicities predominates. In addition, alcohol and ARVs have pronounced effects on membrane-associated drug transporters. Alcohol alters the properties of the lipid bilayer by changing membrane permeability and protein distribution. Since drug transporters critical to pharmacokinetics are integral membrane proteins, alcohol tends to diminish the activity of both the efflux and influx transporters. While excessive alcohol precipitates accelerated hypogonadism, future research needs to be directed to quantifying these effects of alcohol and ARVs in human testicular tissue.

Key Points
Induction or inhibition of drug metabolism and disposition can significantly affect the pharmacokinetics of ARVs and other co-administered drugs.
Alcohol disrupts ARV membrane transporter proteins altering its function.
Many ARVs have substantial side effects, which can be accentuated by concomitant use of alcohol.
The combined toxicities result in disproportionate harm to male reproductive health.

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1 Introduction

HIV/AIDS epidemic is one of the largest global health challenges of current times to date having claimed more than 35 million lives thus far [1]. Reports have shown that the Southern African region has the highest HIV

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prevalence in the world, accounting for one-third of the global burden of HIV [2]. Amidst several countries within this region, South Africa has the highest HIV prevalence [3–5]. Findings also show a high degree of alcohol abuse with high-risk sexual behavior patterns [6–8].

In the management of HIV/AIDS globally, highly active antiretroviral therapy (HAART) has been shown to be effective with the standard antiretroviral regimens consisting of three different drugs from at least two different classes [9]. These classes include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CCR5 antagonists) [10]. However, despite the success of ARVs in reducing morbidity and mortality in people living with HIV/AIDS (PLWHAs), there are still many adverse effects incurred from their chronic use [11].

Over the years, HIV patients are known to struggle with depression [12], with many resorting to chronic alcohol consumption to alleviate its symptoms [13]. Alcohol is a known psychoactive drug believed to act as the "most effective antidepressant for the moment," [14]. The resulting elevated mood, however, is short-lived after which its depressant effects exacerbate the underlying depression. This, in turn, leads to further intake of alcohol, thus perpetuating a vicious cycle [15]. According to WHO (2016), South Africa is the fifth largest 'drinking nation' in Africa [16]. South Africa has previously been rated as one of the nations with the most risky drinking patterns in the world with of score of 4 out of 5 (1 being the least risky pattern of drinking, and 5 the most) [17]. Reported data from the South African national surveys have shown that approximately one-third of the population engage in heavy episodic bouts of drinking [18–20] with a total alcohol consumption of 11.2 L of pure alcohol per capita in subjects over 15 years of age. This therefore makes it by definition one of the biggest drinking nations in the world [16]. The definition of a 'heavy drinking nation' is having a high prevalence with consumption of at least 60 g or more of alcoholic drinks on at least a single occasion within a 30-day period. This is referred to as heavy episodic drinking [21–23]. Furthermore, the high consumption of alcohol in South Africa is closely related to the high risk of contracting HIV [7, 20, 24]. This corresponds to similar patterns globally [22, 25–27]. This may contribute to an understanding of why South Africa has the highest HIV prevalence in the world [28].

Alcohol consumption has been reported to be more frequent among HIV patients with the prevalence of heavy and mild-to-moderate drinking rates approximately 2–2.5 times higher, respectively, when compared with the general population [24, 29]. Though several reports have demonstrated that alcohol consumption contributes to poor ARV adherence among HIV-infected patients, some of this poor compliance may be the result of unintentional non-adherence related to intoxication or attributed to patients' belief of interactive toxicity of alcohol and ARVs [30, 31]. Despite this, the use of alcohol with ARVs has been investigated and shows significant usage. Studies from the USA, France, Ethiopia, and South Africa have shown a prevalence of alcohol consumption among patients undergoing ARV treatment as 63, 84, 36, and 40%, respectively [32–35].

The long-term use of antiretroviral drugs can range from several potentially fatal conditions such as mitochondrial dysfunction and oxidative stress damage to lesser effects such as alluded to below, leading to detrimental effects on the male reproductive system [36–39]. The habitual or chronic consumption of alcohol can adversely influence male sexual potency by deterioration of sperm functional parameters, decrease in sexual hormone levels leading to testicular hypofunction and overall organ damage resulting in lowered male fertility [40–43]. Studies have reported alcohol exacerbating the side effects of certain ARVs with several studies emphasizing the toxicities resulting from these drug–dietary interactions on the liver [44, 45]. Other studies focus on the decline of the immunological and virological responses [32, 46], which in some studies appear to be independent of ARVs acting rather through a direct action of alcohol [32, 47] (refer to Table 1). These drug–dietary habit interactions alter the bioavailability of ARVs with reduced antiretroviral efficacy and acceleration of disease progression [48].

This review therefore serves to critique the current state of knowledge of the underlying pathogenesis of alcohol and ARVs focusing on the testicular and spermatogenic indices as markers of toxicity and adverse interactions. An attempt is made to elucidate the putative pathways regarding steroidogenic and biochemical perturbations seen in the pathogenesis of alcohol and ARVs with regard to its toxic potentials on patients with HIV/AIDS.

2 Search Strategy

A systematic search of literature was conducted on PubMed and Google Scholar electronic databases. The articles and abstracts identified were available in English between the years 1970 and 2017. The following search terms were used with Boolean operators (OR, AND) combining them with: "alcohol AND antiretroviral adherence" OR "alcohol AND testis" OR "antiretroviral AND testis" OR "alcohol AND antiretroviral interactions" OR "alcohol use AND antiretroviral therapy". The identified literature was scrutinized based on potentially relevant

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Table 1 Experimental and clinical evidences linking alcohol with antiretroviral disposition and effect on spermatogenesis

Reference	Type of study	Outcomes	Possible effects on spermatogenesis
Donde [188]	Animal experimental study	Increased visceral adiposity, increased inflammation and greater hepatic neutrophil infiltration	Interference to lipid metabolism can impair male infertility [143]
Miguez et al. [32]	Cross-sectional study	Alcohol use significantly reduces CD4 counts and virological response on HIV patients undergoing antiretroviral therapy	Semen parameters correlate positively with CD4 counts; lower CD4 will negatively influences spermatogenesis [151]
Fabris et al. [189]		Alcohol intake has no significant effect on the virological response following antiretroviral treatments in HIV-infected individuals	May not impact spermatogenesis, because semen parameters correlate positively with CD4 counts [151]
Cooper and Cameron [44]	Retrospective cohort study	Excessive alcohol intake and antiretroviral drugs exacerbate liver damage (increase HCV RNA levels) in HCV and HIV co-infected patients	Chronic HCV infection, liver cirrhosis and other forms of liver diseases have a negative impact on semen parameters and reproductive hormones [190–192]
Míguez-Burbano et al. [193]	Longitudinal observational study	Alcohol was associated with thymus deterioration leading to a poorer viral immune response after antiretroviral initiation	Lower CD4 will negatively influence spermatogenesis [151]
McCance-Katz et al. [46]	Clinical trial	Blood alcohol concentration was reduced following antiretroviral administration. Alcohol intoxication was unaffected. No effect of alcohol on the pharmacokinetics of ritonavir or efavirenz	Low alcohol concentration will have less adverse effect on semen parameters [46]
McDowell et al. [194]	Clinical study	Alcohol consumption increases the bioavailability of abacavir, although the increase is statistically significant, but not clinically significant	Increased bioavailability is not likely to cause testicular damage [194]
Whitfield et al. [195]	Clinical study	Alcohol potentiates pancreatitis in HIV-infected individuals on azidothymidine/dideoxyinosine therapy	Pancreatitis has a negative impact on glucose metabolism [196]. Altered glucose metabolism can negatively influence spermatogenesis [132]
Samet et al. [197]	Cohort study	Heavy alcohol consumption does not lower CD4 counts in HIV-infected individual undergoing antiretroviral therapy	May have a positive impact on semen parameters due to stable CD4 counts [151]
Ghebremichael et al. [198],	Cohort study	Alcohol has no significant effect on CD4+ T cell counts, but of significance, it causes depression in patients undergoing antiretroviral therapy. However, depression negatively impacts CD4+ T cell counts regardless of antiretroviral use	Semen parameters correlate positively with CD4 counts. Lower CD4 counts negatively influence spermatogenesis [151]

HCV hepatitis C virus

titles and abstracts. The retrieved full-text articles were managed using EndNote reference manager version X7.7.1 (Clarivate Analytics, Philadelphia, PA, USA).

2.1 Eligibility Criteria

The following inclusion criteria were adopted for these review:

1. Original research articles published in peer-reviewed journal.
2. Studies showing the effects of alcohol and male reproductive health.
3. Selected studies showing the effects of antiretroviral drugs on male reproductive health.
4. Studies showing the interaction of alcohol or antiretroviral with cytochrome P450 enzymes and drug transporters.
5. Studies showing possible pharmacokinetic and pharmacodynamics interaction between alcohol and antiretroviral therapy.

2.2 Result of Literature Search

The literature search yielded a total of 466 hits which included articles, reports and books. After reading their titles and article abstracts, 261 were selected based on our inclusion criteria. Out of these 261, 150 were selected for full reading. We found only 92 meeting our inclusion

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criteria and were included in this review, while 55 were excluded as they did not meet the inclusion criteria.

3 Testicular Interaction of Alcohol and Antiretroviral Drugs in Relation to Cytochrome P450 and Other Enzymes

Absorption, distribution, metabolism and excretion are four pharmacokinetics stages used to characterize the bodies handling of any xenobiotic (drug) [49]. However, the effect of the drug on the body (pharmacodynamics) is related to its concentration at the target site [50]. CYPs are a family of isoenzymes that play a major role in the metabolism of ARVs as well as alcohol [48]. They are predominantly found in the microsomes of the endoplasmic reticulum and the mitochondria of the liver as well as many other organs throughout the body [51, 52]. Among the CYPs family is a major isoform CYP3A4, which is known to play a vital role in the metabolism of all PIs, NNRTI, some INSTI and FIs [29], with CYP3A4 now metabolizing both ARVs and alcohol [48, 53]. In addition, two major isoforms (CYP2E1 and CYP3A4) have proven involvement in alcohol-induced toxicities [54].

Several ARV drugs can either induce or inhibit CYP3A4 [55]. Alcohol, however, is a potent inducer of CYP3A4 [29]. Interaction of alcohol with CYP3A4 inducing ARVs may therefore result in increased metabolism with subsequent decreased plasma concentration and sub-therapeutic effect of the drug [48]. PIs are inhibitors of CYP3A4 and are classified as type I (atazanavir, lopinavir, saquinavir and tipranavir), type II (indinavir and ritonavir), and spectrally unbound (amprenavir and darunavir) PIs [48]. For type I, the inhibitory effects of these PIs predominate in the liver even in the presence of chronic alcohol induction. However, the reverse occurs in type II and spectrally unbound PIs as the alcohol induction overrides the inhibitory effects of the PIs in these groups [29, 48, 56, 57]. PIs inhibition of CYP3A4 will increase drug bioavailability [29, 48], leading to possible toxicity and testicular dysfunction [38, 58, 59]—refer to Tables 2 and 3. Chronic or binge alcohol consumption will result in CYP2E1 induction (up to 20-fold), which can result in the accelerated metabolism of alcohol to acetaldehyde [60, 61]. Acetaldehyde is a toxic metabolite of alcohol, causing severe damage when not fully detoxified to acetate [62].

Unlike ARVs metabolized by CYP enzymes, all NRTIs undergo intracellular phosphorylation to their active form. INSTIs undergo phase II conjugation reactions such as glucuronidation [63, 64]. Pharmacodynamic interactions involving these agents with alcohol can result in additive or synergistic effects leading to toxicities [65, 66]. Since HAART involves the use of at least two drugs from NRTIs class, the likelihood of testicular adverse events attributed to mitochondrial dysfunction may also be increased [67, 68] (refer to Tables 2, 3).

4 Role of Drug Transporters in Relation to Alcohol and Antiretroviral Interaction on the Testis

The task of drug disposition relies heavily on certain helper proteins called drug transporters, which are primarily bound to the plasma membrane They function to enhance the movement of several poorly bioavailable drugs across biological barriers [69]. However, the pharmacokinetics of xenobiotics can be significantly altered when several co-administered drugs compete for the same transport pathways. This in turn may result in abnormal changes in serum and tissue drug levels, resulting in a change in drug potency as well as increasing adverse drug reactions [70, 71].

There are two main categories of drug transporters, namely the ATP-binding cassette (ABC) transporters and the solute carrier (SLC) transporters [69, 72]. ARVs rely exclusively on these drug transporters for movement across biological membranes [72]. The ABC transporters are responsible for the cellular efflux of xenobiotics [73]. The three well-studied ABC transporters include: permeability glycoprotein (P-gp), multidrug resistance-associated proteins, and the breast cancer-resistant protein (BCRP) among several other minor ones [74]. SLC transporters, however, mediate the influx of xenobiotics with certain SLC members transporting substrates in a bi-directional manner [69, 75]. Studies conducted on both the ABC drug efflux and SLC transporters have shown that they are abundant and expressed in the testicular tissue [74, 76–78]. Several ARVs act as substrates for these transporters. They may then act either as inhibitors or inducers of the ABC and SLC transport system [72, 79–81].

The affinity of ARVs varies from one class to another in terms of their degree of binding. Studies reviewed by Kis et al. [72], Su et al. [78] and Pyles et al. [82] have shown that ARVs have high affinity for certain ABC transporters, especially P-gp, and less so MRP and BCRP. Induction of drug transporters can significantly affect the pharmacokinetics of ARVs and other co-administered drugs [81, 83]. Reduced bioavailability of ARVs was also observed in the seminal fluid of HIV patients treated with NNRTIs [84–89]. Similar instances of reduced bioavailability of PIs were also reported in seminal fluids of HIV-infected individuals when compared with drug concentration in the plasma [84, 90, 91]. Inhibition of efflux transporters can increase tissue drug concentrations exposing organs to possible toxicities [92]. Accumulation of ritonavir was detected in

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Table 2 Summarized studies evaluating the effects of HAART on the testis

Drugs used	Dosage	Experimental type	Duration	Observation	References
ART	Standard dosage	Human observational study	6 months	HIV RNA and HIV DNA were detectable and high in the semen of patients on ART, while HIV DNA was also detected in blood but plasma HIV RNA was reduced to an undetectable level	[182]
HAART (AZT + 3TC + NVP)	1.89 mg/kg AZT, 0.95 mg/kg 3TC, 1.26 mg/kg NVP	Animals (rats)	2 months	Decreased sperm counts and motility, disorder in seminiferous tubular architecture	[115]
HAART (3TC + d4T + NVP)	2.06 mg/kg 3TC, 0.57 mg/kg d4T, 1.54 mg/kg NVP	Animals (rats)	2 months	Decreased sperm counts and motility; seminiferous tubules adversely affected	[183]
HAART (TFV + FTC + LPV/RTV + RAL)	20 mg/kg TFV, 50 mg/kg FTC, 36 mg/kg LPV boosted with 12 mg/kg RTV once daily, last 7 weeks intensified with 200 mg/animal RAL	Animals (cynomolgus macaques)	4 months	Genital tissues were not decreased by HAART continuous shedding of SIV in semen despite efficient HAART	[184]
LPV/RTV	8.3 mg/kg, 16.6 mg/kg	Animals (rats)	21 days	Decreased sperm counts and motility, fewer spermatogenic cells, significant increase in total sperm abnormalities, decreased testicular GSH, SOD, CAT and increased lipid peroxidation	[59]
HAART (TFV + FTC + EFV)	17 mg/kg TFV, 20 mg/kg FTC, 50 mg/kg EFV	Animals (rats)	6 weeks	Significant decrease in sperm motility and viability, testicular glutathione, CAT and SOD significantly reduced, TBARS level also increased significantly in HAART-treated group	[185]
ART	Standard dosage	Human cross-sectional study	6 months	Fertility potential was not impaired since neither ART nor HIV infection could impair Sertoli cell functions and fertility potential of patients. IB to FSH ratio also was not compromised in HIV-infected individuals, as there was no damage caused by ART	[178]
FTC		Human study		FTC distributed well in the seminal plasma than in the blood plasma	[186]
HAART	Standard dosage	Human pilot study	6 months	Low LH, low FSH, and primary and secondary hypogonadism were observed; no patient had low testosterone	[116]
ART	Standard dosage	Human prospective study	2 years and 1 month	Semen parameters impaired and sperm protein composition altered	[177]
HAART (3TC + 4dT + NVP)	2.06 mg/kg 3TC, 0.57 mg/kg 4dT, 1.54 mg/kg NVP	Animals (rats)	28 days	Deleterious histopathological changes observed, tubular atrophy and altered morphometric indices also occurred	[114]
NRTI (ABC)	100, 300 & 600 mg/kg	Animals (rats)	28 days	Abnormality in genetic composition and impaired fertility function observed	[155]
LPV/RTV	22.8/5.8 mg/kg	Animals (rats)	2 weeks	Decrease in total sperm counts and sperm motility, increase in abnormal morphology, decrease in SOD and increase in MDA levels	[58]
NNRTI (NVP)	18 mg/kg 36 mg/kg	Animals (rats)	4 weeks	Decrease in sperm motility and sperm counts, degeneration of seminiferous tubules, increase in lipid peroxidation, decrease in the levels of SOD and CAT; decrease in antioxidant status and increase in oxidative stress	[52]
Multiple antiretrovirals		Case report	16 years	14-day-old male newborn of mother on multiple antiretrovirals for 16 years showed signs of teratogenic effect, mainly sexual ambiguity and karyotype 45, X/46 and XY	[187]
NRTIs (ddI, AZT) PIs (SQV, IDV)		In vitro study on humans		Higher doses of SQV present adverse effects on the sperm motility, mitochondrial potential and acrosome reaction	[38]
NRTIs, NNRTIs and PIs		Human cross-sectional study	5 years and 8 months	Patients receiving NNRTI (NVP) showed higher percentage of motile spermatozoa	[148]

GSH glutathione peroxidase, *GSTs* glutathione S-transferase, *CAT* catalase, *SOD* superoxide dismutase, *TBARS* thiobarbituric acid, *IB* inhibin, *FSH*-follicle stimulating hormone, *ART* antiretroviral therapy, *NRTI* nucleoside reverse transcriptase inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor, *PI* protease inhibitor, *HAART* highly active antiretroviral therapy, *AZT* zidovudine, *3TC* lamivudine, *NVP* nevirapine, *d4T* stavudine, *TFV* tenofovir , *FTC* emtricitabine, *LPV* lopinavir, *RTV* ritonavir, *RAL* raltegravir, *EFV* efavirenz, *ABC* abacavir, *ddI* didanosine, *SQV* saquinavir, *IDV* indinavir

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testicular tissue due to the inhibition of P-gp, MRP1, or MRP2 [77]. Increased concentrations of several NRTIs [84, 90, 93–95] were observed in the seminal fluid of HIV-infected men compared to plasma drug concentration.

Interaction of ARVs with alcohol can significantly result in therapeutic failures or place patients at significant risk for toxicities [46, 65]. Alcohol alters the properties of the lipid bilayer (cell membrane) by changing the membrane permeability, lipid bilayer free volume, and protein distribution and expression, thus bringing about disordered conformational arrangement [96, 97]. The change in membrane properties comes with resultant alteration of protein functions [98, 99]. Since the drug transporters which are critical to pharmacokinetics are integral membrane proteins [100], there is a high probability that alcohol will alter accessibility to the protein-binding sites of ARVs substrates [96, 99]. The overall observable effect of alcohol therefore will be the diminished activity of both the efflux and influx transporters.

5 ARVS: Physicochemical Properties and Interaction with Alcohol

The physicochemical properties of drugs including ARVs play a vital role in their bioavailability at the site of action. These properties include key factors such as lipophilicity, plasma protein binding, degree of ionization, partition coefficient and the molecular weight among others [101, 102]. ARV's penetration into the male genital tract (MGT) can be quantified by its concentration in the seminal fluid. However, interaction with alcohol can significantly affect seminal fluid concentration due to plasma protein binding, the dissociation constant and partition coefficients within plasma [103, 104].

The concentration of serum albumin as produced by the liver is critical to the extent of distribution and bioavailability of ARVs in the body. Chronic heavy drinking will ultimately progress to chronic conditions such as liver cirrhosis, leading to hypoalbuminemia [105, 106]. Alcohol-induced hypoalbuminemia typically results in higher circulating unbound fraction of ARVs [107] with an increased level of ARVs at receptor sites within the MGT, bringing about increased effects as well as potential toxicity. This is especially true for ARVs which are highly protein bound (NNRTIs, PIs and INSTIs) [108, 109] (refer to Table 3).

In addition, the alcohol-induced hypoalbuminemia will alter the blood pH, affecting the extent of drug ionization (depending on the p*K*a of the drug) [110]. The degree of drug ionization will subsequently affect the bioavailability of the drug at the receptor sites. [110]. Acidic ARV drugs have a higher affinity for protein binding and are thus more affected by hypoalbuminemia [108, 110].

6 Impact of Alcohol and Antiretroviral Interactions on the Testicular Structure

The intricate interactions between the extracellular matrix, tubular wall and germinal cells contribute significantly to their normal development [111]. Germinal cell development has been reported to be adversely affected by the increase in the amount of collagen, which can lead to interstitial fibrosis, infiltration and a thickened basement membrane in seminiferous tubules [112, 113]. HAART (as seen in animal experimental models) has demonstrated increased infiltration and cellularity of the interstitial spaces and thickening of the basement membrane, extensive necrosis of the seminiferous tubules and atrophy with loss of maturation stages of spermatocytes [58, 114]. Whether the direct attack of HAART components targets the Sertoli cells (the anchors of the layered arrangement of seminiferous tubules) with resultant loss of support remains to be fully understood. However, exposure to ethanol can further exacerbate the adverse effects of ART, which can either be additive or synergistic [24, 48, 53]. Clinical and animal experimental studies have shown that HAART negatively affects morphometric parameters with reduction in the diameter of the seminiferous tubules, decreases in the number of germ cells in all stages in the spermatogenesis, sharp drop in Leydig cell number and severe injury to the Sertoli cells [114–116]. Table 2 shows the summarized effects of HAART on the testicular parameters.

Taken together, these changes implicate alterations in androgen metabolism and transport that putatively subverts the architecture of the testicular anatomy with consequent loss of germ cells and ensuing fertility problems. Excessive ethanol consumption can adversely impact sperm DNA integrity and chromatin remodeling and may also induce sperm apoptosis through negative interference on antioxidant defense mechanism [117–119]. Treatment of experimental rats with chronic ethanol administration causes thickening, hyalinosis and sclerosis of lamina propria and interstitial tissue infiltration with mast cells [118, 120]. Severe increase in testicular oxidative stress associated with increased reactive oxygen species (ROS) generation and lower cellular antioxidant levels are also features of ethanol induction in rats [121, 122].

7 Impact of Alcohol and Antiretroviral Interactions on Testicular Carbohydrate and Lipid Metabolism

Abnormal carbohydrate metabolism has been associated with antiretroviral regimens [123, 124] with considerable adverse effects on glucose metabolism that precipitates into

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Table 3 Potential pharmacokinetic (PK) and pharmacodynamics (PD) interactions of alcohol with antiretrovirals

ARVs	PK interaction with alcohol	PK mechanism of interaction with alcohol	PD interaction with alcohol	PD mechanism of interaction with alcohol
NRTIs				
Zidovudine	Alcohol reduces its bioavailability	Alcohol induces increased metabolism by CYP enzymes	Alcohol causes similar adverse effects, including hepatomegaly with steatosis	Physiologic interaction
Lamivudine	No significant pharmacokinetic interaction with alcohol	–	Alcohol causes similar adverse effects, e.g., hepatomegaly	Physiologic interaction
Abacavir	Alcohol increases its bioavailability	Alcohol decreases the metabolism of abacavir	Alcohol causes similar effects, e.g., hepatotoxicity	Physiologic interaction
Tenofovir	No significant pharmacokinetic interaction with alcohol	–	Potentiation of adverse effects, e.g., renal impairment	Physiologic interaction
Emtricitabine	No significant pharmacokinetic interaction with alcohol	–	Potentiation of adverse effects, e.g., hepatomegaly	Physiologic interaction
Stavudine	No significant pharmacokinetic interaction with alcohol	–	Potentiation of adverse effects, e.g., hepatotoxicity	Physiologic interaction
Didanosine	No significant pharmacokinetic interaction with alcohol	–	Potentiation of adverse effects, e.g., neuritis, pancreatitis	Physiologic interaction
NNRTIs				
Nevirapine	Alcohol decreases its bioavailability	Alcohol induces its metabolism	Potentiation of adverse effects, e.g., hepatotoxicity	Physiologic interaction
Efavirenz	Alcohol decreases its bioavailability	Alcohol induces its metabolism by liver enzymes	Potentiation of adverse effects, e.g., CNS disturbances	Physiologic interaction
Etravirine	Alcohol decreases its bioavailability	Alcohol induces its metabolism by liver enzymes	Potentiation of adverse effects, e.g., peripheral neuropathy	Physiologic interaction
Delavirdine	Alcohol decreases its bioavailability	Alcohol induces its metabolism by liver enzymes	Potentiation of adverse effects, including hepatotoxicity	Physiologic interaction
Rilpivirine	Alcohol decreases its bioavailability	Alcohol induces its metabolism by liver enzymes	Potentiation of adverse effects including depression disorder	Physiologic interaction
PIs				
Type I				
Atazanavir	Increases alcohol bioavailability	Inhibits alcohol metabolism	Potentiation of adverse effects, e.g., hepatotoxicity	Physiologic interaction
Lopinavir	Increases alcohol bioavailability	Inhibits alcohol metabolism	–	–
Saquinavir	Increases alcohol bioavailability	Inhibits alcohol metabolism	Potentiation of adverse effects, e.g., pancreatitis	Physiologic interaction
Tipranavir	Increases alcohol bioavailability	Inhibits alcohol metabolism	Potentiation of adverse effects including hepatotoxicity	Physiologic interaction
Type II				
Indinavir	Alcohol decreases its bioavailability	Alcohol induces its liver enzyme metabolism	Potentiation of adverse effects including renal damage	Physiologic interaction
Ritonavir	Alcohol decreases its metabolism	Alcohol induces its metabolism by liver enzymes	Potentiation of adverse effects including hepatotoxicity	Physiologic interaction
Spectral unbound				
Fosamprenavir	Alcohol decreases its bioavailability	Alcohol induces its metabolism by liver enzymes	–	–
Darunavir	Alcohol decreases its bioavailability	Alcohol induces its metabolism by liver enzymes	Potentiation of adverse effects including hepatotoxicity	Physiologic interaction
INSTIs				

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Table 3 continued

ARVs	PK interaction with alcohol	PK mechanism of interaction with alcohol	PD interaction with alcohol	PD mechanism of interaction with alcohol
Raltegravir	No significant pharmacokinetic interaction with alcohol	–	Potentiation of adverse effects including liver injury	Physiologic interaction
Dolutegravir	Alcohol reduces its bioavailability	Alcohol stimulates its liver metabolism	Potentiation of adverse effects including liver injury	Physiologic interaction
Elvitegravir	Alcohol reduces its bioavailability	Alcohol-induced liver metabolism	–	–
FIs				
Enfuvirtide	No significant pharmacokinetic interaction with alcohol	–	Potentiation of adverse effects, e.g., pancreatitis	Physiologic interaction
CCR5 antagonist				
Maraviroc	Alcohol reduces its bioavailability	Alcohol induces its metabolism by the liver	Potentiation of adverse effects including hepatotoxicity	Physiologic interaction

ARV antiretroviral, *NRTI* nucleoside reverse transcriptase inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor, *PI* protease inhibitor, *CYP* cytochrome P450, *INSTI* integrase strand transfer inhibitor, *FI* fusion inhibitors, *CCR* chemokine receptor

various pathologies of insulin resistance, glucose intolerance and diabetes mellitus [125, 126]. While carbohydrate (mainly glucose) supplies the energy requirement for most biochemical activities in the body [127], the passive transport of glucose across the blood testes barrier is vital in the process of spermatogenesis [128]. Thus, any disruption in glucose metabolism and/or transport can adversely affect the germinal cells, leading to spermatogenic arrest in the seminiferous tubules [114, 129].

To facilitate the movement of glucose, certain carriers known as glucose transporters (GLUT), localized on plasma membranes of sperms are responsible for mediating these energy sources into the cells, primarily in the form of intracellular ATP [130, 131]. This process is prone to disruptions by toxicants or drugs with deprivation of spermatozoal energy and resultant male subfertility [132–135]. Direct inhibition of GLUT-4 transporter led to insulin resistance in patients on indinavir [136]. Studies in humans and animals shows that metabolic complications arising from ARVs lead to reduction in fecundity due to altered glucose metabolism [132–135].

With interrupted energy supply (due to altered glucose metabolism), protein synthesis in germinal cells become deranged resulting in lipid accumulation in the cytoplasm of germinal cells and interstitial connective tissue of the testes [127, 129]. This pathological process generally associated with HAART results in fat re-distribution, lipohypertrophy and central accumulation which are major complications [137–139]. The mechanism by which this distribution occurs remains unclear [140]. However, there is sufficient evidence that testicular lipid accumulation can impair male fertility [141–143]. In addition to these myriad of metabolic events, alcohol abuse under ARV treatment may well further exacerbate the processes. Ethanol-induced disruption of glucose transporters causes decline in ATP production in the Leydig cells [144] with resultant impairment in the oxidation of glucose. These events have negative implications with regard to androgenesis, as chronic alcoholism can predispose to significantly high levels of testicular cholesterol, free fatty acids, phospholipids and triglycerides resulting in testicular hyperlipidemia [145, 146].

8 Impact of Alcohol and Antiretroviral Interaction on Semen Parameters

Antiretroviral drugs have been reported to give rise to several life-threatening complications; such as mitochondrial dysfunction arising from altered mitochondrial DNA (mtDNA) replication and oxidative stress [68, 147]. The outcomes of these have a negative impact on semen parameters leading to sexual dysfunction [148]. Many

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clinical studies performed in this field have shed light on the effects of ARVs on sperm quality with a majority of them indicating that ARTs can adversely affect seminal parameters. Decrease in sperm mitochondrial DNA was observed among HIV patients on NRTIs antiretroviral [36], and sperm mtDNA deletions were observed among patients on HAART for 12 months [37]. Analyses from an in vitro study had indicated that saquinavir, a protease inhibitor has adverse effect on sperm motility, mitochondrial potential and acrosome reaction [38]. Other findings have also indicated that antiretroviral regimens cause a reduction in sperm counts, decrease in progressive motility [149–151] and decrease in semen volume [152]. Decreased progressive sperm motility/or decreased sperm count are all measures of seminal viability and thus indices of toxicity [153, 154].

Furthermore, animal experimental studies have buttressed the negative influence of ARVs on sperm functional parameters; administration of abacavir, an NRTI for 28 consecutive days, on rats has been reported to cause a decrease in sperm counts and induction of abnormalities in the genetic makeup [155]. Another NRTI, zidovidine, has also been reported to cause structural abnormalities to testicular tissues [156], while lopinavir/ritonavir, a protease inhibitor, was reported to lower sperm quality and induce testicular oxidative damage in rats [59]. In addition, HAART has been reported to cause atrophy of the seminiferous tubules, decrease epithelial height, deplete the Sertoli cells and cause spermatogenic arrest [114].

The impact of moderate (< 1 drink/day over 6 months) to excessive alcohol (≥ 2 drinks/day) consumption among HIV-infected individuals cannot be underestimated [24, 47]. It can adversely impact the absorption and metabolism of ARVs [32] and possibly exacerbate the side effects arising from ART toxicities [45]. Chronic alcohol consumption among HIV/AIDS patients undergoing ART has been reported to increase the serum level of the drug and their toxicities [157]. On exposure to alcohol, human spermatozoa exhibits decreased sperm motility, severe morphological abnormalities and increased DNA fragmentation [158–160]. More evidences from clinical studies have also indicated that long-term consumption of alcohol alters sperm parameters and potentiates testicular pathology [40, 41, 161, 162].

9 Male Hypogonadism in Relation to Testicular Interaction with Alcohol and Antiretroviral Drugs

Male hypogonadism is a clinical condition characterized by insufficient testosterone and/or sperm production owing to a decline in the functional activity of the gonad (testis) [163, 164]. Hypogonadism can be categorized into a primary condition which originates from the testicles, or a secondary condition which is due to modifications at some levels of the hypothalamic–pituitary axis (HPA), resulting in low circulating luteinizing hormone (LH) which is responsible for testosterone secretion [165, 166]. However, sperm production is significantly more affected compared to testosterone production in primary hypogonadism owing to extensive injuries sustained by the testicular seminiferous tubules [167].

Common causes of primary hypogonadism include genetic abnormalities, normal aging (declining testosterone production as men grow older), testicular injuries (torsion), developmental and metabolic abnormalities and anticancer treatment [163, 168]. Secondary causes of hypogonadism include pituitary and hypothalamic disorders, morbid obesity, diabetes mellitus, malnutrition, chronic systemic illnesses (HIV/AIDS), adverse effect of medications and excessive alcohol consumption [163, 169].

Testosterone is widely known to play vital roles throughout the cycle of a male individual, especially from fetal stage to adulthood [170]. It contributes significantly to sexual differentiation [163] and its effects are still evident during late adult life [163, 166]. Androgen (testosterone) deficiency increases with advancing age in all men with an annual estimated rate of about 0.2–4%, while the occurrence rate is about 6% in middle-age men [171]. The prevalence rates of 12, 19, 28 and 49% were also reported in older men in their 50s, 60s, 70s and 80s, respectively [172].

The effect of antiretroviral drugs in relation to male hypogonadism in HIV/AIDS pathogenesis remains controversial and details are still unclear. Hypogonadism worsens with increasing age in men. Several studies have also observed a greater prevalence among HIV-infected men based on HPA impairment and total testosterone levels with a drastic attenuating effect when ARV drugs are introduced [164, 173, 174]. Recent studies have reported an increased prevalence of male hypogonadism among HIV patients on effective ARV treatment [175, 176]. ARVs can adversely impact the testis, especially in primary hypogonadism, by affecting the production of spermatozoa, causing alteration of the structural and genetic makeup and thereby changing the semen parameters [177, 178] (refer to Table 2).

Testosterone is vital in the regulation of sperm production; however, there has been difficulty correlating the number of germ cells produced with testosterone concentration [179]. Ethanol permeates directly into the testes bringing about a decrease in spermatogenesis, lowering the synthesis of testosterone, increasing metabolic clearance of testosterone and resulting in increased estrogen levels [159, 180]. The outcome will lead to secondary

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hypogonadism [181]. In support of the above, chronic alcohol consumption among HIV patients undergoing ART has been reported to show increased serum level of the drug and increased toxicities [157]. On chronic exposure to alcohol, human spermatozoa show decreased sperm motility, severe morphological abnormalities and increased DNA fragmentation [158–160]. Further evidence from clinical studies also confirms the relationship of long-term alcohol consumption and testicular pathology [41, 162].

It is therefore likely that alcohol will counteract any improved gonadal function induced by ARVs via increased testosterone production and will potentiate the adverse effects of hypogonadism on sperm production and quality.

10 Concluding Remarks

Drug–dietary habit interactions between alcohol and antiretroviral drugs in HIV/AIDS patients is a subject of great concern owing to the possibility of accelerated disease progression or adverse effects and toxicities. Alcohol is reported to disrupt ARV membrane transporter proteins altering its function. This will reduce the antiretroviral drug efficacy with worsening disease progression. Alcohol also decreases the male sexual hormone levels, lowers male fertility and causes testicular hypofunction. Many antiretroviral drugs have adverse effects, which can be exacerbated by the excessive use of alcohol . The combined toxicities of these agents act synergistically to diminish male fertility. HIV–AIDS patients on ARV treatment who tend to 'binge drink' may benefit from the use of antidepressants or possibly antidipsotropic agents. This would reduce the toxic effects resulting from ARV–alcohol drug interactions. Future research on the clinical relationship of alcohol–ARVs induced hypogonadism is therefore recommended.

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Compliance with Ethical Standards

Conflict of interest None declared by all the authors.

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BRIDGING TEXT

FROM CHAPTER TWO TO THREE

As reported in chapter two, interactions between alcohol and antiretroviral drugs in HIV/AIDS patients is a subject of great concern owing to the potential of alcohol exacerbating the adverse effects of HAART resulting in disproportionate harm to male reproductive health. This however justifies the need to seek for alternative remedies in order to palliate the ravages arising from the interactions of both drugs.

As illustrated earlier (chapter one), plant-based adjuvants are widely used for the management of various diseases and ailments. They may have the potential to ameliorate adverse effects and toxicities arising from the use of HAART and alcohol. It is in this respect that Chapter three was designed to investigate the effects of virgin coconut oil on testicular microanatomy following treatment with HAART.

CHAPTER THREE

MANUSCRIPT TWO

Coconut Oil Extract Mitigates Testicular Injury Following Adjuvant Treatment with Antiretroviral Drugs

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Coconut Oil Extract Mitigates Testicular Injury Following Adjuvant Treatment with Antiretroviral Drugs

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Increased access to highly active antiretroviral therapy (HAART) has made the management of drug toxicities an increasingly crucial component of HIV. This study investigated the effects of adjuvant use of coconut oil and HAART on testicular morphology and seminal parameters in Sprague- Dawley rats. Twelve adult male Sprague-Dawley rats, weighing 153~169 g were distributed into four groups (A-D) and treated as follows: A served as control (distilled water); B (HAART cocktail- Zidovudine, Lamivudine and Nevirapine); C (HAART + Virgin coconut oil 10 mL/kg) and D (Virgin coconut oil 10 mL/kg). After 56 days of treatment, animals were killed and laparotomy to exercise the epididymis for seminal fluid analyses done whilst testicular tissues were processed for histo-morphometric studies. Result showed a significant decline in sperm motility ($P < 0.05$) and count ($P < 0.0001$) in HAART-treated animals while there was insignificant changes in other parameters in groups C and D except count that was reduced ($P < 0.0001$) when compared with controls. Histomorphological studies showed HAART caused disorders in seminiferous tubular architecture with significant ($P < 0.01$) decline in epithelial height closely mirrored by extensive reticulin framework and positive PAS cells. Adjuvant Virgin coconut oil + HAART resulted in significant decrease in seminiferous tubular diameter ($P < 0.05$), but other morphometric and histological parameters were similar to control or Virgin coconut oil alone (which showed normal histoarchitecture levels). While derangements in testicular and seminal fluid parameters occurred following HAART, adjuvant treatment with Virgin coconut oil restored the distortions emanating thereof.

Key words: Histomorphology, Testis, Antiretroviral therapy, Andrology, Stains, Coconut oil

INTRODUCTION

The introduction of highly active antiretroviral therapy (HAART) in the management of HIV/AIDS have turned the tide from a high morbidity and mortality epidemic into a manageable chronic condition and thus making HAART the effective standard regimen (1,2). HAART could be a double or triple-drug cocktail with two nucleoside reverse transcriptase inhibitor (NRTI) backbones in combination with a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor (PI) or an integrase strand transfer inhibitor (INSTIs) (3). The effective and efficient roll-out of HAART in South Africa, where the vast majority of HIV-infected individuals live, has contributed to the dramatic decline in morbity and mortality due to HIV/AIDS (4).

However, in spite of this success, there are still adverse effects incurred from HAART (5) including hepatotoxicity (6), nephrotoxicity (7) and neurotoxicity (8), following large-scale clinical trials on humans as well as animal experimental studies. In addition, there are concerns that the treatment-related drop in morbidity and mortality may actually be overestimated (4) as most studies relies on health-related quality of life outcomes considering only mortality

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and ignoring treatment-related morbidities.

Several reports have raised the possibility that long-term use of antiretroviral drugs may interrupt normal male reproductive function (9,10). Clinical and animal experimental studies have ascertained the negative influence of HAART on sperm functional parameters, sexual hormones, testicular structure, germinal cell development, as well as testicular carbohydrate and lipid metabolism (9,11). With the high financial burden of sustaining compliance with antiretroviral regimens, there is a major challenge of people living with HIV/AIDS (PLWHAs) (12) most especially among sub-Saharan Africa necessitating majority of them resorting to the use of traditional medicines and products (13,14) for improvement of their well-being. Besides, scientific interest towards the use of medicinal plants has been on the rise due to their perceived effectiveness during treatment, relatively low costs and minimal side-effects (15).

Cocos nucifera (Coconut) is an important fruit that is grown in most tropical and subtropical regions of the world (16) and has been referred to as the 'tree of life due to its' several applications (17). Its part includes the husks, coconut water, coconut meat, coconut milk and coconut oil and have been linked to numerous medicinal properties (18). There are reports of its use as alternative medicinal therapy (19,20) for treatment of diabetes, diarrhea and as anticancer (20) using *in vitro* and *in vivo* techniques.

Virgin coconut oil (VCO) is extracted from fresh coconut (20) and differs from ordinary coconut oil because it contains more biologically active components of polyphenols, tocopherols, sterols and squalene (21). It has captured a lot of interest due to its ability to mitigate oxidative stress-related processes via inhibition of lipid peroxidation (22). Reports of its improvement of semen quality (sperm counts and motility) as well as boosting serum testosterone levels (23) have been acknowledged. Though VCO has many therapeutic values, there are no scientific reports on its ability to mitigate testicular injuries associated with any HAART either in human and/or experimental animal model. This present study therefore investigated the possible ameliorative effects of VCO as adjuvant with ART with the view to mitigating the ravages of HAART on testicular tissues using an animal (male Sprague-Dawley rats) model.

MATERIALS AND METHODS

Twenty adult male Sprague-Dawley rats, weighing 153~169 g were used for this study. These animals were bred and maintained at the Animal House of the Biomedical Resources Unit, University of Kwazulu-Natal. All procedures involving the animals was 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 (24). The protocol for the study was approved by Animal Ethics Committee (protocol reference number: AREC/087/015D). The rats had unrestricted access to food (standard rat pellets) and water.

All the rats were housed in plastic cages (3 rats/cage) having dimensions of 30 long, 20 wide and 13 cm high) and soft wood shavings employed as bedding in the cages. Rats were maintained under standardized animal house conditions (temperature: 28~31°C; light: approximately 12 hr natural light per day; humidity: 50~55%). The drug Zidovudine, Lamivudine, and Nevirapine (Aspen) were procured from Pharmed, Durban, South Africa.

Plant material. The solid endosperm of mature coconuts were commercially purchased from a local store in Durban area and were authenticated at the Department of Life Science, Westville Campus, University of Kwazulu-Natal, South Africa.

Preparation of VCO. The wet extraction method described by Nevin and Rajamohan (22) was used for VCO extraction. Briefly, the solid, matured coconut were crushed and made into viscous slurry, water of about 500 mL was added and squeezed through cheese cloth to obtain coconut milk. The coconut milk produced was left for about 24 hrs to aid the gravitational separation of the milk, which was in accordance with (25,26). Three phases resulted; a lower aqueous phase, a middle emulsion phase, and an upper oily phase. The upper oily phase was then decanted and heated for about 10 min to remove moisture. The resultant VCO was then filtered with a fine sieve, stored in plain bottles at room temperature and used for the experiment.

Experimental design: The animals were randomly distributed into four (4) groups (A-D) and treated as follows:

A (control animals received distilled water).

B (HAART cocktail of Zidovudine, Lamivudine and Nevirapine adjusted to the equivalent animal dose of 1.89, 0.95 and 1.26 mg/kg body weight respectively) (27).

C (HAART + VCO 10 mL/kg).

D (VCO 10 mL/kg).

All treatment was applied daily by oro-gastric gavage and at the end of 56 days all animals were killed 24 hrs after the last treatment under halothane^{R} anesthesia.

Body and testicular weight. The body weights of animals were recorded on the first day before treatment (initial), thereafter on a weekly basis and finally on the last day of experiment. The weight of the testes (TW) was measured by an electronic balance (Mettler Toledo; Microsep (Pty) Ltd., Greifensee, Switzerland). The testes of each rat were measured individually and the average value obtained for each of the two testes were regarded as one observation. The values are expressed in grams (g) for TW.

Semen analysis. The caudal epididymis of the rats were excised and minced with an anatomical scissors, a

drop of epididymal fluid was collected onto a glass slide, covered with cover slip covered with a 22 × 22 mm cover slip and examined under a light microscope (28). The microscopic field was scanned systematically and the motility of each spermatozoa encountered was assessed (29), motility was graded as progressive, non-progressive and dead (30). After assessing different microscopic fields, the relative percentage of motile sperm was estimated and reported to the nearest 5% using the subjective determination of motility (31).

The epididymal tissue was cut into small pieces and diluted with normal saline and sperm count was determined using an automated sperm analyser with a double slide counting chamber. The dilution was mixed thoroughly and both sides of the counting chamber were scored and the average taken. The number of spermatozoa counted was expressed in millions/mL (31).

Histomorphometrical and histochemical studies. Each testis was removed, trimmed of attached tissues and weighed. The testis was fixed in 10% Neutral buffered formalin and processed for histological and histochemical evaluations.

For routine histological study, the testis were dehydrated in an alcohol series of 100%, 90%, 70%, 50%, cleared in xylene, infiltrated and embedded in paraffin and then sectioned (4 μm thick) using a microtome (microm HM 315 microtome, Walldorf, Germany). They were further deparaffinised, stained with hematoxylin and eosin (H&E). For histochemical studies, the tissues were stained with Periodic acid schiff (PAS) technique for the detection of glycogen, neutral polysaccharides and basement membrane; Gordon and Sweet's silver staining method to demonstrate reticular fibers (32). Examination of the stained tissue sections was done by a histopathologist who was blinded to the study protocol.

For morphometric analyses, seven vertical sections from the polar and the equatorial regions were sampled and an unbiased numerical estimation of the following morphometric parameters (diameter and cross-sectional area of the seminiferous tubules, seminiferous epithelial height/thickness) was determined using systematic random scheme (33). The seven vertical sections were selected by a systematic sampling method that ensured fair distribution between the polar and equatorial regions of each testis. The 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 ≥ 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 (33,34). The tubular diameter and height of the seminiferous tubule epithelium was scanned using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at X 100 magnification using image analyser Leica (DMLB) and Leica microsystem software. The diameter of the seminiferous tubule was measured across the minor and major axes, and the mean diameter obtained as stated above. Cross-sectional area (AC) of the seminiferous tubules was determined from the formula, $A_C = \pi D^2/4$ (where π is equivalent to 3.142 and D is the mean diameter of the seminiferous tubules).

Statistical analysis. The morphometric data were analysed using parametric methods. The results are expressed as mean standard error of mean. These were then subjected to within and between group differences using one way ANOVA, followed by Dunnett's multiple comparison test, which was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA.

RESULTS

Organ-body weight changes. There was a general insignificant increase in body weight of animals in all groups but this increase was least in group D compared with the control group A. Significant decrease ($p < 0.05$) in percentage body weight of group D animals compared with control was observed (34.64 ± 3.80 vs. 51.61 ± 5.49). The testicular weight (TW) of all the treated groups B, C and D were similar to that of the control. There was insignificant difference ($P > 0.05$) in the relative organ weight (TW/BW × 100) in all the treated groups (B, C and D) when compared with control group A (Table 1).

Changes in semen parameters. There was a highly significant decrease ($p < 0.0001$) in mean sperm count in animals treated with HAART (0.74 ± 0.30 × 10^6/mL), HAART + VCO (0.93 ± 0.34 × 10^6/mL) and VCO (1.86 ±

Table 1. Body weight, testicular weight and TW/BW ratio in groups

Grp	Initial Bw (g)	Final Bw (g)	Weight Diff (g)	Difference (%)	TW (g)	TW/BW × 100
A	216.70 ± 5.93	328.70 ± 16.80	112.0 ± 13.05	51.61 ± 5.49	3.30 ± 0.01	1.01 ± 0.05
B	232.70 ± 13.86	364.00 ± 24.64	131.3 ± 11.46	56.34 ± 2.44	3.40 ± 0.11	0.93 ± 0.05
C	240.70 ± 8.33	325.70 ± 18.19	85.0 ± 11.68	35.18 ± 4.10	3.36 ± 0.29	1.03 ± 0.03
D	222.70 ± 4.67	300.00 ± 13.00	77.3 ± 9.39	34.64 ± 3.80*	3.41 ± 0.10	1.14 ± 0.15

*Statistically significant at $p < 0.05$.

0.54×10^6/mL) respectively when compared with control ($5.67 \pm 0.26 \times 10^6$/mL). In addition HAART significantly lowered the progressive sperm motility of group B animals when compared with the control ($p < 0.05$) (46.67 ± 6.67% vs. 71.33 ± 4.67%). An insignificant increase in progressive motility was observed in group D animals ($p > 0.05$) when compared with the control group (74.00 ± 7.02% vs. 71.33 ± 4.67%). On the other hand, a significant increase in non-progressive motility ($p < 0.05$) was observed in group B when compared to control group A (31.33 ± 4.67% vs. 16.67 ± 3.33%). Similarly, adjuvant HAART + VCO restired sperm motility to almost control values with % non-progressive motility and dead spermatozoa almost similar to VCO-alone and control groups (Fig. 1).

There were no animal deaths recorded during the experimental period.

Morphological parameters. The diameter of seminiferous tubules in HAART + VCO animals showed a significant decrease ($p < 0.05$) whereas HAART-alone animals showed insignificant decrease ($P > 0.05$) compared with the control group A (266.10 ± 6.23 μm vs. 294.50 ± 8.04 μm). Geminal epithelial thickness (ET) of seminiferous tubules showed a significant decrease in HAART group compared to that in control ($P < 0.01$). However, in adjuvant HAART + VCO and VCO-alone groups recorded no significant alterations in this parameter. The cross-sectional areas of seminiferous tubules of group C treated with a combined dose of HAART + VCO was significantly lowered ($p < 0.05$) when compared with the control group A (55.88 ± 2.60 vs. 68.59 μm^2 ± 3.79 μm^2) (Fig. 2).

Histological and histochemical results. Results of testicular section staining using H&E, PAS and Gordon and Sweet's silver methods revealed varying effects of treatment on the tissues. Testicular cross section of control and VCO groups showed essentially well preserved cyto-architecture and microanatomy of the testes with seminiferous tubules (ST) populated by spermatogenic cells at various stages of development. The interstitial spaces were normal with Leydig cells clearly visible and no infiltrations. In many of the ST of HAART-alone and HAART + VCO

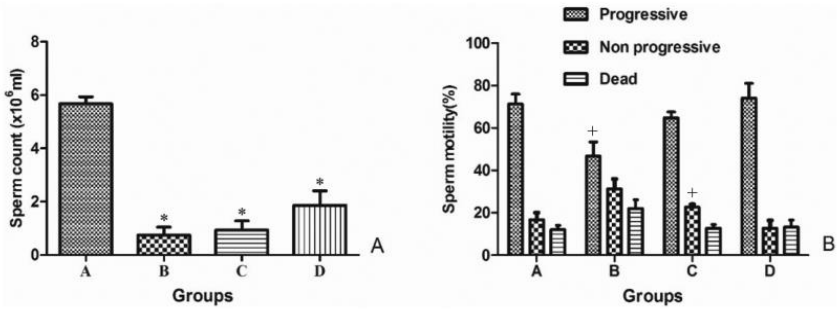

Fig. 1. Effect of VCO and HAART treatment on sperm count and motility in Sprague-Dawley rats after 8 week-treatment period. Bars indicate the mean ± SEM; *$P < 0.0001$; $^{+}P < 0.05$.

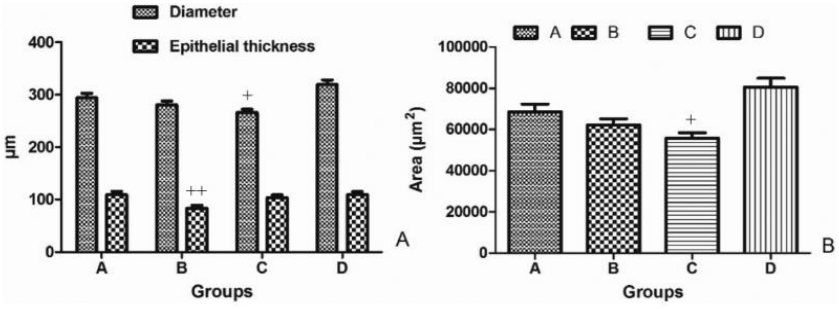

Fig. 2. Effect of VCO and HAART treatment on diameter, epithelial thickness and area in Sprague-Dawley rats after 8 week-treatment period. Bars indicate the mean ± SEM; $^{+}P < 0.05$; $^{++}P < 0.001$.

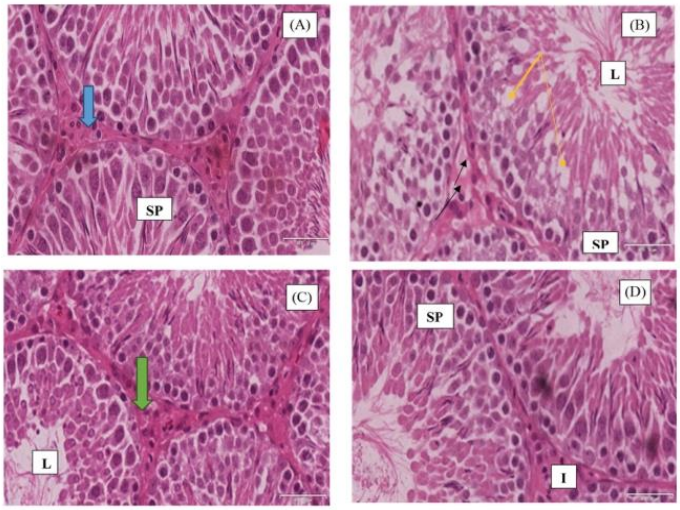

Fig. 3. Micrograph of testicular histological section of groups (A), (B), (C) and (D) (H & E). Control animals (A) and (D) (VCO) shows seminiferous tubules with normal architecture and spermatogenic cells (SP). The interstitial spaces (I) with Leydig cells are normal. The lumen (L) is also populated by immotile spermatozoa. Note the hypoplastic changes with vacuolations (arrowed) in some of the seminiferous tubules in slide (B). There are also reduced spermatogenic cell series in tubules. In (C) (HAART + VCO), partial restoration in some seminiferous tubules was evident.

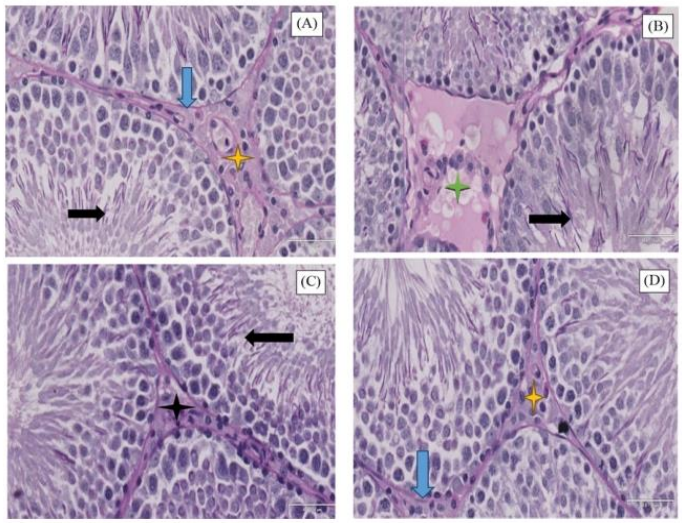

Fig. 4. Micrograph of testicular section of groups (A), (B), (C) and (D) (PAS). The basal membrane of the seminiferous tubule (blue arrow) has normal thickness, the basement membrane and the upper series of germinal epithelium exhibits a positive PAS reaction. The intensity of PAS is higher in (B) and (C). Note in slide (B) the few number of Leydig cells and the peritubular interstitial tissue presenting weak reaction with PAS (green star *). In (C) there is also a strong peritubular interstitial tissue reaction with PAS compared with other slides (black star *). Yellow star (*) in (A) and (C) there is normal peritubular interstitial tissue reaction with PAS. Black arrow shows acrosome stained with PAS.

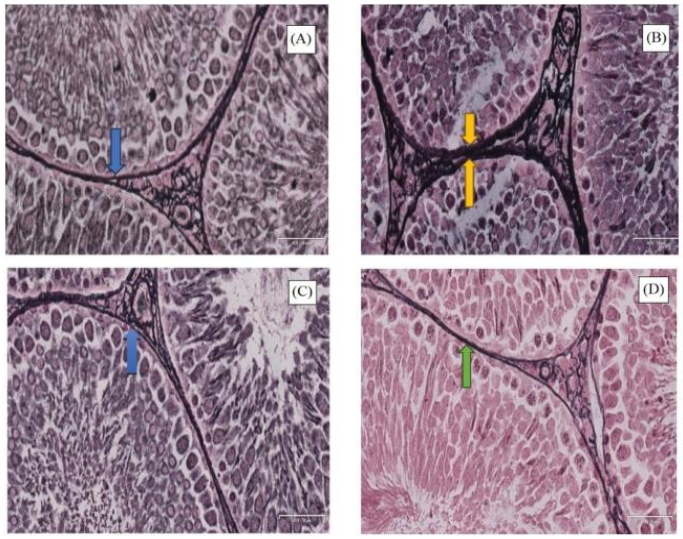

Fig. 5. Micrograph of testicular section of groups (A), (B), (C) and (D) (Gordon and Sweet's) demonstrating reticular fibres (arrowed). Note the thickened basement membrane in (B) with fine threads around (A) and (D). Slides (A) and (D) show well preserved reticular connective tissue (black coloured stain).

groups, there were gross hypoplastic changes in many ST of the former with additional vacuolar changes more numerous in the later. These observations relates with the reduced and disorganized spermatogenic cells in many ST of HAART-treated animals with only basal spermatogonia in some ST. There were less architectural distortions in adjuvant VCO + HAART testis (Fig. 3).

In the current study, the cytoplasm of Leydig cell, peritubular interstitial tissues and basement membrane reacts positively with PAS. PAS-stained sections reveal glycogen presence in the tissues and is depicted by pink color in the sections. Sections from control animals showed clear and positive PAS-reaction with pink-stained thin basement membranes around ST as well as upper series of the seminiferous epithelium and acrosomal caps of matured spermatozoa. There were significant increase in PAS positive particles in slides B and C compared with control and VCO groups. While the PAS stain demonstrates glycogen, indications for distortion in ST cells were evident in the intensity as well with HAART-group exhibiting most staining intensity (Fig. 4).

Silver impregnation with the Gordon and Sweet's method revealed the presence of unique dark-stained reticulin fibres around ST basement membranes and connective tissues in the interstitial spaces in slides from groups A and D. In HAART-treated group, extensive meshwork of thickened, dark-stained fibres were clearly visible around ST basement membranes and sections from group C also exhibited similarity with HAART group but to a lesser degree indicating restoration to control and VCO groups. The massive loss of germ cells (primary and secondary spermatocytes) in HAART group compared with HAART + VCO is visibly present with only basal spermatogonia cells present in the former (Fig. 5).

DISCUSSION

In the last decade, the positive impact of combination antiretroviral therapy on the reduction of HIV-associated mortality and morbidity cannot be underscored despite challenging pitfalls with therapy. Besides, the intolerable side effects of HIV medications can emanate from failure due to drug-drug interactions, organ specific toxicity and/or non-adherence (35). In other to mitigate some of the unwanted side effects of HAART, PLWHAs in sub-Saharan Africa rely on medicinal plants rich in biologically active components for alleviation of some of the toxicities of HAART (36). Reports by (37) and (38) corroborates the use of complimentary alternative medicine for alleviation of pain in PLWHAs. Many herbal extracts are known to exert protective action against noxious effects of toxicants/drugs on organs by their ability to decrease oxidative damage-mediated pathologies (40). VCO has been reported to have active and large amounts of polyphenols and tocopherols which are powerful counter measures against lipid peroxi-

dation (39,41) in tissues. Therefore, as compelling evidence suggests that HAART amplifies oxidative stress status in PLWHAs (42,43), the current study therefore demonstrates the possible protective role of VCO in the mitigation of HAART's ravages on testicular parameters.

We report, for the first time, that adjuvant treatment with VCO mitigates the intensive histopathological changes and loss of considerable number of germ cells in seminiferous tubules of rats due to HAART. The decrease in seminiferous epithelial layers may be linked to inhibitive processes against spermatogonium B that elongates the G-1 phase of spermatogenic cycle (44). In our experiment, adjuvant treatment of VCO had a protective effect on the seminiferous epithelium as demonstrated by the lowering of degeneration and restitution of normal epithelial lining. Testicular sections of HAART-treated animals showed extensive fibrosis around basement membranes of ST (seen with silver impregnation) which are suggestive that disturbances between the Sertoli cells and basement membrane cells (myoid) may have been drastically altered with consequent fibrotic reaction and possible hyalinization (45).

Decreased ET and ST diameter are a result of reduced metabolic activity of the germinal cells as well as cell numbers with the consequence that interstitial spaces widen with oedematous appearance (46). Several studies that have examined HAART effects on semen parameters and other morphometric features of the testis (9,47), have supported our overall result indicating HAART-induced distortions do occur by changes in morphology and morphometric indices of the testis. These ravages were ameliorated by VCO possibly due to its rich antioxidative properties as previously stated by (48). Putatively, pathways for the generation of reactive oxygen species leading to oxidative stress and toxicity of HAART relies on mitochondria-related perturbations that manifests in many side effects such as hepatic failure and lactic acidosis (49). The 'mitochondrial dysfunction hypothesis' reviewed in (50) is believed to operate via energy deprivation, mitochondrial oxidative stress and consequent mitochondrial DNA damage. With depleted energy reserve (observed by significantly reduced progressive motility and increased non-progressive motility in HAART-treated group), mitochondria are unable to execute the needed forward propulsive motion necessary to achieve fertilization and NRTIs and PIs have been implicated as major culprits (49,51). Whilst this protocol did not report perturbations in antioxidant enzymes, previous animal studies on VCO (48) supports the ability of VCO to increase the activity of antioxidant enzymes (e.g. catalase, superoxide dismutase, etc.) in tissues with consequent decline in formation of lipid peroxidation products. Spermatozoa motility is dependent on a robust energy supply and a jealously-guarded polyunsaturated fatty acid membrane prone to oxidative stress mediated damage. With adjuvant VCO + HAART, these negative effects were mitigated in this study.

There was no significant difference in the percentage weight difference of the animal in the control and treatment group except animals in the group administered with only VCO. This might have been due to the fact that VCO contains saturated fatty acids which may likely cause softening of stools and diarrhea that may have caused weight loss (52), although we did not measure any parameter in this regard.

In the current study, VCO at 10 mL/kg dose resulted in an increase in parameters including ST diameter (though not significantly different with control). Though progressive sperm motility was higher and non-progressive motility lowest compared to other groups, these values were not significant. However, the positive indices reflects the ability of VCO to ameliorate toxicant injuries to the testis. Previous reports have pointed to the unsaponifiable components of VCO like vitamin E and polyphenols playing a beneficial role in reducing cholesterol levels and lipid peroxidation (53). It is very likely that this postulation may be operational in our scenario.

The increased PAS positive particles (which are carbohydrate components) in cells seen in sections of tissues from groups C and D alongside the thickened basement membrane and silver stained reticulin network in HAART-treated groups all points towards a metabolic disorder of testicular cells. While Sertoli cells provide the bare cytoskeletal framework for ST, they also assist in phagocytosis of dead/or degenerating spermatogenic cells (47). Many of the ST in sections from HAART group showed Sertoli cells with dense congregations as inclusions. While we did not report assay of testosterone in this study, the results from derangements in seminal and morphological data following antiretroviral therapy evidently implicates altered testicular androgen levels hence impairment in spermatogenic processes as discussed in (9). Calvin *et al*. (54) have suggested that the metabolic pathway of testosterone synthesis requires protection against lipid peroxidation of which the polyphenols present in VCO have been recognized as a powerful counter measure. Taken together, these observations tallies with morphometric data and thus supports the positive role of VCO in this experiment.

In summary, HAART regime as used in this protocol can lead to testicular damage and sperm abnormalities and VCO extract has potential of ameliorating this deleterious effects and protecting the testis through its antioxidant properties. Further studies are needed to understand the precise mechanistic pathway through which this action is dissipated on testicular tissue.

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CONFLICT OF INTEREST

None declared by all authors.

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BRIDGING TEXT

FROM CHAPTER THREE TO FOUR

In chapter three, treatment with HAART showed a decline in sperm concentration and motility, extensive degeneration of germinal cells, distortion in the seminiferous epithelium coupled with derangement in testicular histo-morphology and morphometric indices. However, adjuvant treatment with VCO mitigated the adverse effects of HAART with restoration of testicular structure and function. This required further evaluation of the key components for determining testicular integrity and function from an ultrastructural, physiological and biochemical view point. In Chapter four, we investigate the effects of HAART induced toxicities on testicular ultrastructure, hormonal and biochemical markers in Sprague-Dawley rats following treatment with the adjuvant VCO.

CHAPTER FOUR

MANUSCRIPT THREE

Adjuvant potential of virgin coconut oil extract on antiretroviral therapy-induced testicular toxicity: An ultrastructural study

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ORIGINAL ARTICLE

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Adjuvant potential of virgin coconut oil extract on antiretroviral therapy-induced testicular toxicity: An ultrastructural study

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Summary

The effects of Virgin coconut oil as an adjuvant to highly active antiretroviral therapy (HAART) were investigated on the testicular ultrastructure and biochemical markers in rats. Twenty male Sprague-Dawley rats, weighing 153-169 g were divided into four groups and treated as follows: control A (distilled water), B (HAART), C (HAART+Virgin coconut oil 10 ml/kg) and D (Virgin coconut oil [VCO] 10 ml/kg). Testicular segments were evaluated using transmission electron microscopy. Serum was assayed for testosterone, luteinising hormone, follicle stimulating hormone and testicular tissue for malondialdehyde and glutathione. Ultrastructure of basement membrane (Bm), mitochondria and spermatocytes was normal in the control group. HAART-treated group showed significant increase ($p < .01$) in Bm thickness with significant decrease in Leydig cell nuclear diameter ($p < .05$) and volume ($p < .01$) when compared with control group. Mitochondrial cristae appear collapsed, and Sertoli cells showed cytoplasmic vacuolations. HAART+VCO group showed improved ultrastructural details in Bm, and Sertoli cell and Leydig cells show abundant lipid droplets. Virgin coconut oil-treated group showed thinning of Bm with otherwise normal ultrastructural features of organelles. HAART-treated group showed significant increase ($p < .01$) in testosterone levels. There was no significant effect on malondialdehyde and glutathione levels. Virgin coconut oil improved testicular morphology and reversed HAART-induced ultrastructural alterations. Further studies on putative mechanism are required.

KEYWORDS
Highly active antiretroviral therapy, Leydig cells, sertoli cells, ultrastructure, virgin coconut oil

1 | INTRODUCTION

HIV/AIDS epidemic is one of the major global health challenges of current times having claimed more than 25 million lives so far (Wouters, Masquillier, Ponnet, & le Roux, 2014). It has been reported that Southern Africa is the region with the highest prevalence of HIV infection in the world accounting for one-third of the global burden of HIV (Williams et al., 2015). In the management of HIV/AIDS globally, highly active antiretroviral therapy (HAART) was shown to be effective with a drastic decrease in the incidence of AIDS. It typically constitutes a double- or triple-drug cocktail with a backbone of two nucleoside reverse transcriptase inhibitor (NRTI) in combination with a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor (PI) or an integrase strand transfer inhibitor (INSTI) (Arenas-Pinto et al., 2015). The advent of HAART has significantly improved the quality of lives of people living with HIV/AIDS (PLWHA); however, its use has been associated with a plethora of adverse events (Nagiah, Phulukdaree, & Chuturgoon, 2015). The long-term use of HAART has shown negative consequences on male fertility in clinical practice and animal experimental studies (Azu et al., 2014). The use of HAART

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has demonstrated extensive apoptotic degeneration of germinal cells and metabolic disarray of testicular cells. Its involvement in the generation of free radicals leading to oxidative stress damage cannot be overemphasised.

The heavy financial burden tied to sustaining adherence to antiretroviral therapy, coupled with its rising toxicities, has posed significant challenges leading to PLWHAs settling for traditional medicines and products (Shah, 2007). Though HAART is perceived globally as a standard therapy, it is still considered as one of the many alternative therapies for managing HIV/AIDS. However, in Africa, traditional remedies have been considered a long-established form of health care and a way of life, and many of these products are generally safer than potent synthetic pharmaceuticals, locally available and cost-effective (Jaarin, Norliana, Kamisah, Nursyafiza, & Mohd, 2014).

Recently Virgin coconut oil (VCO) has become popular due to its perceived high therapeutic values. It has been reported to improve the semen quality, boost serum testosterone levels and protect against testicular toxicities (Dosumu, Duru, Osinubi, Oremosu, & Noronha, 2010). Our previous study focusing on the testicular histomorphology and seminal fluid parameters demonstrated the ameliorative potential of VCO against the ravages induced by HAART (Ogedengbe et al., 2016). Studies have also acknowledged its neuroprotective and memory enhancing abilities (Rahim, Lim, Mani, Abdul Majeed, & Ramasamy, 2017), hepatoprotective potential, reduction of body weight (Gunasekaran et al., 2017) and its capacity to ease parturition as well as possible prevention of birth defects (Ab Rahman et al., 2007).

Furthermore, its ability to retain most of its unsaponifiable component coupled with the possession of several biologically active components such as polyphenols and tocopherol amongst others has contributed immensely to its alleviation of oxidative stress damage and improvement of antioxidant status(Nevin & Rajamohan, 2006). Despite extensive work reported on HAART, ultrastructural studies demonstrating testicular morphological features are scarce. This study investigates the effects of testicular ultrastructure, reproductive and biochemical markers in Sprague-Dawley rats following the use of Virgin coconut oil as an adjuvant to HAART.

2 | MATERIALS AND METHOD

Twenty (20) adult male Sprague-Dawley rats weighing 153~169 g were used for this study. These animals were bred and maintained at the Animal House of the Biomedical Resources Unit, University of KwaZulu-Natal. All procedures involving the animals 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 (Barthold, Bayne, & Davis, 2011). The protocol for the study was approved by Animal Ethics Committee (protocol reference number: AREC/087/015D). The rats had unrestricted access to food (standard rat pellets) and water. All the rats were housed in plastic cages (three rats/cage) having dimensions of 30 long, 20 wide and 13 cm high, and softwood shavings employed as bedding in the cages. Rats were maintained under standardised animal house conditions (temperature: 28~31°C; light: approximately 12 hr natural light per day; humidity: 50%~55%). The drugs zidovudine, lamivudine and nevirapine (Aspen) were procured from Pharmed, Durban, South Africa, with batch numbers A844552, A847223 and A849377 respectively.

2.1 | Plant material

The solid endosperm of mature coconuts was commercially purchased from a local store in Durban area and was authenticated at the School of Life Sciences, Westville Campus, University of KwaZulu-Natal, South Africa.

2.2 | Preparation of VCO

The wet extraction method described by Nevin and Rajamohan (2006) was used for VCO extraction. Briefly, the solid, matured coconut was crushed and made into a viscous slurry, the water of about 500 ml was added and squeezed through cheesecloth to obtain coconut milk. The coconut milk produced was left for about 24 hr to aid the gravitational separation of the milk, which was in accordance with Nour, Mohammed, Yunus, and Arman (2009). Three phases resulted; a lower aqueous phase, a middle emulsion phase and an upper oily phase. The upper oily phase was then decanted and heated for about 10 min to remove moisture. The resultant VCO was then filtered through a fine sieve, stored in plain bottles at room temperature and used for the experiment.

2.3 | Experimental design

The animals were randomly distributed into four (4) groups (A-D) and treated as follows:

A (control group animals received distilled water).

B (HAART cocktail) using human therapeutic equivalent doses (600, 300 and 400 mg/day of zidovudine, lamivudine and nevirapine, respectively, were dissolved in 100 ml of distilled water and adjusted to the equivalent animal dose of 1.89, 0.95 and 1.26 mg/kg body weight respectively) (Ogedengbe et al., 2016; Umar et al., 2008).

C (HAART + VCO 10 ml/kg).

D (VCO 10 ml/kg) (Yeap et al., 2015).

All treatment was applied daily by orogastric gavage, and at the end of 56 days, all animals were killed 24 hr after the last treatment under excess halothane anaesthesia.

2.4 | Electron microscopy technique

This procedure was carried out as previously described by Trindade, Simões, Silva, Macedo, and Spadella (2013) and Hashish (2015). The testes were removed and sliced into one cubic millimetre segments. The segments were fixed in 2.5% glutaraldehyde and post-fixed in 0.5% osmium tetroxide (OsO_4), washed with 0.1M sodium cacodylate buffer (PH 7.2), dehydrated in graded series of acetone (30%, 50%, 75% and 100%), infiltrated with propylene oxide–resin and embedded in Spurr's resin. Following polymerisation, five semithin sections (1 μm

thick) of each animal were cut using Leica Ultracut R Ultramicrotome (Reichert, Austria) and stained with 1% toluidine blue for light microscopy. Ultrathin sections (50-70 nm thick) were cut on the cutting edge of glass knives using Leica EM UC7 Ultramicrotome (Austria), mounted on copper grids and stained with uranyl acetate and lead citrate (Hayat, 1989). The ultrastructural analyses of each section were examined under JEOL JEM-1010 transmission electron microscopy (TEM) at magnification 3,000 to 50,000 in the Microscopic and Microanalysis Unit (MMU) of the University of KwaZulu-Natal, Westville Campus.

2.5 | Measurement of the tubular basement membrane

Evaluation of the basement membrane thickness was carried out as previously described by Pop et al. (2011) and Shokri, Hemadi, and Aitken (2012). A total of ten seminiferous tubules were randomly selected using the same magnification with five random measurements performed on each of the tubules.

2.6 | Measurement of Leydig cell diameter and nuclear volume

A total of 10 Leydig cell nuclear diameters were measured per animal. The volume of Leydig cell nucleus was calculated using two different methods based on the shape of Leydig cell nucleus. The first method was used for the measurement of rounded nuclei using the formula:

$V = 4/3\pi r^3$ (Castro, Berndtson, & Cardoso, 2002).

The second method was for the measurement of oval nuclei using the revolution of spheroid formula: $V = P/6\ AP^2$.

where P = short diameter and A = long diameter (El-Sokkary, 2001; Lewiński, Vaughan, Champney, Reiter, & Smith, 1984). Measurements of the basement membrane thickness and Leydig cell nuclei were carried out using iTEM 5.0 Universal TEM Imaging Platform (Olympus Soft Imaging Solutions, Münster, Germany).

2.7 | Serum analysis of free testosterone, luteinising and follicle-stimulating hormonal levels

The blood was collected from the heart and allowed to clot for 2 hr at room temperature, and it was then centrifuged for 15 min at (1,000 x *g*). The collected supernatant/serum samples were kept in the deep freezer (−20°C) until measurement of testosterone hormone. The free testosterone, luteinising hormone (LH) and follicle stimulating hormone (FSH) levels were analysed by ELISA method with rat free testosterone, rat luteinising hormone and rat follicle stimulating hormone ELISA Kits respectively (Elabscience Biotechnology, Wu Han, P, R, C., China).

2.8 | Measurement of testicular tissue malondialdehyde level

The testicular tissue was homogenised in 0.2 M sodium phosphate buffer (7.8 pH) and then centrifuged for 15 min at 15,000 x *g* at 4°C. The supernatant was collected and used for the measurement of lipid peroxidation. The procedure was carried out using a complex formed from the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) as described by Chenni et al. (2007) with slight modification. Into an assay mixture containing 200 μl of 8.1% sodium dodecyl sulphate (SDS), 750 μl of 20% acetic acid (pH, 3.5), 2 ml of 0.25% TBA and 850 μl of distilled water, 200 μl of sample or MDA standard series (0, 7.5, 15, 22.5, and 30 μM) was added in a Pyrex screw-capped test tube. The mixture was heated at 95°C for 60 min in a sand bath, cooled down to room temperature and absorbance was read at 532 nm in a spectrophotometer (Synergy HTX multi-mode reader, VACUTEC, USA). Thiobarbituric acid reactive substance (TBARS) concentrations of samples were extrapolated from MDA standard curve.

2.9 | Measurement of testicular tissue reduced glutathione concentration

Reduced glutathione concentration was measured in tissue according to methods modified from Ellman (1959). The sample was first precipitated with 10% TCA and then centrifuged at 2,000 rpm for 10 min at 25°C. Reaction mixture contained 100 μl of supernatant, 50 μl of 0.5 mMDTNB and 150 μl of 0.2 M sodium phosphate buffer (pH, 7.8). Absorbance was measured at 412 nm using a spectrophotometer (Synergy HTX multi-mode reader, VACUTEC, USA) after 15 min incubation at 25°C, and glutathione (GSH) concentrations of samples were extrapolated from a standard curve of GSH.

2.10 | Statistical analysis

The results were expressed as mean ± standard error of mean. These were then subjected to within and between group differences using one-way ANOVA, followed by Dunnett's multiple comparison test, which was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA.

3 | RESULTS

3.1 | Control group

Ultrastructural examination of the control group rats (group A) showed normal testicular architecture with germinal epithelium in different stages of maturation progressing in an orderly manner indicating active spermatogenesis. This group is characterised by a high proportion of Sertoli cells and spermatogenic cells. Furthermore, the absence of morphological defects is evident in the seminiferous epithelium.

3.1.1 | Basement membrane, sertoli cells and spermatogonia

As shown in Figure 1a, the basement membrane shows normal diameter with the presence of several peritubular myoid cells

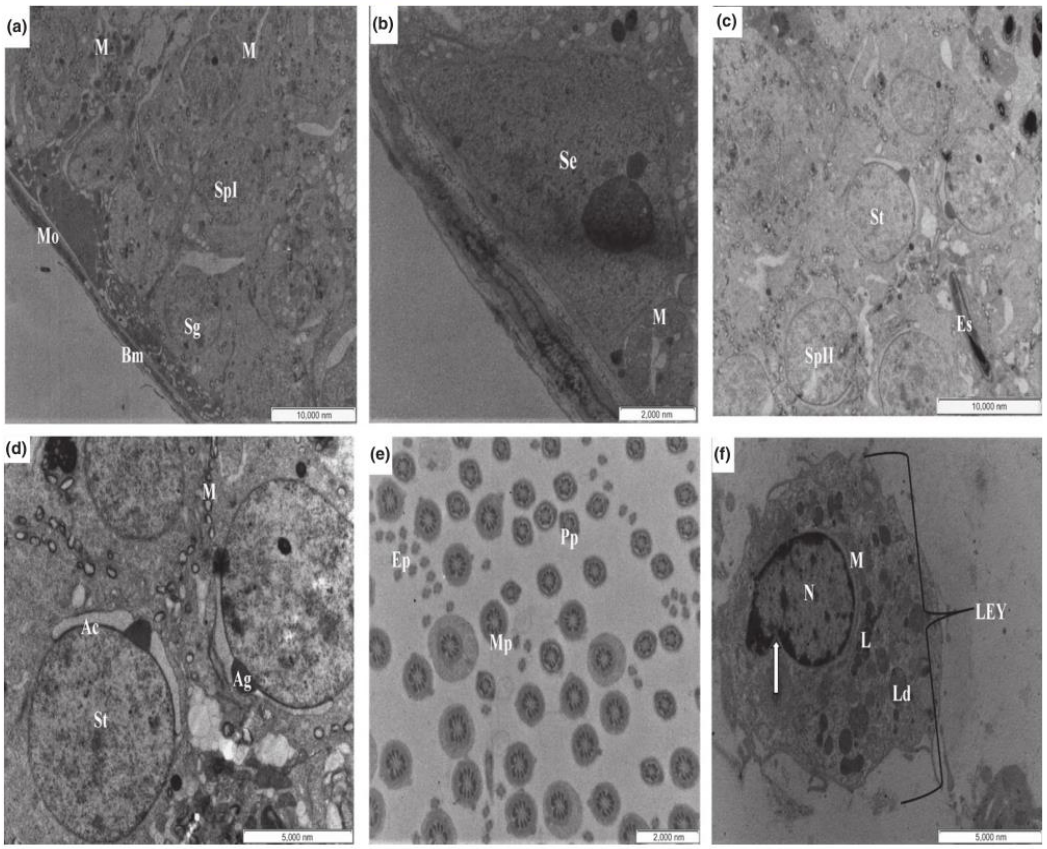

FIGURE 1 Ultrastructure of group A (control) depicts normal testicular architecture with germinal epithelium demonstrating different stages of spermatogenesis in an orderly manner. (a) reveals seminiferous tubules with basement membrane (Bm), myoid cells (Mo), abundant mitochondria (M), spermatogonium (Sg) and primary spermatocyte (SpI). (b) shows the Sertoli cell (Se) with prominent nucleolus; (c) shows secondary spermatocyte (SpII); and (d) shows early spermatid (St), acrosomal granule (A_G) and acrosomal cap (A_C). (e) shows several flagella of sperm cells containing a core of microtubules which includes the middle piece (Mp), principal piece (Pp) and end piece (Ep). (f) shows the interstitial cells of Leydig (LEY) with nucleus (N) denoting slight indentation (white arrow), lipid droplet (Ld) and lysosome (L)

surrounding the basal lamina. Spermatogonia, as well as the basal ends of Sertoli cells (Figure 1b), were found in normal relationship to the basal lamina of the basement membrane. Abundant mitochondria, smooth endoplasmic reticulum, ribosomes and a variable amount of lipid inclusions were noticeable in the Sertoli cell cytoplasm.

3.1.2 | Primary and secondary spermatocytes

Electron micrograph of Figure 1a and c) shows primary and secondary spermatocytes of the control group appearing rounded with distinct nuclei and characterised by a varied pattern of chromatin condensation. The cytoplasm of spermatocytes appears granular and shows scattered oval mitochondria and ribosomes with no manifestation of morphological abnormalities.

3.1.3 | Spermatids and spermatozoa

The early spermatids are rounded in configuration and well-defined in the adluminal compartment of the seminiferous tubules (Figure 1c and d). The nuclei appear to be rich in euchromatin. The cytoplasm is dispersed with abundant mitochondria. Ribosomes, smooth endoplasmic reticulum and lysosomes are also visible in this region. Acrosomal formation which is initiated by the appearance of the acrosomal granule in acrosomal vesicle is noticeable, while the acrosomal vesicle becomes enlarged over one part of the nuclear hemisphere to form an acrosomal cap. Electron micrograph of Figure 1c shows elongated spermatids, Figure 1e shows multiple spermatozoa with their tails. The tail region was divisible into middle, principal and end pieces. Each piece depicts a typical flagellar axoneme, which is composed of an array of microtubules, featuring nine doublets

radially arranged around two single central ones. Mitochondria sheaths are seen only throughout the middle piece. The end pieces are smaller in size and consist of the flagellar axoneme and the surrounding cell membrane.

3.1.4 | Interstitium

The interstitium (Figure 1f) presents normal morphology of the Leydig cells which are arranged in clusters of polymorphic cells. The nucleus shows a slight indentation, and it appears richer in euchromatin. It is also lined by a thin rim of heterochromatin. A great abundance of lipid droplets along with numerous mitochondria possessing well-developed cristae was seen in this region. Several other organelles such as lysosomes and smooth endoplasmic reticulum are well distributed in the Leydig cell cytoplasm of the control group.

3.2 | Group B (HAART only)

Testicular sections of HAART-treated group present several ultrastructural abnormalities.

3.2.1 | Basement membrane, sertoli cells, spermatogonia and spermatocytes

The ultrastructure of the HAART-treated group (Figure 2a) showed an increased thickness of the basement membrane (Figure 5a). Some of the Sertoli cells appear irregular and possess a large pale nucleus with prominent nucleolus resting on the basement membrane. There are degenerative changes in the Sertoli cell cytoplasm in the form of apoptosis with vacuolisation as well as loosening and disorientation of the spermatogenic epithelium. Cellular alteration and severe germ cell atrophy were demonstrated in this group as several spermatogonia appear not to be in contact with the basement membrane. Spaces were evident separating adjacent cells. Many of the spermatocytes showed discontinuous nuclear membrane with a high number of intercellular vacuoles in the cytoplasm. Several mitochondria underwent collapse of cristae were scattered amongst the cytoplasm of germinal cells (Figure 2a-b).

3.2.2 | Spermatids and spermatozoa

In Figure 2b, rounded spermatids are present with oval-shaped nucleus. Ultrastructure of elongated spermatids and mature spermatozoa shown in Figure 2c and d presents several deformities including increased acrosomal and subacrosomal swelling, the absence of the acrosome and ruptured nuclear membrane in the head of matured spermatozoa with some tail deformities.

Flagella of sperm cells can also be seen (Figure 2e), but are fewer than in the control group. There is evidence of ballooned tails with defective mitochondria sheaths in middle pieces. Cross section of several principal and middle pieces shows complete axonemal disorientation.

3.2.3 | Interstitium

Electron micrograph of Figure 2f showed shrunken nucleus with decreased diameter and volume (Figure 5b and c).The nucleus is also condensed with clumped chromatin, while its indentation appears deeper when compared with the control group. There is increased vacuolation in the cytoplasm. The mitochondria are fewer with severe alterations evident in some of them. Lysosomes and lipid droplets appear less conspicuous in the cytoplasm.

3.3 | Group C (HAART+VCO)

Testicular sections of group C showed improved ultrastructural appearance when compared with HAART-treated group B.

3.3.1 | Basement membrane, sertoli cells, spermatogonia and spermatocytes

As shown in Figure 3a and b, the ultrastructure of group C shows decrease in basal membrane thickness (Figure 5a) compared with the group B (HAART only), similar to that of the control group. Sertoli cell possesses a large euchromatic nucleus. The Sertoli cell cytoplasm shows more mitochondria and fewer vacuoles compared with the HAART only treated group. The spermatogenic epithelium shows some improvement as cells become more compact, although there are some cases of disruption leading to loosened disorientation of the epithelium. Spermatogonia display some contact with the basement membrane. The population of germinal cells appears more numerous and closely packed with little space when compared with the HAART-treated group. There is evidence of restoration of nuclear membrane of several germinal cells. Structural alterations in this region appear to be mild as the introduction of VCO triggers conspicuous recovery to the cells of the spermatogenic epithelium.

3.3.2 | Spermatids and matured spermatozoa

In Figure 3c, rounded spermatids display a large oval euchromatic nucleus with acrosomal cap (blue arrow) visible in some of them. Ultrastructural appearance in this region was improved as there were few cases of cytoplasmic vacuolisation. Organelles such as mitochondria appear to be abundant in the cytoplasm. There was drastic increase in the number of mature spermatozoa when compared with the HAART-treated group B. The heads of several of these spermatozoa exhibited improved ultrastructural appearance and in similar to that of the control group. The flagella of mature sperm cells were of normal appearance although a few cross sections displayed some mitochondria and axonemal degeneration (Figure 3d and e).

3.3.3 | Interstitium

The addition of VCO to HAART-treated rats shows great improvement in the interstitium (Figure 3f). The Leydig cell nucleus also

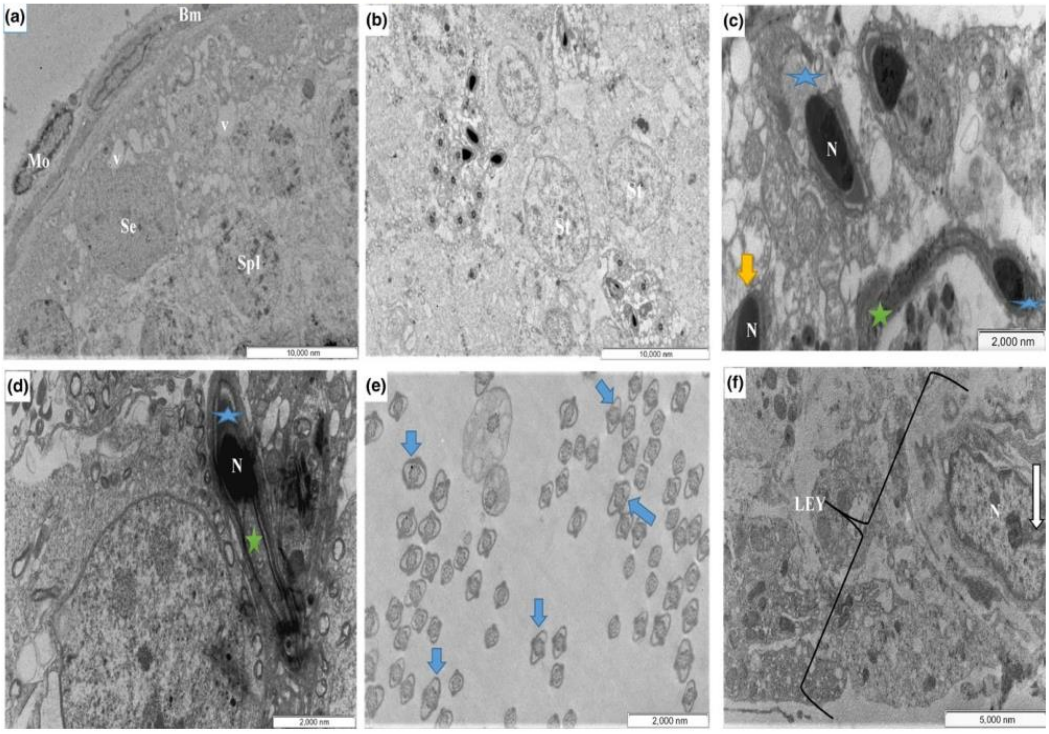

FIGURE 2 Ultrastructural micrograph of seminiferous tubule portion of group B highly active antiretroviral therapy ([HAART] only). Observation from this group presents several ultrastructural abnormalities and disorganised germinal cells. (a) showed increased thickness in basement membrane (Bm) and myoid cell (Mo), Sertoli cells (Se) with vacuolations (V). (b) shows spermatids; (c & d) shows morphological deformities in the head (blue star, yellow arrow) and tail regions (green star) of spermatozoa. (e) shows ballooned mitochondrial with disorganised axonemes in cross section of tail in section. (f) shows degenerative changes in Leydig cells, nucleus appears shrunken (N) with deep indentation (white arrow)

shows recovery from its shrunken state seen in group B, but its nuclear indentation remains deep. Effects on interstitial cells of Leydig are evident by the abundant numbers of cytoplasmic organelles (ribosomes and lipid droplets) when compared with the HAART only treated group.

3.4 | Group D (VCO)

3.4.1 | Basement membrane, sertoli cells, primary and secondary spermatocytes

The testicular sections of VCO only treated group show ultrastructural appearance similar to the control group with slight differences. The basement membrane thickness appears to be the thinnest amongst all groups. The Sertoli cells were seen resting on the basement membrane, having irregular shape nuclei with prominent nucleolus and fine granular chromatin (Figure 4a). The cytoplasm showed normal mitochondrial density. The germinal epithelium also presented with a normal architectural pattern showing different stages of maturation. Primary and secondary spermatocytes had normal appearance.

3.4.2 | Rounded and elongated spermatids, matured spermatozoa

Rounded spermatids presented several oval euchromatic nuclei. Acrosomal formation resulted in the development of prominent acrosomal caps which were seen covering the hemispheres of each spermatid. The cytoplasm of these rounded spermatids also showed abundant mitochondria with few vacuoles. Other organelles such as ribosomes and lysosome were present in normal numbers (Figure 4a-b).

Figure 4c showed several elongated spermatids, especially in the late stage of development with normal pyriform-shaped nuclei. Mature spermatozoa also displayed well-developed heads and tails. The tail region showed abundant cross sections of complete principal, middle and end pieces with no defects (Figure 4c-d).

3.4.3 | Interstitium

The ultrastructure of Leydig cells of rats treated with VCO only (Figure 4e) did not differ significantly from that of the control group and group C (HAART + VCO). A slight indentation was present in the

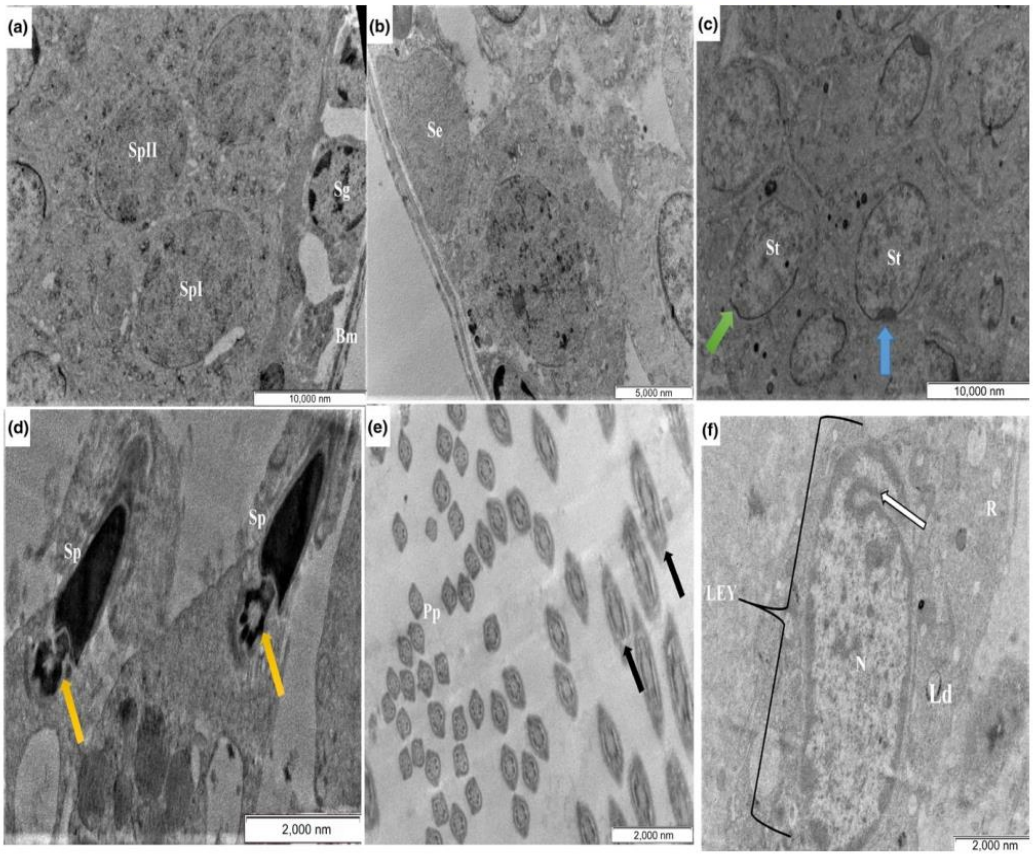

FIGURE 3 Ultrastructural micrograph of seminiferous tubule portion of group C (HAART + [VCO]Virgin coconut oil). Observation from this group presented an improvement in the ultrastructural appearance. (a) there is increase in germinal cells, and they appear more compacted, reduced basement membrane thickness (Bm). (b) shows Sertoli cell; (c) shows the presence of acrosomal cap (blue arrow), while green arrow shows the presence of incomplete acrosomal formation. Several pieces of the tail show improved ultrastructural changes in (c-e) with less amount of deformities, yellow and black arrows for tail deformity in some middle pieces with mitochondria degeneration; and green arrow shows disorganised axoneme. (f) shows Leydig cell with abundant lipid droplets (Ld) supporting numerous spermatocytes, nucleus (N) presenting deep indentation (white arrow)

Leydig cell nucleus, with the cytoplasm containing several mitochondria, free ribosomes and some lipid droplets.

3.5 | Changes in basement membrane, Leydig cell nuclear diameter and volume

Figure 5a shows a moderately significant increase ($p < .01$) in the mean basement membrane thickness of HAART-treated group B (2.40 ± 0.19 μm) when compared with the control group (1.30 ± 0.04 μm). There were no significant differences ($p < .05$) in the mean basement membrane thickness of HAART + VCO group C (1.2 ± 0.04 μm) and VCO group D (1.2 ± 0.03 μm) when compared with the control group. Figure 5b shows a significant decrease ($p < .05$) in the mean Leydig cell nuclear diameter in HAART-treated group B (7.50 ± 0.56 μm) compared with the control group (9.60 ± 0.36 μm). The mean Leydig cell nuclear diameter for groups C and D is 8.50 ± 0.54 μm and 10.06 ± 0.50 μm respectively. Figure 5c shows a moderately significant decrease ($p < .01$) in the mean Leydig cell nuclear volume in HAART group (15.61 ± 3.69 μm^3) compared with the control group (55.72 ± 9.63 μm^3). The mean Leydig cell nuclear volume for HAART + VCO group C and VCO group D is 29.09 ± 5.37 μm^3 and 71.43 ± 10.78 μm^3 respectively.

3.6 | Changes in serum testosterone, luteinising and follicle-stimulating hormonal levels

Figure 6a reported a moderately significant increase ($p < .01$) in the mean serum testosterone level of HAART-treated group B (437.0 ± 3.416 pg/ml) when compared with the control group (375.1 ± 12.23 pg/ml). The mean serum testosterone of the HAART + VCO group C and VCO group D is 344.9 ± 13.53 pg/ml and 378.2 ± 11.39 pg/ml respectively. On the other hand, result of the mean serum luteinising hormone (Figure 6b)

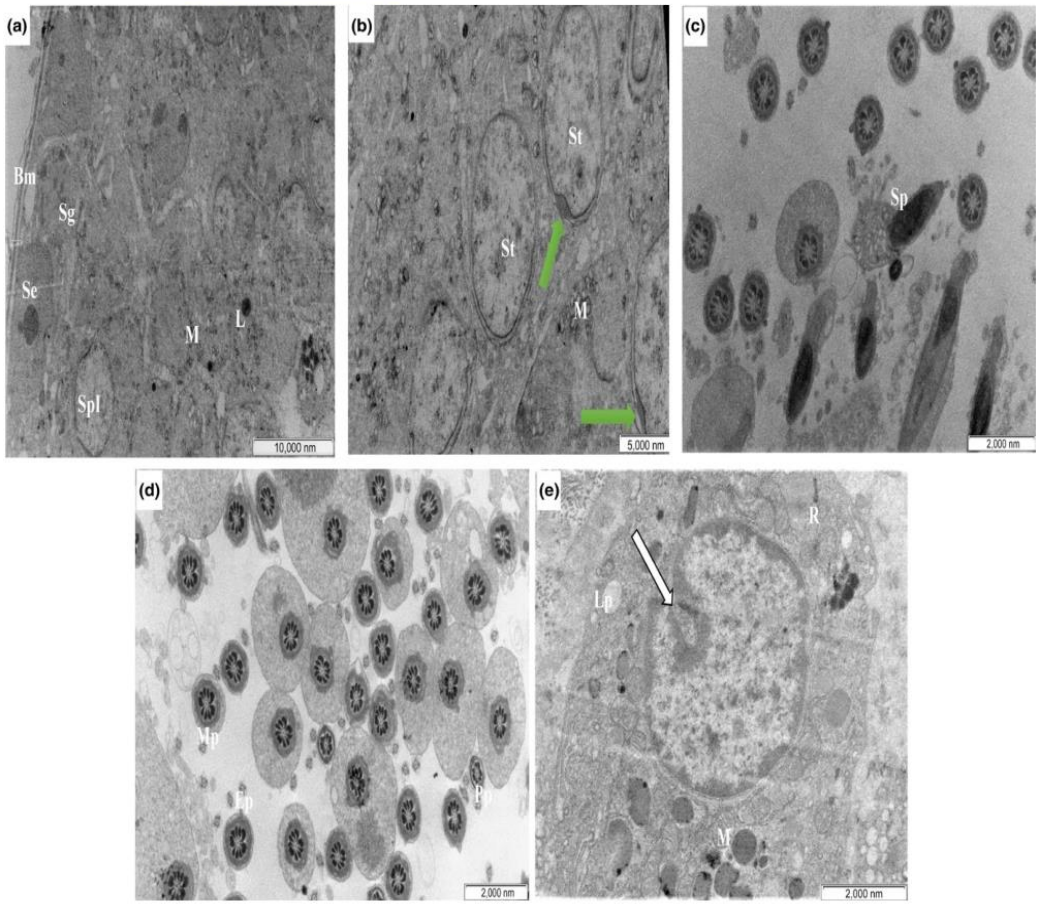

FIGURE 4 Virgin coconut oil -treated group D showed thinned BM with normal ultrastructural features of organelles (mitochondria, ribosomes, lipid droplets) all showing essential characteristics like control group. Green arrow showing acrosomal cap (A_C) of rounded spermatid. Leydig cells showed slight indentation with several organelles, abundant mitochondria (M), lipid droplets (Lp) and ribosomes (R)

was similar in all groups. The mean serum LH for group A, B, C and D was 220.0 ± 4.8 mIU/ml, 220.0 ± 1.1 mIU/ml, 220.0 ± 0.97 mIU/ml and 220.0 ± 1.8 mIU/ml respectively. There were no cases of statistical significance ($p < .05$) across groups.

Figure 6c shows the mean increase in FSH concentration in the VCO-treated group D (7.7 ± 4.12 ng/ml) vs. the HAART-treated group B (1.4 ± 1.99 ng/ml). The mean FSH for group A and C was (2.3 ± 1.93 ng/ml) and (4.9 ± 2.10 ng/ml) respectively. There was no statistical significance ($p > .05$) when experimental groups B, C and D were compared with control group A.

3.7 | Changes in testicular tissue malondialdehyde and reduced glutathione concentrations

As shown in Figure 7a, the mean testicular tissue malondialdehyde showed highest and lowest peak concentrations in the HAART-treated group B (638.4 ± 220.6 μmol/L) and VCO only (428.4 ± 108.0 μmol/L), respectively, when compared across the groups. An ANOVA showed no significant difference between groups at the $p < .05$ level. Group C HAART + VCO showed no significant alterations when compared with all groups.

Figure 7b shows the mean glutathione concentrations of the testicular tissue. The lowest and highest concentrations were recorded in the HAART-treated group B (0.023 ± 0.001 mM) and VCO only (0.030 ± 0.003 mM) respectively. The mean glutathione concentrations for control group A and HAART + VCO group C were 0.026 ± 0.001 mM and HAART + VCO was 0.025 ± 0.002 mM respectively. An ANOVA showed no significant difference between groups at the $p < .05$ level.

4 | DISCUSSION

Studies have shown that the basement membrane plays an essential role in sustaining the structural and functional integrity of the

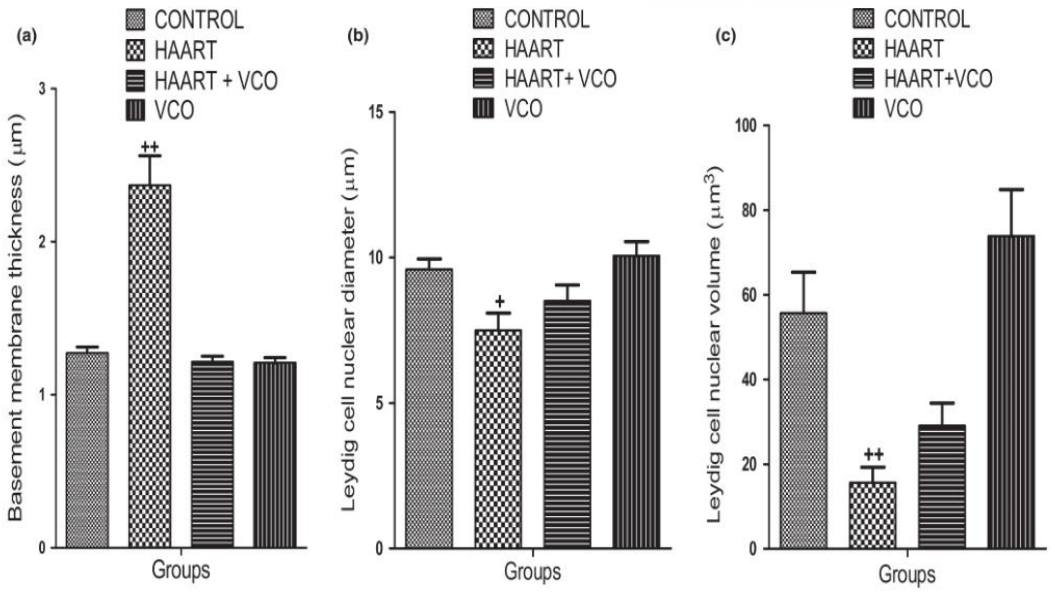

FIGURE 5 Effect of Virgin coconut oil and highly active antiretroviral therapy (HAART) treatment on (a) basement membrane, (b) Leydig cell nuclear diameter and (c) Leydig cell nuclear volume. Bars indicate the mean ± SEM. $^{+}p < .05$, $^{++}p < .01$

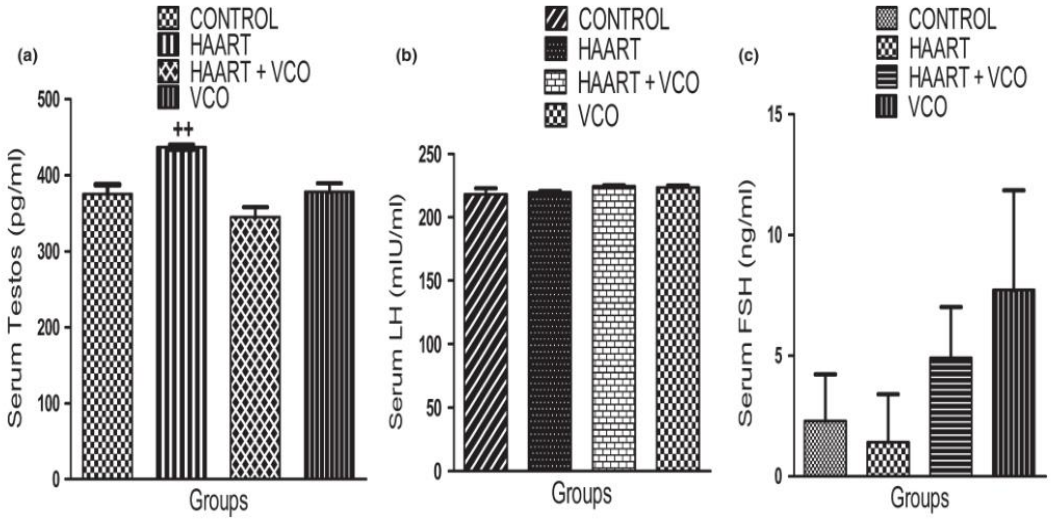

FIGURE 6 Effect of Virgin coconut oil and highly active antiretroviral therapy (HAART) treatment on serum testosterone (a) serum luteinising hormone (b) serum follicle stimulating hormone (c) in Sprague-Dawley rats after 8 weeks treatment period. Bars indicate the mean±SEM. $^{++}p < .01$

testis (LeBleu, MacDonald, & Kalluri, 2007). Impairment of the membrane will severely compromise testicular functionality (Dobashi et al., 2003). In the present study, the testicular ultrastructure of the HAART-treated rats displayed an abnormally significant increased thickness of basement membrane (refer to Figure 5a). This actually conforms to our previous work and another study indicating that HAART increases carbohydrate components and contributes to extensive fibrosis around the basement membrane (Azu et al., 2014; Ogedengbe et al., 2016). As the entire basement membrane is made of extracellular matrix, interactions between the matrix, tubular wall and germinal cells will contribute significantly to normal testicular development (Favorito, Hidalgo, Pazos, Costa, & Sampaio, 2005).

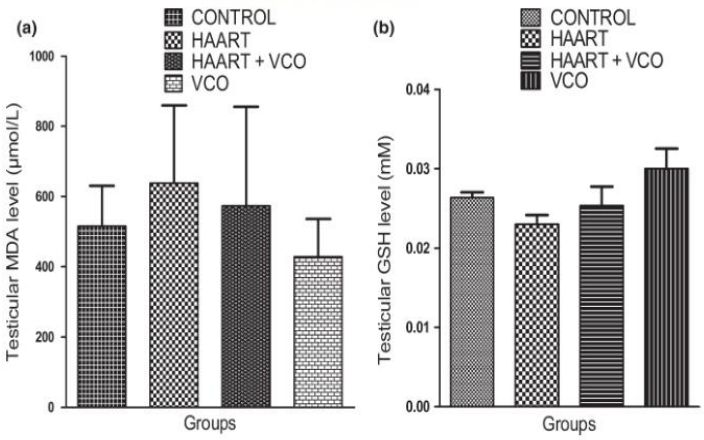

FIGURE 7 Effect of Virgin coconut oil and highly active antiretroviral therapy (HAART) treatment on testicular malondialdehyde level (a), testicular reduced glutathione (b) in Sprague-Dawley rats after 8 weeks treatment period. Bars indicate the mean±SEM. There were no cases of statistical significance ($p > .05$) across groups

Furthermore, the presence of type IV collagen (most abundant protein in the basement membrane) has been fundamental in the maintenance of its stability (LeBleu et al., 2007), and germinal cell development can be adversely affected by its overproduction by myoid and Sertoli cells (Dobashi et al., 2003). This in turn leads to interstitial fibrosis, infiltration and a thickened basement membrane in the seminiferous tubules (Clark, Garland, & Russell, 2000). According to Gulkesen, Erdogru, Sargin, and Karpuzoglu (2002), overexpression or degradation of the extracellular matrix is a major cause of male infertility in humans. Experimental animal studies on HAART have confirmed increased infiltration, thickened membrane, extensive necrosis and atrophy with disruption of the normal maturation stages of spermatogenesis (Azu et al., 2014).

The role of Sertoli cell in the process of spermatogenesis is very crucial in creating a conducive environmental milieu which is important for the development of germ cells. It also provides hormonal secretions and several nutrients for the normal development of germ cells up to the maturity stage (Monsees et al., 2000). In the current study, Sertoli cell of the HAART-treated group showed degenerative changes such as cytoplasmic vacuolations and mitochondrial cristae appear collapsed in section. Disorganised spermatogenic cell series were also evident in the lumina of seminiferous tubules.

Previous research has shown that the spermatogenic cell population is regulated by germ cell apoptosis to a size appropriate for the supportive role of Sertoli cell (Shokri et al., 2012). Furthermore, the Sertoli cells have been considered a prime target for several reproductive toxicants which can cause impairment of the Sertoli cell function, leading to increased degradation of germ cell and consequent testicular dysfunction (Monsees et al., 2000). Severe degenerative changes seen in the cytoplasm of Sertoli and germinal cells of HAART-treated animals (group B) are a clear indication of the over-riding effect of HAART-induced distortions on the supportive role of Sertoli cells. The presence of vacuoles in Sertoli cells has been reported as being one of the first and most common morphological features following injury. This is succeeded by germ cell degeneration, disorganisation or exfoliation (Creasy, 2001). Severe vacuolisation in the Sertoli and germinal cell cytoplasm, alongside degeneration of spermatogenic cells in the seminiferous tubules coincide with the ravages of HAART on Sertoli cell.

The contribution of the cytoskeleton in the efficient functioning of Sertoli cell is considered critical. These roles include Sertoli–germ cell attachment, germ cell movement from basal region to the lumen which helps to preserve the normal architecture of the germinal epithelium keeping it in its normal compact shape. In addition, transfer of nutrients will also be disrupted when germinal cells are detached from Sertoli cells resulting in the death and collapse of spermatogenic cells (Creasy, 2001). The looseness or disorientation of the cells of the germinal epithelium in HAART-treated animals is a clear indication of perturbations secondary to Sertoli cell injury.

Follicle stimulating hormone (FSH) has been known over the years as an important marker for Sertoli cell function and spermatogenesis. Its action on Sertoli cell has also been reported to increase spermatogenic output (Jensen et al., 1997). The decrease in serum FSH concentration as demonstrated in the HAART-treated animals when compared across groups corroborates the negative influence of HAART has on Sertoli cell functions.

The present study highlights several ultrastructural abnormalities such as nuclear membrane discontinuations, loss or absence of acrosomal cap, ballooned mitochondria, head and tail deformities of spermatozoa and flagella abnormalities depicting mitochondria and axoneme disorientation with defective mitochondrial sheaths. In support of our results, Doaa, Ashraf, Ahmed, and Amal (2014) observed disorganisation of the axonemes, outer fibrous sheaths and swollen and distorted mitochondrial sheaths on exposure to certain toxicant.

Antiretroviral drugs have been reported to give rise to several life-threatening complications such as mitochondrial dysfunction arising from altered mitochondrial DNA (mtDNA) replication and generation of reactive oxygen species (ROS) leading to oxidative stress (Day & Lewis, 2004). In this study, the damage to mitochondria is probably due to elevated ROS production leading to membrane damage with increased permeability to HAART. This is accompanied by disruption of normal oxidative phosphorylation and cytoplasmic swelling which

eventually results in disruption of cristae, collapse of internal mitochondrial structure and energy deprivation (Tolomeo et al., 2003).

In addition, HAART can adversely impact sperm DNA integrity and chromatin remodelling. It may also induce excessive sperm apoptosis through negative interference on antioxidant defence mechanism (Saleh et al., 2003). Spermatozoal vulnerability to oxidative damage by HAART toxicity relies on the high proportion of polyunsaturated fatty acids in the plasma membrane, alongside low cytoplasmic level of scavenging enzymes (Pace, Lawrence, Behr, Parsons, & Dias, 2005). Hashish (2015) also indicated that abnormal Sertoli cell may contribute to the defective nuclear shaping of spermatids. Moreover, several authors have reported morphological abnormalities leading to excessive damage to spermatozoa in HIV-infected patients undergoing HAART (Barboza et al., 2004; Nicopoullos, Almeida, Vourliotis, & Gilling-Smith, 2011).

The ultrastructural examination in this present study reveals severe morphological alteration of the interstitium in the HAART-treated group. Ultrastructure of the Leydig cells showed degenerative changes with fewer mitochondria and less dense cytoplasmic matrix. The Leydig cell nucleus also showed a shrunken appearance with significant decrease in nuclear diameter and volume (Figure 5b and c). The close alliance between Leydig cells and blood vessels is an indication that these cells can become highly endangered by exogenous toxicants (Elshennawy & Elwafa, 2011). Blanco et al. (2009) reported a similar result of Leydig cell nuclear shrinkage, while de Souza Predes, Monteiro, Matta, Garcia, and Dolder (2011) observed fewer mitochondria with dense cytoplasmic matrix. Furthermore, steroidogenesis has been reported to take place within the Leydig cells of the testis, and the production of testosterone is influenced by the activity of LH on the Leydig cells. Thus LH depletion will result in reduced testosterone production (Ichihara, Kawamura, Nakano, & Pelliniemi, 2001). However, the highest testosterone concentration was found in the HAART-treated animals which appears discordant with the degenerative changes such as decreased Leydig cells, reduction in Leydig cell nuclei diameters and volumes seen in the interstitium of this group at ultrastructural level. Though the mechanism behind these findings remains unclear, a single testosterone level measurement is insufficient to indicate hypogonadism (Bhasin et al., 2006; Dube et al., 2007). It is also possible for mitochondria dysfunction to play a role here, as it is associated with an increase in fat-free mass. This in turn increases the free testosterone level following the initiation of HAART (Dube et al., 2005, 2007). Our findings were consistent with several other hormonal studies on HAART. Collazos, Martinez, Mayo, and Ibarra (2002) reported an increased testosterone level in HIV patients undergoing HAART. Dube et al. (2007) also observed an increase in free testosterone after the initiation of antiretroviral in HIV patients.

Luteinising hormone concentrations were similar in all groups. The concentration of LH appears normal and similar to that of the control group owing to its production from the anterior lobe of the pituitary glands being unaffected (Babu, Sadhnani, Swarna, Padmavathi, & Reddy, 2004).

Lipid peroxidation has been cited as one of the leading detrimental consequences of free radicals and ROS formation (Gaweł, Wardas, Niedworok, & Wardas, 2003). Malondialdehyde (MDA) is an oxidative stress marker that can be used to measure the extent of lipid peroxidation (Gaweł et al., 2003). Ultrastructural injury as seen in many tissues often arises from intense oxidation of lipid membranes culminating in impaired function of cellular organelles (Milei et al., 2007). In the current study, we detected the highest level of MDA level in HAART-treated group signifying an occurrence of lipid peroxidation. Although not significant, our result still corroborates previous reports by Oyeyipo et al. (2015) and Adaramoye, Akanni, Adewumi, and Owumi (2015) indicating an increased MDA levels after treatment with antiretroviral drugs.

Reduced GSH is also an important antioxidant which plays a critical role in the prevention of cellular damage from ROS (Jurczuk, Moniuszko-Jakoniuk, & Rogalska, 2006). It also quantifies its potential defensive capacity to detoxify many xenobiotics. The lowest concentration of testicular GSH was found in the HAART-treated (group B), which was improved by the introduction of VCO (group C) and showed the highest concentration in the VCO only (group D), demonstrates the antioxidative potential and powerful countermeasures of VCO against lipid peroxidation in tissues (Marina, Che Man, Nazimah, & Amin, 2009; Ramos, Alía, Bravo, & Goya, 2005; Yeap et al., 2015).

However, we report, for the first time, the role of VCO in alleviating HAART-induced testicular ultrastructural alterations. Our previous work on the testicular histomorphology and seminal fluid parameters revealed that VCO mitigated HAART-induced alterations in male Sprague-Dawley rats (Ogedengbe et al., 2016). Accumulating evidence has also shown that VCO is effective in improving semen parameters (Dosumu et al., 2010), enhances insulin secretion, lower lipid levels in serum and tissues, and could alleviate other pathological conditions (Rahim et al., 2017).

In the current study, VCO was effective in improving the ultrastructure of the testis and was able to reverse most ravages induced by HAART. However, we suggest that the positive indices through its improved antioxidant defensive capacity reflect its ability to mitigate against HAART injuries to the testis. Aside from the antioxidative properties, several other mechanisms of action have been reported to be responsible for the protective function of VCO. Virgin coconut oil relies on its high unsaponifiable lipid components like vitamin E and polyphenols, tocotrienols, tocopherols, β carotene and phytosterol in stabilising cell membranes by preventing alterations in membrane lipid polarity and fluidity (Jaarin et al., 2014; Nevin & Rajamohan, 2006). Moreover, VCO prevents mitochondrial damage owing to it being highly saturated and not prone to oxidation (Lemieux, Bulteau, Friguet, Tardif, & Blier, 2011).

5 | CONCLUSION

Virgin coconut oil supplementation as demonstrated in this study reversed testicular ultrastructural alterations induced by HAART owing to its ability to mitigate oxidative injury possibly through its antioxidative capacity. Further studies on the precise pathways and mechanisms involved in the interaction between HAART and VCO are warranted.

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CONFLICT OF INTEREST

None declared by all author.

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BRIDGING TEXT

FROM CHAPTER FOUR TO FIVE

The negative impact of HAART on the testis has been reported in chapters three and four. VCO as an adjuvant with HAART, has demonstrated its antioxidant potential in mitigating most of the ravages induced by HAART. It is in this regard that the next chapter was designed to investigate the impact of concomitant use of alcohol with antiretrovirals owing to the high prevalence of alcohol use amongst HIV infected patients on treatment. We took into consideration the possibility of exacerbated toxicities arising from the concomitant use of these two agents. In addition, we introduced VCO due to its antioxidant potential and the possibility of ameliorating toxicity that may arise from the use of alcohol with HAART. Hence chapter five investigated VCO as an adjuvant to the deleterious effects of alcohol with HAART on the cyto-architecture and functioning of the testis in an animal model.

CHAPTER FIVE

MANUSCRIPT FOUR

Virgin Coconut Oil Extract Mitigates Testicular Induced Toxicity of Alcohol Use in Antiretroviral Therapy

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VIRGIN COCONUT OIL EXTRACT MITIGATES TESTICULAR INDUCED TOXICITY OF ALCOHOL USE IN ANTIRETROVIRAL THERAPY

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Abstract

The consumption of alcohol by people living with HIV/AIDS is associated with a graver prognosis. Long term use of antiretrovirals may have certain health challenges that may be aggravated by concomitant alcohol use. This study investigated Virgin coconut oil (VCO) as an adjuvant to the deleterious effects of highly active antiretroviral therapy (HAART) and alcohol on the cyto-architecture and functioning of the testis. Forty adult male Sprague-Dawley rats, weighing 165~176g were divided into eight groups and treated according to protocol. Testicular histology, stereological parameters, seminal fluid, testosterone, luteinizing hormone, follicle stimulating hormone, the antioxidants marker malondialdehyde (MDA) and antioxidant glutathione (GSH) were examined. The use of ethanol alone and ethanol + HAART showed extensive degeneration in the seminiferous epithelium, decreased semen quality, disorganised basement membrane and widened, hypocellular interstitium. GSH was significantly decreased in the ethanol alone treated group with no significant effect on Testosterone, LH, and MDA levels. Adjuvant treatment with VCO at low dose (2.5ml/kg/bw) improved sperm motility with a partial restoration of the histopathological alterations. High doses of VCO (5.0ml/kg/bw) showed greater improvement with respect to sperm counts, increased FSH hormonal and GSH antioxidant levels and a well preserved testicular cytoarchitecture.

Keywords: Alcohol, HAART, Virgin coconut oil, Seminiferous epithelium, Semen quality

Introduction

The frequency of alcohol use amongst people living with HIV/AIDS (PLWHA) undergoing antiretroviral treatments has been recognized to pose a serious clinical management dilemma with dire implications for global health (Kekwaletswe and Morojele, 2014b, Soboka et al., 2014b). This has been linked to some fundamental altered therapeutic indices which addresses HIV virology, pathogenesis and response to antiretroviral treatments (Kumar et al., 2012b). The global expansion of access to highly active antiretroviral therapy (HAART) has recorded significant improvement in the management of HIV/AIDS (Arenas-Pinto et al., 2015b). HAART has a positive impact on AIDS patients' survival with a sharp decline in morbidity and reduction in new HIV cases (Bendavid et al., 2012, Chang et al., 2015b). Despite its high success rate however, there are still many adverse effects incurred from their chronic use (Nagiah et al., 2015b).

In clinical practice and animal experimental studies, the long term use of HAART has shown negative consequences on the male reproductive system (Azu et al., 2014). These range from several potentially fatal conditions such as mitochondrial dysfunction and oxidative stress damage to several lesser detrimental effects (Pavili et al., 2010a, Ahmad et al., 2011b). The habitual or heavy consumption of alcohol has been recognized to impair sexual potency in men (La Vignera et al., 2013b). These undesired effects include deterioration in semen quality and sperm functional parameters, sexual hormonal impairment and overall organ damage resulting in decreased male fertility (Ramlau-Hansen et al., 2010a, Rai and Rai, 2016a). There is increasing evidence that alcohol can further exacerbate the side effects of many antiretrovirals (ARVs) with these interactions altering the bioavailability, leading to reduced ARV efficacy and acceleration of disease progression (Cooper and Cameron, 2005b, Braithwaite and Bryant, 2010b, Kumar et al., 2012b).

The use of traditional remedies as complementary and alternative therapies has been widely embraced across Africa and many developed countries in the world (Braun et al., 2010b). Herbal medicines and products often provide a better alternative to synthetic pharmaceuticals (Shah, 2007b). They are less liable to cause toxic effects, locally accessible and inexpensive (Jaarin et al., 2014b). Certain herbal remedies also show some degree of efficacy against HIV infections (Mills et al., 2005, Zou et al., 2012), as well as ameliorating testicular damage caused either by alcohol (Kurus et al., 2009, Dosumu et al., 2012b) or HAART (Ogedengbe et al., 2016, Jegede et al., 2017).

Virgin coconut oil (VCO) is a natural oil which has in recent times gained tremendous popularity globally owing to its wide range of health benefits. It has been reported to significantly improve semen quality as well as testosterone levels that are adversely affected by alcohol (Dosumu et al., 2012b). Our previous study also demonstrated the mitigating potential of VCO on HAART induced testicular ravages (Ogedengbe et al., 2016). Other health benefits of VCO include neuroprotective and memory enhancing

abilities (Rahim et al., 2017), a cardioprotective potential (Kamisah et al., 2015), antidiabetic capacity (Akinnuga et al., 2014), hepatoprotective potential (Zakaria et al., 2011) and ability to help reduce body weight (Gunasekaran et al., 2017).

In addition, VCO contains more unsaponifiable components. Its richness in phenolic compounds such as flavonoids and polyphenols amongst other compounds, effectively increases its antioxidant capacity and provides protection against oxidative stress damage (Nevin and Rajamohan, 2006). However despite extensive work on VCO, there are no scientific reports to date demonstrating its potential to ameliorate testicular injuries involving alcohol with antiretroviral therapy. This study therefore investigates the possible role of VCO as an adjuvant to HAART and alcohol in limiting adverse cyto-architectural and functional changes in the testis.

2.0 Materials and Methods

2.1 Plant material

The solid endosperm of mature coconuts were commercially purchased from a local store in the Durban area and was authenticated at the Department of Life Science, Westville Campus, University of KwaZulu-Natal, South Africa

2.2 Preparation of VCO

The wet extraction method described by Nevin and Rajamohan (2006) was used for VCO extraction. Solid mature coconuts were crushed and made into viscous slurry. 500 mls water was added to thin down slurry and squeezed through a cheese cloth to obtain the coconut milk. The coconut milk produced was left for 24 hours to aid gravitational separation in accordance with Onsaard et al. (2005a) and Nour et al. (2009a). Three phases resulted; a lower aqueous phase, a middle emulsion phase, and an upper oily phase. The upper oily phase was then removed and heated for 10-15 minutes to remove visible water and as much moisture as possible. The resultant pure VCO was then filtered through a fine metallic sieve to remove any residual fine particulates and stored in plain bottles for 2 weeks at room temperature ready for use.

2.3 Animals and treatments

Forty adult male Sprague-Dawley rats weighing 165g to 176g were used for this study. The animals were bred and maintained at the Animal House of the Biomedical Resources Unit, University of KwaZulu-Natal. All procedures involving the animals was 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 (Council, 2010). The protocol for the study was approved by Animal Ethics Committee (protocol reference number: AREC/087/015D). The rats had unrestricted access to food (standard rat pellets) and water. All the rats were housed in plastic cages (3 rats/cage) having dimensions of 30 cm long, 20 cm wide and 13 cm high) and soft wood shavings employed as bedding in the cages.

Rats were maintained under standardized animal house conditions (temperature: 28~31^{o}C; light: approximately 12 hr. natural light per day; humidity: 50~55%). The drug Zidovudine, Lamivudine, and Nevirapine (Aspen) were procured from Pharmed, (Durban, South Africa) with Batch numbers A844552, A847223 and A849377 respectively. Absolute Ethanol (99%) was procured from LABOQUIP (Johannesburg, South Africa - Batch 15/082).

The animals were randomly distributed into eight (8) groups (A-H) of five (5) rats per group as indicated below.

Animals received treatments 5 days in a week, with 2 days off for the entire 8 weeks study period.

Group A: control animals receiving only distilled water

Group B: received Ethanol only (5ml/kg BW of 20% w/v) (Erukainure et al., 2011b)

Group C: received Ethanol + HAART. The HAART cocktail using human therapeutic equivalent doses (600, 300 and 400 mg/day of Zidovudine, Lamivudine, and Nevirapine respectively was dissolved in 100 ml of distilled water and adjusted to the equivalent animal dose of 1.89, 0.95 and 1.26 mg/kg body weight respectively) (Umar et al., 2008, Ogedengbe et al., 2016).

Group D: Ethanol + HAART (Reversal group)

Group E: Ethanol + HAART + VCO_1 (2.5 ml/kg body weight)

Group F: Ethanol + HAART + VCO_2 (5 ml/kg body weight)

Group G: Ethanol + VCO_1

Group H: Ethanol + VCO_2

Treatment was administered by oro-gastric gavage. At the end of 56 days the animals were killed 24 hours after the last treatment under halothane® anesthesia except for Group D animals which served as the reversal group. In this group HAART and Ethanol were discontinued to ascertain whether their effects were reversible when withdrawn. These animals were killed on day 84 following withdrawal of all treatment for 28 days.

2.4 Body and testicular weight

The body weights (BW) of animals were recorded on the first day before treatment (initial) and thereafter on a weekly basis until the day of sacrifice. Testicular weight (TW) was measured by an electronic balance (Mettler Toledo; Microsep (Pty) Ltd, Greifensee, Switzerland). The testes of each rat were measured individually, and the average value obtained for each of the two measurements was regarded as one observation. The values were expressed in grams (g) for TW.

2.5 Semen analysis

The caudal epididymis of one tesis were excised and minced with an anatomical scissors. A drop of epididymal fluid was placed onto a glass slide, covered with 22×22 *mm* cover slip and immediately examined under a light microscope (Leica DM500, CH-9435 Heerbrugg, Switzerland) (Organization,

1999). The total field was scanned systematically and spermatozoa motility assessed (Rizk and Sallam, 2012) . Motility was graded as progressive, non-progressive and immotile (Vasan, 2011a). The percentage of motile sperm was then estimated and reported to the nearest 5% using a subjective determination of motility (Keel and Webster, 1990).

A portion of the macerated caudal epididymis was diluted with normal saline (1:9) and sperm count determined using Biorad® automated cell counter 1450101TC 20TM with a double slide counting chamber. The dilution was mixed thoroughly and both sides of the counting chamber were scored and the average of the two counts were taken and sperm count expressed in millions/ml (Keel and Webster, 1990).

2.6 Histopathology of the testes

The testes were removed and weighed. The left testis was used for biochemical analyses and the right testis fixed in 10% Neutral buffered formalin and processed for histology. For routine histology, the testis tissues was subject to systematic serial sectioning at 4µm intervals using a microtome (Microm HM 315, Germany) and stained with hematoxylin and eosin (H&E). Examination was done by a histopathologist blinded to the study protocol.

2.7 Morphometric analyses

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 of the seminiferous tubules (diameter and cross-sectional area) was determined using systematic random sampling (Gundersen and Jensen, 1987) that ensured fair distribution between the polar and equatorial regions of each testis. The 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 was to eliminate longitudinal profiles which might exhibit different degrees of damage along their length and show irregular shrinkage as previously reported (Christensen and Peacock, 1980, Gundersen and Jensen, 1987). The tubules were scanned using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at X 100 magnification using image analyzer Leica (DMLB) and Leica microsystem software. The diameter of the seminiferous tubule was measured across the minor and major axes, and the mean diameter obtained. Cross-sectional area (Ac) of the seminiferous tubules was then calculated from the formula; $A_C = \pi D^2/4$ (where π is equivalent to 3.142 and D is the mean diameter of the seminiferous tubules).

2.8 Stereological study

The procedure was carried out by point counting method in order to calculate the volumes of the germinal epithelium (GE), lumina (L) and testicular interstitium (I). This was done in accordance with the method

previously described by Weibel (1979), Howard and Reed (2004). Four sections per testis and six fields per section were randomly chosen for analysis. Fields were sampled from images captured using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany) and measured at X 100 magnification using image analyzer Leica (DMLB) and Leica microsystem software.

Volume densities of testicular components (GE, L and I) were determined by randomly superimposing a transparent grid comprising 160 test points per image arranged in a quadratic array (Freitas et al., 2011a). The ratio of the number of point intersections on the grid overlying each tissue component (P_N) to the total number of the points of the grid (P_T) was considered as the volume density (V_V) of the component, with the formula:

$V_V = P_N / P_T$ (Bielli et al., 2001b)

V_V values for GE, L and I were multiplied by 100 and expressed in percentages (%).

The absolute volume (AV) of each testicular components was evaluated based on the previous method of Howard and Reed (2004) with modification. This was obtained by multiplying the corresponding volume densities with the testicular weight (TW). Values were expressed in ml.

2.9 Serum analysis of free testosterone, luteinizing and follicle stimulating hormonal levels

Blood was collected from the heart and allowed to clot for 2 hours at room temperature. It was then centrifuged for 15 minutes at (1000xg). The collected supernatant (serum) was kept in the deep freezer (-80^0C) and all hormone measurements done within 6 months. The free testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were analyzed by ELISA method using rat free-testosterone, rat luteinizing hormone and rat follicle stimulating hormone ELISA Kits (Catalog numbers: E-EL-R0389, E-EL-R0026 and E-EL-R0391 respectively - Elabscience Biotechnology, Wu Han, P, R, C., China).

2.10 Measurement of testicular tissue malondialdehyde level

The testicular tissue was homogenized in 0.2M sodium phosphate buffer (7.8 pH) and then centrifuged for 15 mins at 15000xg. The supernatant was collected and used for the measurement of lipid peroxidation. The procedure was carried out as described by Chenni et al. (2007) with slight modification. This procedure uses the complex formed from the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). Into an assay mixture containing 200µL of 8.1% sodium dodecylsulfate (SDS), 750 µL of 20% acetic acid (pH, 3.5), 2mL of 0.25% TBA and 850µL of distilled water, 200µL of sample of MDA standard series (0, 7.5, 15, 22.5, and 30 µM) was added in a Pyrex screw-capped test tube. The mixture was heated at 95^OC for 60min in a sand bath, cooled down to room temperature and the absorbance read at 532nm using spectrophotometer - Synergy HTX multi-mode reader, VACUTEC,

USA. Thiobarbituric acid reactive substances (TBARS) concentrations in the samples were extrapolated from the MDA standard curve.

2.11 Measurement of testicular tissue reduced glutathione concentration

Reduced glutathione concentration was measured in tissue according to methods modified from Ellman (1959). The sample was first precipitated with 10% TCA and then centrifuged at 2000rpm for 10 min at 25^0C. Reaction mixture contained 100 µL of supernatant, 50 µL of 0.5mM DTNB and 150 µL of 0.2 M sodium phosphate buffer (pH 7.8). After 15 min incubation at 25^0C, the absorbance was measured at 412 nm using a spectrophotometer (Synergy HTX multi-mode reader, VACUTEC, USA) and GSH concentrations extrapolated from a standard GSH curve.

2.12 Statistical analysis

The morphometric and stereological data were analyzed using standard parametric tests. The results are expressed as means ± standard error of the mean. These were then subjected to one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

3.0 **Results**

Table 1: Body weight, testicular weight and TW/BW ratio in groups

Groups	**Initial BW (g)**	**Final BW (g)**	**Weight Diff (g)**	**Difference (%)**	**TW (g)**	**TW/BW x 100**
A	219.6 ± 5.17	351.4 ± 16.19	131.8	60.02	3.4 ± 0.06	0.97
B	224.8 ± 2.54	347.8 ± 10.17	123.0	54.76	3.7 ± 0.18	1.06
C	225.0 ± 2.39	340.2 ± 6.30	115.2	51.20	3.2 ± 0.23	0.94
D	217.5 ± 2.75	387.4 ± 15.72	169.9	78.13	3.6 ± 0.22	0.93
E	218.2 ± 2.82	315.4 ± 14.41	97.2	44.54	3.5 ± 0.19	1.12
F	225.2 ± 2.94	336.8 ± 5.70	111.6	49.67	3.3 ± 0.08	0.98
G	217.2 ± 2.27	335.4 ± 13.92	118.2	54.54	3.2 ± 0.07	0.95
H	222.6 ± 3.67	328.6 ± 19.02	106.0	47.97	3.2 ± 0.17	0.97

Values are expressed as mean ± SEM, (all values compared were $P>0.05$)

A: Control; B: Ethanol; C: Ethanol + HAART, D: Ethanol + HAART (Reversal group); E: Ethanol + HAART + VCO_1; F Ethanol + HAART + VCO_2; Group G: Ethanol + VCO_1; Group H: Ethanol + VCO_2. Groups compared as follows: B, C, D, E, F. G & H vs A; C, G & H vs B; D, E, F vs C

3.1 Organ-body weight changes

A general insignificant increase was observed in the body weight of all groups. This increase was least in group E, with group D showing the highest. The testicular weight (TW) and relative organ weight (TW/BW × 100) of all groups were similar with no between group differences reaching statistical significance (P<0.05) (Table 1).

Table 2: Seminal fluid analysis: Epididymal sperm count and motility

Groups	Sperm counts (10^6/ml)	Sperm motility (%)		
		Progressive	Non progressive	Immotile
A	4.80 ± 0.53	66.80 ± 3.77	18.80 ± 2.33	14.40 ± 1.92
B	0.79 ± 0.23$^{\gamma}$	26.00 ± 2.19$^{\gamma}$	49.20 ± 3.20$^{\gamma}$	25.20 ± 2.50
C	1.30 ± 0.31$^{\beta}$	16.00 ± 2.76$^{\gamma}$	42.80 ± 1.50$^{\gamma}$	41.20 ± 4.08$^{\gamma}$
D	2.30 ± 0.57	36.00 ± 3.41$^{\gamma\rho}$	28.40 ± 1.17$^{\alpha\phi}$	35.60 ± 2.99$^{\beta}$
E	3.50 ± 0.66	64.40 ± 2.99$^{\phi}$	5.60 ± 0.98$^{\gamma\phi}$	30.00 ± 3.58
F	4.10 ± 0.64$^{\lambda}$	68.40 ± 3.37$^{\phi}$	6.40 ± 1.17$^{\gamma\phi}$	25.20 ± 3.56
G	2.60 ± 0.58	56.00 ± 5.22$^{\Theta}$	6.00 ± 0.89$^{\gamma\Theta}$	37.60 ± 5.46$^{\beta}$
H	3.90 ± 0.71$^{\varepsilon}$	52.40 ± 3.82$^{\Theta}$	11.20 ± 1.50$^{\Theta}$	36.40 ± 3.87$^{\beta}$

Values are expressed as mean ± SEM. A: Control, B: Ethanol, C: Ethanol + HAART, D: Ethanol + HAART, (Reversal group), E: Ethanol + HAART + VCO_1, F: Ethanol + HAART + VCO_2, Group G: Ethanol + VCO_1, Group H: Ethanol + VCO_2. Groups compared as follows: B, C, D, E, F. G & H vs A; C, G & H vs B; D, E, F vs C

$^{\alpha, \delta, \lambda}$ Statistically significant at $P<0.05$

$^{\beta, \varepsilon, \rho}$ Statistically significant at $P<0.01$

$^{\gamma, \Theta, \phi}$ Statistically significant at $P<0.001$

3.2 Changes in Sperm counts

A significant decrease in the sperm counts was observed between groups B (Ethanol) and A (Control) (P<0.001) and between groups C (Ethanol + HAART) and A (Control) (P<0.01). While adjuvant treatment with VCO significantly increases sperm count in group H compared to group B (P<0.01). Similarly, the co-administration of VCO in group F resulted in a significant increase in sperm count when compared to group C (P<0.05) (Table 2).

3.3 Changes in Sperm motility

Progressive motility

A significant decrease was observed in the progressive motility of groups B, C & D when compared with the control group A ($P<0.001$). VCO co-administration in groups G and H significantly increases motility compared to the ethanol only group B ($P<0.001$). Varying degrees of increases were observed in the progressive motilities of the reversal group D, the VCO co-treatment groups E and F, when group compared to group C (Ethanol + HAART) (Table 2). However all were at the $P<0.01$ level.

Non-progressive motility

Varying degrees of statistically significant increase was observed in the non-progressive motility of group B, C ($P<0.001$) and D ($P<0.05$) when compared with the Control group A. In addition, VCO co-treatment resulted in a significant decrease ($P<0.001$) in this parameter in groups E, F and G when compared with the Control group A. Similarly, the co-administration of VCO significantly lowered the non-progressive sperm motility in group G and H ($P<0.001$) when compared with Ethanol group B. A significant decrease ($P<0.001$) was also seen in reversal group D, as well as VCO co-treatment groups E and F when compared with group C (Ethanol + HAART) (Table 2).

Immotile sperms

Ethanol and HAART co-treatment groups C and D, as well as VCO co-treatment groups G and H showed significant increase in immotile sperms when compared with Control group A ($P<0.01$) (Table 2).

Table 3: Morphometric measurements of seminiferous tubules

Groups	**D (µm)**	**$A_C = \pi D^2/4$ x (10^3 µm^2)**
A	295.10 ± 8.86	68.98 ± 4.14
B	335.10 ± 9.99	88.86 ± 5.40
C	333.40 ± 11.58	88.25 ± 5.76
D	334.00 ± 11.00	88.49 ± 5.79
E	332.70 ± 12.33	88.03 ± 6.61
F	318.00 ± 4.99	79.61 ± 2.42
G	342.40 ± 13.15	93.34 ± 7.57
H	302.30 ± 16.30	73.68 ± 6.67

Values are expressed as mean ± SEM. ($P >0.05$)

A: Control; B: Ethanol; C: Ethanol + HAART, D: Ethanol + HAART (Reversal group); E: Ethanol + HAART + VCO_1; F Ethanol + HAART + VCO_2; Group G: Ethanol + VCO_1; Group H: Ethanol + VCO_2. Groups compared as follows: B, C, D, E, F. G & H vs A; C, G & H vs B; D, E, F vs C

3.4 Morphometric evaluation of the seminiferous tubules

Tubular diameter and cross sectional area of treatment groups B, C, D, E, F, and G appear to be slightly higher when compared with the control group A, whereas group H was similar to the control. There was no statistically significant difference ($P<0.05$) between any of the groups (Table 3).

Table 4: Stereological measurements (Volume density and absolute volume) of the germinal epithelium, lumen and interstitium

	Germinal epithelium		Lumen		Interstitium	
Groups	**Vv (%)**	**Av (ml)**	**Vv (%)**	**Av (ml)**	**Vv (%)**	**Av (ml)**
A	71.81 ± 1.25	2.37 ± 0.04	17.63 ± 1.07	0.58 ± 0.03	10.56 ± 0.73	0.35 ± 0.02
B	$46.16 \pm 1.94^{\gamma}$	$1.82 \pm 0.08^{\gamma}$	$38.97 \pm 1.96^{\gamma}$	$1.54 \pm 0.08^{\gamma}$	14.88 ± 1.37	$0.59 \pm 0.05^{\gamma}$
C	$47.81 \pm 1.81^{\gamma}$	$1.34 \pm 0.05^{\gamma\phi}$	$36.50 \pm 2.80^{\gamma}$	$1.08 \pm .011^{\gamma\phi}$	$15.69 \pm 1.47^{\alpha}$	$0.43 \pm 0.04^{\delta}$
D	$44.81 \pm 1.65^{\gamma}$	$1.81 \pm 0.08^{\gamma\phi}$	$40.59 \pm 1.72^{\gamma}$	$1.64 \pm 0.08^{\gamma\phi}$	14.59 ± 0.71	$0.59 \pm 0.03^{\gamma\lambda}$
E	$59.34 \pm 1.33^{\gamma\phi}$	$2.19 \pm 0.06^{\phi}$	$26.88 \pm 1.28^{\beta\rho}$	$1.00 \pm 0.06^{\gamma}$	13.78 ± 1.20	$0.51 \pm 0.04^{\alpha}$
F	$66.53 \pm 1.17^{\phi}$	$2.26 \pm 0.05^{\phi}$	$21.28 \pm 1.29^{\phi}$	$0.72 \pm 0.04^{\rho}$	12.19 ± 0.99	0.42 ± 0.04
G	$59.34 \pm 1.11^{\gamma\Theta}$	$1.85 \pm 0.04^{\gamma}$	$24.09 \pm 1.15^{\Theta}$	$0.75 \pm 0.04^{\Theta}$	$16.56 \pm 0.69^{\beta}$	$0.51 \pm 0.02^{\alpha}$
H	$63.53 \pm 1.03^{\beta\Theta}$	$2.07 \pm 0.07^{\alpha}$	$23.00 \pm 1.11^{\Theta}$	$0.75 \pm 0.05^{\Theta}$	13.47 ± 0.72	$0.44 \pm 0.02^{\delta}$

Values are expressed as mean ± SEM. A: Control, B: Ethanol, C: Ethanol + HAART, D: Ethanol + HAART, (Reversal group), E: Ethanol + HAART + VCO_1, F: Ethanol + HAART + VCO_2, Group G: Ethanol + VCO_1, Group H: Ethanol + VCO_2. Groups compared as follows: B, C, D, E, F. G & H vs A; C, G & H vs B; D, E, F vs C

$^{\alpha, \delta, \lambda}$ Statistically significant at $P<0.05$

$^{\beta, \varepsilon, \rho}$ Statistically significant at $P<0.01$

$^{\gamma, \Theta, \phi}$ Statistically significant at $P<0.001$

3.5 Changes in the germinal epithelium (GE)

Volume density

A significant decrease ($P<0.001$) was observed in the GE volume density of Group B, C, D, E, and G respectively, as well as group H ($P<0.01$) when compared with the control group A. Adjuvant treatment with VCO in group G and H significantly increased this parameter ($P<0.001$) when compared with Ethanol group B. In addition, a significant increase ($P<0.001$) was observed in the GE volume density of group E and F when compared with group C (Ethanol + HAART) (Table 4).

Absolute volume

A significant decrease ($P<0.001$) was observed in the GE absolute volume of Group B, C, D, and G respectively, as well as group H ($P<0.05$) when compared to the control group A. A significantly decrease ($P<0.001$) was observed in Ethanol and HAART co-administered group C when compared with Ethanol alone group B. Furthermore, the absolute volume of GE in reversal group D as well as VCO co-treatment group E and F were significantly higher ($P<0.001$) when compared with group C (Ethanol + HAART) (Table 4).

3.6 Changes in the Lumen

Volume density

A significant increase was observed in the luminal density of groups B, C, D and E ($P<0.01$) compared to control group A. Meanwhile, the adjuvant co-treatment with VCO in groups G and H were significantly lower ($P<0.001$) in this parameter when compared with Ethanol group B. Group E and F showed a statistically significant reduction in this value ($P<0.01$) when compared to group C (Table 4).

Absolute volume

The absolute volume of lumen showed a significant increase ($P<0.001$) in group B, C, D and E when compared with the control group A. Ethanol and HAART co-administration in group C, as well as VCO co-treatment in group G and H respectively were significantly lower ($P<0.001$) in the value of this parameter when compared with Ethanol treated group B. Reversal group D also showed in increase ($P<0.001$), whereas VCO co-treatment group F showed a significant decrease ($P<0.01$) in luminal volume compared to group C (Table 4).

3.7 Changes in interstitium

Volume density

A significant increase was observed in the interstitial volume density of group C and G when compared to control group A ($P<0.05$, $P<0.01$ respectively) (Table 4).

Absolute volume

A significant increase was observed in groups B, D ($P<0.01$), E & G ($P<0.05$) when compared with the control group A. Groups C and H were significantly lower ($P<0.05$) than Ethanol group B, whereas reversal Group D was significantly higher than group C ($P<0.05$) (Table 4).

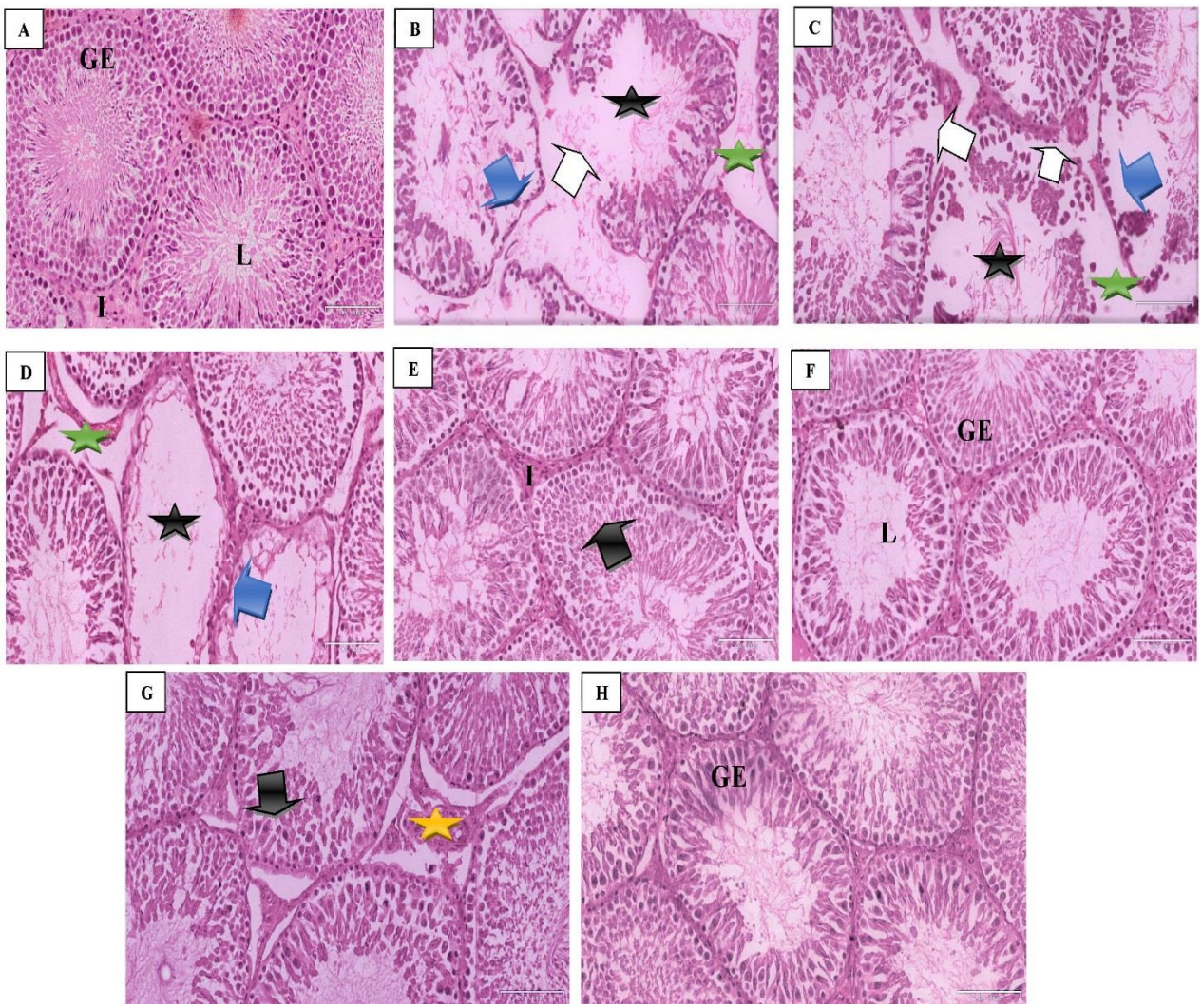
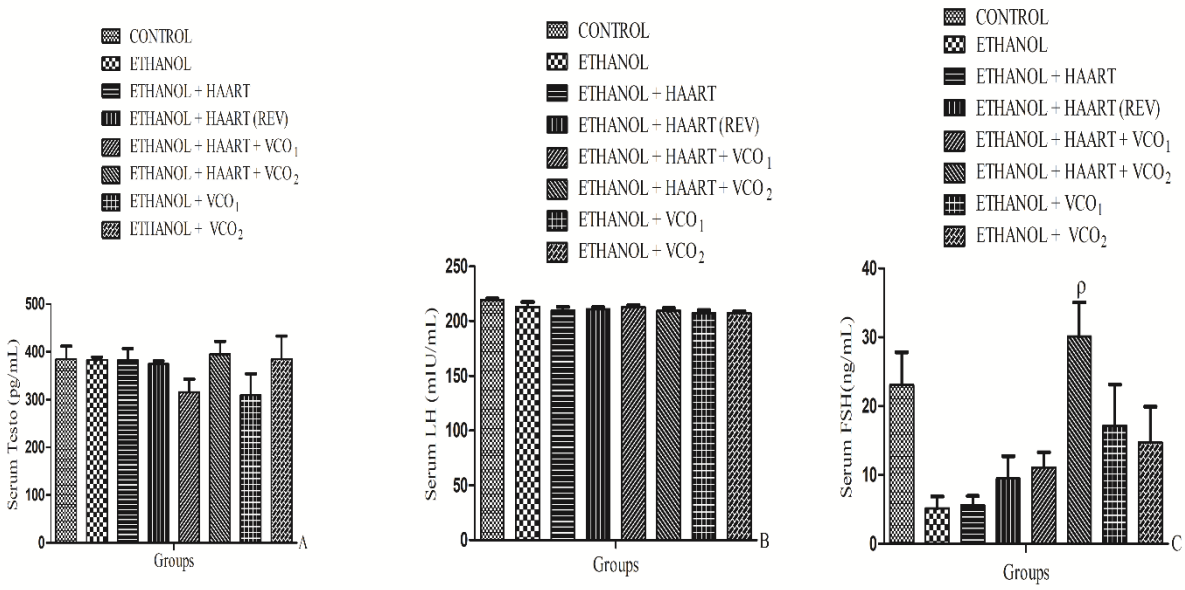


Figure 1: Testicular sections of control animals 'A' showed well-preserved cyto-architecture with normal cellular composition in germinal epithelium (GE). The interstitial (I) spaces filled with leydig cells were normal. The lumen (L) is also populated by immotile spermatozoa. Group 'B', 'C' & 'D' showed massive loss of germ cells (blue arrow), alteration of basement membranes (white arrow), widening of lumen (black star) , widened and hypocellular interstitium (green star). Significant improvement in in cellular densities of germinal epithelium (black arrow) and instersitium was seen in group 'E' with some vacuoles. There is partial restoration of basal membrane alteration. Group G also showed improvement in cellular density of germinal epithelium with widened interstitium having some degree of hypocellularity (yellow star), restoration of basal membrane was evident. Group F and H appear similar to the control group (H&E).

3.8 Histopathological examination of testicular tissue

Testicular cross sections of Control, group F (Ethanol + HAART + VCO_2) and group H (Ethanol + VCO_2) are normal, with well-preserved cyto-architecture and minimal histological change was observed in any of these groups. Seminiferous tubules in these groups are well-populated by different stages of the spermatogenic series. Basement membranes are of normal appearance, interstitial spaces are normal and populated by normal Leydig cells with no cellular infiltrations (Fig 1 A, F, H). Seminiferous tubules of

Ethanol treated group B, Ethanol + HAART group C with reversal group D showed massive loss of germ cells, widening of tubular lumen, widening of interstitial spaces with severe degrees of hypocellularity. Disordered basement membrane was also evident in these groups (Fig 1 B, C, D). Group E (Ethanol + HAART + VCO_1) and G (Ethanol + VCO_1) showed significant improvement with increased cellular densities of germinal and interstitial cells when compared with group B, C & D. Architectural distortions were less, with fewer hypoplastic changes and vacuolations. Basement membrane arrangement was partially restored in groups E and G (Fig 1E, 1G).



Figiure 2: Effect of VCO, alcohol and HAART treatment on Serum testosterone (A), Serum luteinizing hormone (B) and Serum follicle stimulating hormone (C) in Sprague-Dawley rats after 8 weeks treatment period. Bars indicate the mean ± SEM. Groups compared as follows: B, C, D, E, F. G & H vs A; C, G & H vs B; D, E, F vs C

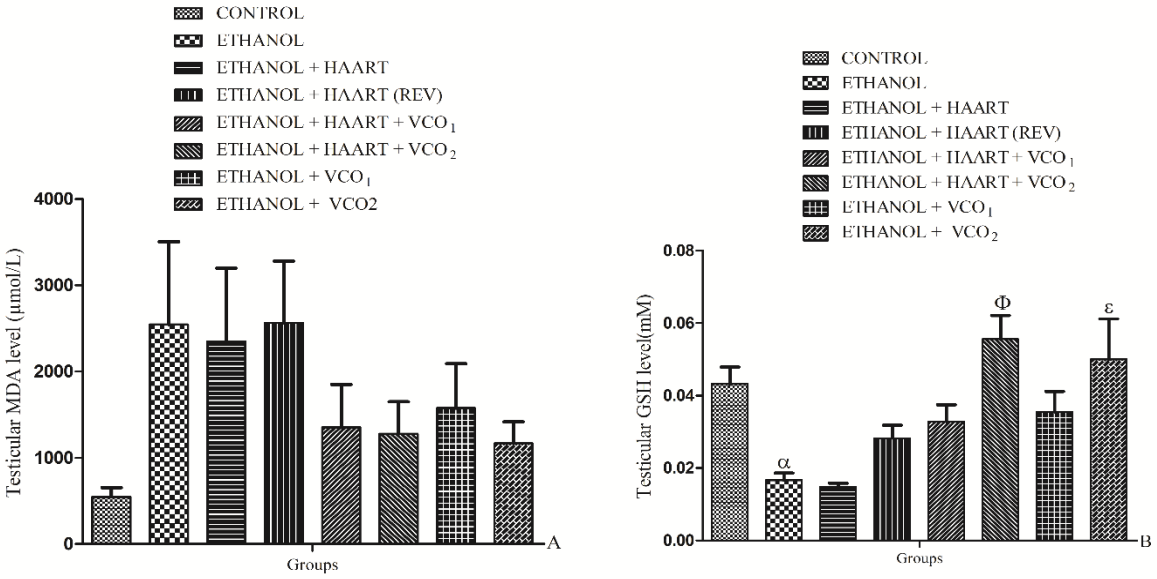
ρ Statistically significant at $P<0.01$

3.9 Changes in serum testosterone, luteinizing and FSH hormonal levels

Figure 2A shows the mean and standard error of mean (95%CI error bars). The highest and lowest mean serum testosterone levels were observed in group F (395.2 ± 26.46 pg/mL) & G (309.8 ± 44.30 pg/mL) respectively. The mean serum testosterone for groups A, B, C, D, E & H were 385.1 ± 27.04 pg/mL, 383.2 ± 6.16 pg/mL, 382.23 ± 24.51 pg/mL, 374.4 ± 6.44 pg/mL, 316.2 ± 26.79 pg/mL & 388.3 ± 47.68 pg/mL respectively. There was no statistical significance between groups at P<0.05.

The mean serum luteinizing hormone (Figure 2B) showed no significant difference between groups. The mean serum LH for group A, B, C, D, E, F, G & H were 219.2 ± 1.34 mIU/mL, 212.9 ± 4.49 mIU/mL, 209.8 ± 3.21 mIU/mL, 210.8 ± 1.81 mIU/mL, 212.6 ± 1.90 mIU/mL, 209.3 ± 2.41 mIU/mL, 207.0 ± 3.10 mIU/mL & 207.2 ± 1.87 mIU/mL respectively (mean and standard error).

A significant increase (P<0.01) was observed in the mean FSH concentration of group F (30.16 ± 4.89 ng/mL) compared to group C (5.58 ± 1.37 ng/mL) (Figure 2C). The mean FSH concentrations for groups A, B, D, E, G & H were (23.06 ± 4.78 ng/mL), (5.19 ± 1.70 ng/mL), (9.49 ± 3.24 ng/mL), (11.12 ± 2.20 ng/mL), (17.14 ± 6.01 ng/mL) and (14.70 ± 5.24 ng/mL) respectively (mean and standard error).



Figiure 3: Effect of VCO, alcohol and HAART treatment on Testicular malondialdehyde level (A), Testicular reduced glutathione (B) in Sprague-Dawley rats after 8 weeks treatment period. Bars indicate the mean ± SEM**.** Groups compared as follows: B, C, D, E, F. G & H vs A; C, G & H vs B; D, E, F vs C

$^{\alpha}$ Statistically significant at $P<0.05$

$^{\rho}$ Statistically significant at $P<0.01$,

$^{\phi}$ Statistically significant at $P<0.001$

3.10 Changes in Testicular tissue malondialdehyde and reduced glutathione concentrations

As shown in figure 3A, the testicular tissue malondialdehyde showed high concentrations in group B (25.47 ± 9.57 x 10^2µmol/L), C (23.50 ± 8.50 x 10^2µmol/L) & D (25.64 ± 7.17 x 10^2µmol/L) (mean and standard error). Low peak concentrations of testicular tissue malondialdehyde was observed in groups A (5.47 ± 1.07 x 10^2µmol/L), E (13.54 ± 4.97 x 10^2µmol/L), F (12.78 ± 3.74 x 10^2µmol/L), G (15.75 ± 5.17 x 10^2µmol/L), and H (11.67 ± 2.50 x 102µmol /L). There was no statistical significance (P<0.05) between groups.

Figure 3B shows the mean glutathione concentrations in testicular tissue. A significant decrease ($P< 0.05$) was observed in Ethanol group B when compared with the control group A (0.017 ± 0.002mM versus 0.043 ± 0.005mM). In addition, adjuvant treatment with VCO caused a significant increase ($P< 0.01$) in GSH in group H when compared with Ethanol group B (0.050 ± 0.011Mm vs 0.017 ± 0.002mM). Similarly, VCO co-treatment in group F significantly increased ($P< 0.001$) GSH when compared with group C (0.056 ± 0.007mM vs 0.015 ± 0.001mM). The mean glutathione concentrations for group D, E and G are 0.028 ± 0.004mM, 0.033± 0.005, and 0.036 ± 0.006 respectively (mean and standard error).

DISCUSSION

In the current study, we reported that the administration of Ethanol or its concomitant use with HAART significantly reduced sperm counts, progressive sperm motility, and increased the percentage of non-motile sperms. The concomitant use of Ethanol and HAART also caused a significant increase in numbers of immotile spermatozoa. Previous studies have shown that the long term use of HAART, as well as the chronic consumption of alcohol correlate positively with a decrease in sperm functional parameters (Sermondade et al., 2010a, Jegede et al., 2017). It is well established that mitochondrial energy metabolism is critical to the efficient functioning of sperm cells which can be compromised when exposed to toxicants (Song et al., 2014b, Piomboni et al., 2012a). Testicular and spermatogenic indices can also be adversely affected by antiretroviral drugs via altered mitochondrial DNA and oxidative stress mechanisms (Day and Lewis, 2004a). Alcohol on the other hand elicits a myriad of mitochondrial alterations which in turn results in the generation of reactive oxygen species (ROS) and oxidative stress damage (Song et al., 2014b). Increased ROS has been correlated with low sperm counts, poor sperm motility and morphology (Agarwal et al., 1994). Since spermatozoal motility wholly depends on a robust cellular energy supply, mitochondrial dysfunction due to oxidative stress will ultimately cause axonemal damage and sperm immobilization (De Lamirande and Gagnon, 1995, Bansal and Bilaspuri, 2011).

Lipid peroxidation is a metabolic process that results in the oxidative degradation of lipids via attacks by free radicals and excess ROS formation (Vasilaki and McMillan, 2011). Malondialdehyde (MDA) is a good marker for determining the extent of lipid peroxidation (Gaweł et al., 2003). Intense oxidation of lipid membranes via increased ROS mediated damage can alter the structure and function of the cell membrane (Vasilaki and McMillan, 2011). This further predisposes cellular organelles to functional impairment (Milei et al., 2007). Thus, interference to lipid metabolism can impair male fertility (Yildiz et al., 2006b).

Our result shows that both ethanol and HAART caused an increase in MDA levels. This indicates testicular occurrence of lipid peroxidation. Although not statistically significant, our result still supports previous findings indicating an increased in testicular MDA levels after administration of alcohol

(Dosumu et al., 2012b, Sanghishetti et al., 2012) or antiretroviral drugs (Adaramoye et al., 2015, Oputiri and Elias, 2014).

Reduced glutathione (GSH) is also an important antioxidant which has the capacity to respond to toxic substances. It also protect the body from cellular damage caused by free radicals or excess ROS formation (Jurczuk et al., 2006a). Ethanol significantly decreased testicular GSH consequently reducing antioxidant availability thus, increasing free radical activities and disrupting the redox balance. It has been reported by Rahman et al (2012) that an imbalance in redox activity which results in oxidative stress is highly deleterious to tissue functionality. This also confirms the decreased spermatogenesis observed (Agarwal et al., 2014). The improvement observed in F and H demonstrates VCO potential in increasing testicular antioxidant enzyme activities, with concomitant decrease in MDA levels via scavenging for excess ROS. This culminates in an increase in sperm count and motility. Our result corroborates previous studies correlating decreased MDA levels and increased antioxidant levels to increased sperm count and motility (Dosumu et al., 2012a, Atig et al., 2012).

In the present study, histopathological results and stereological measurements showed extensive degeneration of spermatogenic cells with thinning and disruption of the basement membrane in ethanol alone as well as it co-treatment groups C and D. Decreased volume density and absolute volume of germinal epithelium, widened lumen with widened hypocellular insterstium were also observed in these groups. This may be due to the morphological alterations of the mitochondria which contributes to apoptosis and necrotic cell death resulting in degradation of the germinal epithelium and interstitial cells (Dosumu et al., 2014b). The consequence of prolonged toxic xenobiotics on actively dividing cells during spermatogenetic cell division ultimately resulting in cessation, may also play a significant role in germinal cell degeneration (Vidal and Whitney, 2014). Our result agrees with previous studies which reported that the induction of HAART (Azu et al., 2014, Ogedengbe et al., 2016) or alcohol (Dosumu et al., 2014b, Akang et al., 2015) causes severe effects on germinal cells leading to extensive necrosis and disruption of spermatogenensis. Decreased seminiferous tubular diameter, widened lumen, and hypocellular insterstitium was also observed.

The basement membrane contributes significantly in maintaining the structural and functional integrity of the testis (LeBleu et al., 2007a). Alteration to the basement membrane will lead to impaired functionality of the testis (Richardson et al., 1998, Dobashi et al., 2003a). Our recent review (Ogedengbe et al., 2017), and previous studies (Gurtovenko and Anwar, 2009b, Ingólfsson and Andersen, 2011a) have reported that alcohol alters the lipid bilayer (cell membrane) affecting membrane permeability, protein distribution and expression. Results from this study supports observations of these toxic effects of alcohol.

The susceptibility of sperms to oxidative damage as seen in B, C and D relies on the high proportion of polyunsaturated fatty acids in the plasma membrane alongside low cytoplasmic level of scavenging

enzymes (Pace et al., 2005a). VCO retains high levels of biologically active unsaponifiable components such as vitamin E and polyphenols which maintains membrane stability and prevents membrane damage from oxidative stress damage and membrane lipid peroxidation (Nevin and Rajamohan, 2006, Jaarin et al., 2014b). Phospholipids with high concentration of polyunsaturated fatty acids (linoleic and a-linoleic acids) play an essential role in male fertility as they are important components of spermatozoa membranes (Gholami et al., 2011). Enhanced sperm membrane stability is correlates positively with fertility (McKinnon et al., 2011). Testicular sections of groups E, F, G and H which show some normalization as seen in the volume densities and absolute volumes of germinal epithelium reflect the ability of VCO to attenuate histopathological changes and loss of germ cells due to alcohol or its concomitant use with HAART.

In the current study, there were no significant differences in the mean serum testosterone concentrations between groups, though the mechanisms behind this observation remains unclear. In addition, LH concentration remain unchanged when compared across groups. This may be due to LH production from the anterior lobe of the pitituary glands being unaltered (Babu et al., 2004a). Similar findings in LH levels have been reported in other studies of alcohol (Dosumu et al., 2014b) or antiretrovirals (Collazos et al., 2002) effects on the testis.

Follicle stimulating hormone (FSH) is considered a biologic marker for accessing Sertoli cell function. Its action on Sertoli cell has also been reported to increase spermatogenic potential of the testis (Jensen et al., 1997b). The significant increase in mean serum FSH concentration serum as observed in VCO co-treatment group F when compared Ethanol + HAART-group C corroborates the positive impact of VCO on Sertoli cell functions.

It is posited that the concomitant use of alcohol with HAART will likely act synergistically to potentiate HAART toxicities (Kumar et al., 2012b). Although we did not report any specific role played by metabolic enzymes (Cytochrome P450) and drug transporters in relation to testicular interactions of alcohol and antiretrovirals, our result still tallies with this observation. The concomitant use of ethanol and HAART showed accentuation of adverse effects such as an increase in immotile sperms, reduced sperm motility, decreased absolute volume of germinal epithelium and greater histopathological perturbation when compared to the ethanol alone treated group. All these ravages depict testicular exacerbation of antiretroviral drugs with alcohol use. Furthermore, the withdrawal of ethanol and HAART for 28 days in group D did not show any significant improvement when compared with Ethanol and HAART co-treatment group C suggesting the possibly irreversible nature of damage.

In conclusion, the use of HAART alone or concomitant use with alcohol has a deleterious effect on semen parameters, testicular structure and its function. VCO however, miltigates these ravages and augments

spermatogenesis through its antioxidant properties. however further studies are still required to fully quantify these effects.

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CONFLICT OF INTEREST

None declared by all author

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CHARTER SIX

SYNTHESIS, CONCLUSION AND RECOMMENDATION

6.1 Synthesis

The use of HAART in the treatment of HIV/AIDS is aimed at maintaining maximal sustainable viral suppression, the restoration and preservation of immunologic function, the reduction in HIV-associated morbidity and the prolongation of survival and the prevention of HIV transmission (Arenas-Pinto et al., 2015a, Conklin and Pineda, 2017). However the enthusiasm generated by HAART has been diminished by the severe consequences of prolonged treatment. These appear to result in varying degrees of damage to mitochondrial DNA mediated via oxidative stress mechanisms (Pavili et al., 2010b, Nagiah et al., 2015c). While HAART has drastically reduced HIV related morbidity and mortality among PLWHAs, the adverse outcomes which include various metabolic complications and multi-organ toxicities arising from long term use have been shown to severely compromise the quality of life (QOL) of these patients (Oguntibeju, 2012, Kaushik et al., 2014).

Alcohol consumption has shown a significant increase amongst HIV-infected individuals who are on antiretroviral drugs (Roux et al., 2008, Kekwaletswe and Morojele, 2014a). However the chronic alcohol use can result in gonadal dysfunction including structural testicular changes and a decrease in serum testosterone (Emanuele and Emanuele, 1998) in these patients. Alcohol increases the metabolic clearance rate of testosterone and increases conversion of androgens into estrogens (Muthusami and Chinnaswamy, 2005). Ethanol (drinking alcohol) and its metabolite acetaldehyde cause a reduction in LH binding to Leydig cells and inhibition of the enzymes responsible for the synthesis of sex hormones (Adler, 1992, Grover et al., 2014). Thus ethanol acts as a Leydig cell toxin (Radhakrishnakartha et al., 2014). Alcohol appears to exert a dual effect on the hypothalamic– pituitary–gonadal axis by directly inhibiting testicular steroidogenesis and blocking the release of LH-releasing hormone from the hypothalamus (Emanuele and Emanuele, 1998, Emanuele and Emanuele, 2001). Alcohol causes a significant decrease in the percentage motility, straight-line velocity and curvilinear velocity of sperm in human seminal fluid. It also causes a significant decrease in the number of spermatozoa with normal morphology and an increase in irreversible tail defects (Donnelly et al., 1999, Ramlau-Hansen et al., 2010b).

Current knowledge of the efficacy, dosing and toxicity of available HAART regimens is related to the plasma kinetics on these drugs (Di Mascio et al., 2009) and does not directly factor in their concentration in seminal fluid and testicular tissue. This justifies the need to evaluate testicular morphological and functional changes for a better assessment and improved drug delivery. Hence, we investigated the testicular cyto-architecture and functional changes in experimental animal model with specific focus on the epididymal sperm, morphological and morphometric perturbations. We also looked at ultrastructural alterations in the testicular tissue, reproductive hormonal changes and biochemical

parameters following the administration of alcohol and HAART and the role of virgin coconut oil extract (VCO) in affecting these parameters.

Our results showed that VCO alone and its adjuvant treatment with HAART induced body weight loss in all experimental animals relative to the control animals, while treatment with HAART alone increases the body weight. We also observed a reduction of body weight in the ethanol-HAART plus VCO (low dose) treated animals with an increase in the body weight of the ethanol plus HAART-reversal animals. While the loss of weight can be attributed to the presence of medium chain saturated fatty acids in VCO which boost metabolism for immediate energy (Hargrave et al., 2005, Assunçao et al., 2009), initiation of HAART may also have contributed to the low pace of weight gain. This is due to alteration in glucose metabolism leading to generalized lipid accumulation (Aurpibul et al., 2012, Razi et al., 2012).

Results of epididymal seminal fluid analysis has shown the positive influence of VCO in mitigating the deleterious consequences caused by HAART and ethanol. The use of HAART alone, ethanol alone, or the concomitant use of HAART and ethanol reduces sperm counts, decreases progressive motility and increases non-progressive motility, it also causes an increase in the number of immotile sperm. Adjuvant treatment with VCO mitigates these ravages with significant improvements in the sperm counts and motility. In this protocol, the positive benefits of VCO on sperm counts and motility was seen when the treatments groups were compared with each other. The groups treated with VCO plus ethanol, VCO plus ethanol with HAART showed improved indices when compared with ethanol alone or with the ethanol plus HAART alone

Compelling evidence suggests that the long term use of HAART with the chronic consumption of alcohol correlates positively with a decrease in functional semen parameters (Sermondade et al., 2010a, Jegede et al., 2017). It is also well established that testicular and spermatogenic indices can be adversely affected by antiretroviral drugs via altered mitochondrial DNA and oxidative stress mechanisms (Day and Lewis, 2004a). In addition, alcohol elicits a myriad of mitochondrial alterations which in turn results in the generation of reactive oxygen species (ROS) which are the chief arbiters of oxidative stress damage (Song et al., 2014b). Since sperm motility is wholly dependent on a robust cellular energy supply, mitochondrial dysfunction due to oxidative stress arising from the negative interactions of HAART or /HAART with ethanol will ultimately cause axonemal damage and sperm immobilization (De Lamirande and Gagnon, 1995, Bansal and Bilaspuri, 2011).

As observed in this study, VCO has demonstrated its potential in ameliorating HAART and ethanol ravages on semen quality. This has been attributed to the high phenolic content of VCO contributing to its antioxidant capacity with a positive correlation on semen quality (Marina et al., 2009b, Illam et al., 2017), thereby making the shift in the antioxidant-pro-oxidant balance to be in favour of the antioxidant effect of VCO. Since HAART use may be associated with an increased desire to bear children amongst the HIV-

infected community - possibly through having a more positive outlook for the future (Bah'him et al., 2010), there is a distinct need to have a clear understanding of how to maximize the fertility potential of the spermatozoa while still under HAART (Azu, 2012).

In the present study, histopathological results showed extensive degenerative changes due to ethanol and HAART co-administration in the seminiferous tubules of the treated animals. These changes were mitigated by adjuvant treatment with VCO. Qualitative light microscopy data of testicular sections of HAART alone treated animals showed gross hypoplastic changes, increased vacuolization with reduced and disorganized spermatogenic cells. Ultrastructural examination also highlighted several HAART abnormalities such as nuclear membrane discontinuations, loss or absence of acrosomal cap, ballooned mitochondria, head and tail deformities of spermatazoa and flagella abnormalities depicting mitochondria and axoneme disorientation with defective mitochondrial sheaths. Similarly, histopathological results of testicular sections of animals treated with ethanol alone, as well as concomitant treatment with HAART showed massive loss of germ cells, widening of tubular lumen, widening of interstitial spaces with severe degrees of hypocellularity, and disordered basement membrane.

The intense histopathological and ultrastructural changes observed in the germinal epithelium of HAART and ethanol exposed testis is an indication that the concomitant use of these drugs cause significant damage to mitochondria most likely due to elevated reactive oxygen species (ROS) production. This leads to membrane damage with increased permeability to ethanol, HAART and a host of potential toxins, which is further disrupts normal oxidative phosphorylation. This results in cytoplasmic swelling and disruption of cristae, collapse of internal mitochondrial structure and consequent disruption in energy balance (Tolomeo et al., 2003). All of these sequelto gross morphological alteration of the mitochondria resulting in apoptosis and necrotic cell death of virtually all cells in the spermatogenic series (Dosumu et al., 2014a).

In addition, the prolonged use of HAART and ethanol potentially results in cessation of normal active cell division during spermatogenetic cell division, leading to massive loss of germ cells (Vidal and Whitney, 2014). The administration of the adjuvant VCO in our study was able to attenuate these extensive histopathological and ultrastructural alterations as wells as to restore the considerable loss of germ cells in the seminiferous tubules due to ethanol and HAART.

The role of Sertoli cell in the process of spermatogenesis is crucial in creating a conducive environmental milieu important for growth and maturation of germ cells. It provides physical support and several key nutrients critical for the normal development of germ cells up to the stage of maturity (Monsees et al., 2000). The spermatogenic cell population is known to be regulated by germ cell apoptosis to a size appropriate for the supportive role of Sertoli cell (Shokri et al., 2012). Despite this, the Sertoli cells still

remain a prime target for several reproductive toxicants which can cause impairment leading to increased degradation of germ cell and consequent overall decline in testicular function (Monsees et al., 2000).

In our study, Sertoli cell ultrastruture of the HAART-treated animals showed degenerative changes such as cytoplasmic vacuolations and the mitochondrial cristae were collapsed in many sections. Disorganized spermatogenic cell series were also evident in the lumina of seminiferous tubules. The degenerative changes seen in the cytoplasm of Sertoli and germinal cells of HAART-treated animals are a indication of the toxic effects of HAART-induced toxicity on the supportive role of Sertoli cells. The presence of vacuoles in Sertoli cells is one of the first and most common morphological feature following toxicological insult. This is followed by germ cell degeneration or disorganization (Creasy, 2001) coinciding with the ravages of HAART.

The contribution of the cytoskeleton to the efficient functioning of Sertoli cell are critical includeing Sertoli-germ cell attachment, germ cell migration from the basal regions of the seminiferous tubules (ST) to the apical or luminal compartment with preservation of the germinal epithelial architecture – maintaining their normal compact shape. In addition, transfer of nutrients will also be disrupted when germinal cells are detached from Sertoli cells resulting in death and collapse of spermatogenic cells (Creasy, 2001). The looseness or disorientation of the cells of the germinal epithelium in HAART-treated animals is a clear indication of perturbations secondary to Sertoli cell injury.

The histopathological and ultrastructural examination undertaken in this study reveal severe morphological alterations of the testicular interstitium in animals on HAART and ethanol treatment. The ultrastructure of the Leydig cells of the HAART alone treated animals showed degenerative changes with fewer mitochondria and less dense cytoplasmic matrix. The Leydig cell nuclei in these animals showed a shrunken appearance with decreased nuclear diameters and volumes. In addition, the histopathological results from the testis of the HAART alone, ethanol alone or ethanol plus HAART co-treatment groups, showed widening of the interstitial spaces with severe degrees of hypocellularity. The close alliance between Leydig cells and blood vessels is an indication that these cells can become highly endangered by exogenous toxicants such as HAART and ethanol. This results in severe deterioration of the Leydig cells and the general hypocellularity seen in the interstitium (Elshennawy and Elwafa, 2011). However our study reflects the ability of VCO in mitigating these ravages with a resultant increase in Leydig cell number, nuclei and volume.

The basement membrane plays an essential role in sustaining the structural and functional integrity of the testis due to it being made of extracellular matrix (LeBleu et al., 2007b). Whereas interactions between the matrix, tubular wall, and germinal cells contribute significantly to normal testicular development (Favorito et al., 2005), impairment of the basement membrane will severely compromise testicular function (Dobashi et al., 2003b). In this study, histochemical evaluation with PAS technique indicated

high intensity staining for glycogen in the testicular sections of the HAART treated animals. This is an indication of distortion in the basement membrane of the seminiferous tubules. Staining with silver impregnation and testicular ultrastructure of the HAART-treated animals also displayed a significantly abnormal increase in the thickness of the basement membrane. This was similar to reports by Santoro and Azu (Santoro et al., 1999, Azu et al., 2014). The presence of type IV collagen - which is the most abundant protein in the basement membrane, has been fundamental in the maintenance of its stability (LeBleu et al., 2007b). However its over-production by myoid and Sertoli cells can adversely affect germinal cell development owing to disturbances between the Sertoli cells and myoid cells of basement membrane (Dobashi et al., 2003b). HAART can stimulate the over-production of type IV collagen as well as its carbohydrate components, thus contributing to extensive fibrosis and possible hyalinization around the basement membrane (Clark et al., 2000, Azu et al., 2014). This is a major cause of male infertility in human (Gulkesen et al., 2002).

Furthermore, our result show that treatment with ethanol alone, or its concomitant use with HAART resulted into disordered basement membrane. Alcohol can alter the lipid bilayer (cell membrane) thus affecting membrane permeability, protein distribution and expression (Gurtovenko and Anwar, 2009a, Ingólfsson and Andersen, 2011b). We reported decreased thickness and less architectural distortion in the basement membrane of testes of the animals given adjuvant VCO plus treatment with HAART. VCO co-administered with ethanol, or co-administered with ethanol plus HAART showed some restoration of disorganized basement membrane.

The susceptibility of spermatozoa to oxidative damage as seen in the HAART and ethanol treated animals rely on the high proportion of polyunsaturated fatty acids in the plasma membrane alongside low cytoplasmic level of scavenging enzymes (Pace et al., 2005b). Poly-unsaturated fatty acid react with oxygen to form peroxides which decompose to aldehydes, ketones, and other volatile decomposition products. Administration of the adjuvant VCO was able to reverse negative changes in spermatozoal cell membranes as reflected by the positive indices. This was due largely to its very high content of saturated fatty acids (approximately 92% - mainly lauric and myristic acids) stabilizing the membrane against oxidation (Henna Lu and Tan, 2009). It also retains high levels of biologically active unsaponifiable components such as vitamin E and polyphenols which also maintains membrane stability and prevent membrane damage from the ravages of oxidative stress and membrane lipid peroxidation (Nevin and Rajamohan, 2006, Marina et al., 2009b).

Morphometric data showed a reduction in seminiferous tubule diameter, epithelial thickness, Leydig cell diameter and Leydig cell nuclear volume of the HAART alone treatment animals compared to the control animals. The decreased epithelial thickness and seminiferous tubular diameter are a result of reduced metabolic activity of the germinal cells as well as cell numbers. As a consequence the interstitial spaces

widen with an edematous appearance (Hesari et al., 2015). Several studies that have examined HAART effects on semen parameters and other morphometric features of the testis have supported the overall result indicating HAART-induced distortions do occur by changes in morphology and morphometric indices of the testis (Azu et al., 2014, Jegede et al., 2017). Increased basement membrane thickness as earlier mentioned, are indicative of morphological disorders in spermatogenesis (Favorito et al., 2005, Azu et al., 2014). Decreased Leydig cell diameter and volume reflect the negative influence of HAART.

Stereological evaluation from this study has also shown that administration with ethanol alone, or with ethanol plus HAART co-treatment decreases volume density and absolute germinal epithelium volume. Increase in volume density and absolute luminal volume with increase in the absolute volume of interstitium were also observed. As earlier mentioned, alteration in mitochondria morphology due to HAART or ethanol induction can instigate degradation and possible death of germinal cells (Shaha et al., 2010).

Adjuvant treatment of VCO with HAART had a protective effect on the seminiferous epithelium as demonstrated by the reduction of degeneration of the normal epithelial lining and subsequent restitution. Adjuvant administration of VCO with ethanol or with ethanol plus HAART, showed significant improvement in the parameters of volume density and absolute volume of germinal epithelium with a reduction in the pathologically widened lumen and widened interstitial spaces which are better seen at higher doses of VCO (5ml/kg/bw) compared to the lower dose (2.5ml/g/bw).

Steroidogenesis has been reported to take place within the Leydig cells of the testis and the production of testosterone is influenced by the activity of LH on the Leydig cells. Thus LH depletion will result in reduced testosterone production (Ichihara et al., 2001). In the current study, the serum testosterone levels were significantly increased only in the HAART alone treated group. The serum testosterone levels of all other treatment groups did not reach statistical significance. The high testosterone concentration found in the HAART-treated animals appears discordant with the degenerative changes that were seen in the interstitium at ultrastrutural level - such as decreased Leydig cells nuclei diameters and volumes. Though the mechanism behind these findings remain unclear, a single testosterone level measurement is insufficient to indicate hypogonadism (Bhasin et al., 2006, Dube et al., 2007). It is also possible for mitochondrial dysfunction to be responsible here since it is known to be associated with an increase in fat-free mass. This in turn increases the free testosterone level following the initiation of HAART (Dube et al., 2005, Dube et al., 2007). Our findings were consistent with several other hormonal studies on HAART. For example, Collazos et al. (2002) reported an increased testosterone level in HIV patients undergoing HAART; Dube et al. (2007) also observed an increase in free testosterone after initiation of antiretrovirals in HIV patients.

In addition, we observed that LH concentrations remained unchanged when compared across all groups. This may be due to unaltered LH production from the anterior lobe of the pituitary glands (Babu et al., 2004b). Similar findings in LH levels have been reported in other studies examining the effects of alcohol (Dosumu et al., 2014a) or antiretrovirals (Collazos et al., 2002) on the testis.

Follicle stimulating hormone (FSH) is well known as an important marker for Sertoli cell function and spermatogenesis. Its action on Sertoli cell indicate it functions to increase spermatogenesis (Jensen et al., 1997b). Our result also show a decrease in serum FSH concentration in the HAART treated animals relative to controls. This explains the negative effects of HAART on Sertoli cell function and the improvement seen with adjuvant VCO administration with HAART treatment. We also observed a significant increase in the mean FSH concentration of adjuvant VCO with ethanol plus HAART co-treatment groups relative to the ethanol plus HAART group. This provides a mechanistic explanation of the positive impact of VCO on Sertoli cell functions.

Lipid peroxidation is a metabolic process that results in the oxidative degradation of lipids by excess ROS formation leading to attacks by highly reactive free radicals (Vasilaki and McMillan, 2011). Malondialdehyde (MDA) is a marker for determining the extent of lipid peroxidation in tissues (Gaweł et al., 2003). Intense ROS oxidation of lipid membranes mediate the damage to cell membranes that can alter structure and function (Vasilaki and McMillan, 2011). This further predisposes cellular organelles to functional impairment (Milei et al., 2007). Thus, interference with lipid metabolism can seriously impair male fertility (Yildiz et al., 2006b). Our result shows that both ethanol and HAART cause an increase in MDA levels. This indicates testicular lipid peroxidation. This corroborates findings by other researchers reporting increased testicular MDA levels after administration of alcohol (Dosumu et al., 2012b, Sanghishetti et al., 2012) or antiretroviral drugs (Adaramoye et al., 2015, Oputiri and Elias, 2014).

Reduced glutathione (GSH) is also an important antioxidant which plays a critical role in the prevention of cellular damage from ROS (Jurczuk et al., 2006b). It quantifies its potential defensive capacity against many xenobiotics. The current study reports that ethanol decreases testicular GSH concentration with consequent reduction in antioxidant availability thus increasing free radical activity and disrupting the redox balance. According to Rahman et al (2012), an imbalance in redox activity which results in oxidative stress is highly deleterious to tissue function. This explains the decrease in spermatogenesis observed (Agarwal et al., 2014). The improvement observed in the adjuvant VCO plus ethanol and in the adjuvant VCO with ethanol plus HAART, demonstrates VCO's potential in increasing testicular antioxidant enzyme activities. This is manifested by a decrease in MDA levels due to scavenging of excess ROS (Marina et al., 2009a, Yeap et al., 2015). The nett result is in an increase in sperm count and motility. Our result corroborates previous studies correlating decreased MDA levels and increased antioxidant levels with increased sperm count and motility (Dosumu et al., 2012a, Atig et al., 2012).

The concomitant use of alcohol with HAART will likely act synergistically to potentiate HAART toxicities (Kumar et al., 2012b). Although our experiments do not report any specific role played by key metabolic enzymes (Cytochrome P450) and drug transporters in relation to testicular interactions of alcohol and antiretrovirals, the results are still consistent with this proposition. The use of ethanol and HAART showed accentuation of adverse effects such as an increase in immotile spermatozoa, reduced sperm motility, decreased absolute volume of germinal epithelium and greater histopathological perturbation when compared to the ethanol alone treated group. These finding indicate exacerbation of testicular toxicity by antiretroviral drugs in the presence of alcohol. Thus HIV infected patients on antiretrovirals and alcohol use stand at increased risk of testicular damage and alcohol use should therefore be avoided. In these circumstances, the use of virgin coconut oil mitigates many serious adverse effects directed at the testis.

6.2 Conclusions

The use of HAART has positively influenced AIDS patients' survival with improved quality of life of PLWHAs. However, its associated organ toxicities as well as adverse effects have long been recognized to pose a serious clinical management dilemma with dire implications for global health.

Our results clearly suggest that both alcohol and HAART significantly reduced male fertility evidenced by poor quality of semen, deranged testicular morphology seen at an ultrastructural level. This is in addition to the many other derangements in the reproductive hormonal and biochemical milieu.

The use of Virgin coconut oil mitigates the deleterious effects of ethanol and HAART thereby significantly preserving and promoting testicular function. In addition, the study has however shown that the damaging effects of combined ethanol and HAART are probably irreversible.

6.3 Recommendations

In consonance with the findings of the mitigating effects of VCO on the adverse effects and toxicities resulting from concomitant use of HAART and alcohol, HIV-infected patients stand to benefit from the widespread use of VCO and enjoy an improved quality of life. Patients on antiretroviral therapy are advised to reduce alcohol consumption in order to minimize side effects and toxicity. For patients with problematic drinking behaviour who in addition have been prescribed antidepressants and antidisoptropic agents, the addition of VCO may be of great clinical value. However, further studies are still required to investigate the molecular underpinnings underlying testicular mitochondrial damage, for better understanding of the precise roles played by key metabolic enzymes and drug transporters during testicular interactions of alcohol and antiretrovirals. In addition VCO may be beneficial as an immune booster and antioxidant with other desirable medicinal properties. However the present high cost has the propensity to prohibit more widespread advocacy and use. Therefore ways to reduce cost to consumer

would be of tremendous benefit in encouraging widespread use with all the potential benefits seen resulting of this study.

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Appendix

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 August 2015

Mr Oluwatosin Olalekan Ogedengbe (215039780)
Department of Clinical Anatomy
School of Laboratory Medicine and Medical Sciences
Westivlle Campus

Dear Mr Ogedengbe,

Protocol reference number: AREC/087/015D
Project title: Testicular structure following interaction of alcohol in antiretroviral therapy: Role of virgin coconut oil extract in an experimental animal model

Full Approval – Research Application

With regards to your revised application received on 08 July 2015. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

CONDITION:

1. **A maximum of 12 animals (3 x 4 groups = 12) for the pilot study.**
2. **A maximum of 40 animals for the main / full study (5 x 8 group = 40)**

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 04 August 2016.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Dr Shahidul Islam
Chair: Animal Research Ethics Committee

/ms

Cc Superivsor: Dr OO Azu
Cc Dean / Head of School: Professor W Daniels
Cc Registrar: Mr Baatile Poo
Cc NSPCA: Ms Lebo Sentle
Cc BRU – Dr Sanil Singh

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