Testicular Morphological and Biochemical Perturbations in Experimental Animals under Antiretroviral Therapy and the Role of Naringenin, a Bioactive Flavonoid

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A Thesis submitted to Discipline of Clinical Anatomy

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Durban, South Africa

In fulfilment of the Requirement for the Degree of

Doctor of Philosophy in Clinical Anatomy

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July, 2018.

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 February 2016 to April 2018, under the supervision of _____ and _____ for the award of Doctor of Philosophy Degree in Clinical Anatomy.

Declaration

I, Dr ADANA, Misturah Yetunde declare as follows:

1. That the work described in this thesis has not been submitted to UKZN or any other tertiary institution for purposes 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 design and submission of the proposal for ethics approval by the University Animal Research Ethics Committee.
- I was whole responsible for carrying out the experiments, data and sample collection.
- I performed all sample analyses, collate and did all the analyses
- I was responsible for the writing of all the manuscripts and the thesis.

3. This thesis does not contain other person's writing, 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 though the general information attributed to them has been referenced.
- Where their exact words have been quoted, then it has been properly referenced in the relevant section.

…… …….……

Signed Date

Dedication

This work is dedicated to Almighty Allah for His mercies that has sustained me through this life-changing journey.

Acknowledgements

My sincere appreciation goes to my supervisor _____ who gave me the opportunity to be his mentee and guided me through to the completion of this programme. He will remain an important character in my success story. My profound gratitude goes to my co-supervisor _____, who has been supportive morally and academically. I appreciate the postdoctoral fellow who contributed immensely to the design and execution of the research work- Dr. Edidiong Akang. I also appreciate Dr. Peter Aniekan who was always there to assist. I say a big thank you to my colleagues in the Morphology and Andrology Group (MAG) namely Dr Ayoola Isaac Jegede, Dr Ismail Onanuga, Dr Ogedengbe Oluwatosin, Mr Ugochukwu Offor, Dr Koffi Kouame, Mr Thato Nkwagatse and Dr Aung Khaing Zaw. I appreciate Mr Salem Kharwa of blessed memory and Mr James for their support.

I am indeed grateful to the former Dean of Research, Professor Chimbari for his good and encouraging words. He made my stay in UKZN worthwhile and I learnt immensely from him. I acknowledge the College of Health Sciences for its magnanimity in training foreign postgraduate students like myself. I am grateful to the immediate past academic research leader, Dr Mitchell Gordon for her support. I will forever be thankful.

Mr and Mrs Yekini Debo Bukoye are my vehicles to this world. They shaped me right from birth into this confident and independent lady I have become. They have been supportive morally, financially and emotionally trusting me in my decisions. They have so much confidence in me and that gives me more self-assurance. Mum, even when you are no more, you cushioned the effect of the turbulence. I pray that Almighty Allah be pleased with you and forgive your shortcomings. Daddy, this is your success. May you live long in health to witness many more successes (amin). Words cannot express my gratitude.

To my siblings, my succour- Ifetayo (sister like mother), Adebola (a wonderful brother), and Adeola (sister like no other), I thank you for the unconditional love you have shown me and I was able to push through the days knowing I meant so much to you. I was away but you were frequently popping up in my mind. I will continue to love you.

To the entire Adana family, I am thankful for your warmth and acceptance. In them I found comfort and succour. They tolerated so much and accepted me for who I am. I thank you for all your advices and words of encouragement. This PhD would not have been a reality without your support. Grandpa and grandma, I say "eku aduroti". May Almighty Allah reward you immensely (amin).

My mentor, Professor Moyosore Salihu Ajao started this journey with me and I enjoyed his support consistently throughout the programme. Thank you for having so much believe in me. My friends- friends like sisters, Amina Muhammed (my No 1 cheerleader), and Mardiyyah Shittu - thank you for your love and encouragements. Guess what, I made it!

To the men that occupy a special place in my heart Dr Nurudeen Sulaiman Adana, the dearest of the dears and Sa'id Shinaayomi Adana, the sweetest of the sweets, you sacrificed the most during this PhD programme. You gave me this opportunity and supported me all the way. Your love for me has only increased despite my absence. I thank you for accepting to take the challenge with me. I owe this success to you. May Almighty Allah increase us in *barakah* (blessings) and *sekinah* (tranquillity).

To my creator, the greatest, the lord of the worlds, master of the universe, I have experienced your *ramah* (mercy) and you have granted me *taofeek* (success), I say *Alhamdulillah!*

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Abbreviations

3β HSD	3 beta hydroxysteroid dehydrogenase
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ARV	Antiretroviral
ATP	Adenosine triphosphate
BW	Body weight
CAT	Catalase
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

HIV	Human immunodeficiency virus
HPG	Hypothalamic pituitary gonadal
INSTIs	Integrase strand transfer inhibitors
IVF	In vitro fertilization
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LMPA	low melting point agarose
MDA	Malondialdehyde
MFI	Male factor infertility
MGT	Male genital tract
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
PEP	Post Exposure Prophylaxis
PIs	Protease Inhibitors
PLWHA	People living with HIV and AIDS
PrEP	Pre-exposure Prophylaxis
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase

Abstract

Declining male fertility is one of the neglected concerns of people living with HIV/AIDS in spite of a dual outlook of a social and a health dilemma. This issue of infertility is particularly relevant as majority of affected individuals are in their reproductive years. This thesis examines the impacts of the Fixed Dose Combination (FDC) of Highly Active Antiretroviral Therapy (HAART) Tenofovir/ Emtricitabine and Efavirenz (TDF/FTC/EFV) on the male reproductive capacity. It also explores the protective potentials of a bioactive flavonoid, Naringenin in testicular perturbations. The study was motivated by two major research questions namely: (1) what are the impacts of the recently approved first line antiretroviral therapy for adults FDC, TDF/FTC/EFV on the testes? (2) What is the role of Naringenin in alleviating testicular perturbations induced by HAART? Previous studies point to the negative impacts of the older generation of FDC of HAART on the semen quality and histomorphometry of the testes following a long-term use. The study addresses both the long-term and short-term use of antiretroviral drugs as observed in pre-exposure prophylaxis (PrEP) and post exposure prophylaxis (PEP). The research assesses the impacts of the drugs on the reproductive capability as well.

Findings from this study support the argument that the negative effects of the drugs were consequent upon both the short-term and long-term use. To illustrate this hypothesis, the study was conducted in two distinct phases. The first phase which lasted a total of 28 days considered the duration of PEP or PrEP. The second phase which lasted a total of ten weeks captured all the stages of spermatogenesis in rats. In this phase male Sprague Dawley rats were exposed to fertile females after the treatments. The study thus advances an understanding of the mechanism of HAART-induced testicular injury. A therapeutic dose of TDF/FTC/EFV adjusted for animal weight was aministered on a total of 48 animals randomly divided into 6 equal groups each with a different treatment as follows; Group A: Control (Distilled water);

Group B: HAART (TDF/FTC/EFV), Group C: Naringenin, 40 mg/kg; Group D: Naringenin, 80 mg/kg; Group E: HAART + Naringenin, 40 mg/kg; Group F: HAART+ Naringenin, 80 mg/kg. At the end of each phase, harvested testes were subjected to histomorphometry and ultrastructural analysis. The caudal epididymis was assessed for semen parameters and sperm mitochondrial DNA (mtDNA) fragmentation. Biochemical parameters such as serum levels of reproductive hormones (Luteinizing hormone and Testosterone) and intratesticular antioxidant enzyme activities were assayed. Contrary to prior beliefs, this research reveals that the immediate effects following short-term use of HAART are far more deleterious. This finding is consequent upon the significant drop in the sperm count ($p<0.001$) and sperms with normal morphology ($p<0.001$) compared to ($p<0.01$) in the long-term. Histomorphometric analysis also revealed a significantly shrunken seminiferous tubule following a short-term use. These outcomes were associated with an increase in the mtDNA fragmentation in group B when compared to control ($p<0.05$). Naringenin reversed abnormalities in groups E and F, displaying better semen parameters in both count and motility. Serum levels of testosterone were altered in both phases. The overall effects of all these changes were observed in the pregnancy rate which reduced in group B when compared to all the other groups.

This study established that HAART has deleterious effects on the testicular microanatomy and function. These effects may impact on steroidogenesis and ultimately spermatogenesis. It consequently impairs fertility while Naringenin promises to be a potential complimentary adjuvant especially in the short term therapies.

Keywords: HAART, semen parameters, reproductive hormones, testicular ultrastructure, 3 beta hydroxysteroid dehydrogenase

Isifingqo (Isizulu Abstract)

Le ngqungquthela ikhanyisa ngemithelela ye-Fixed Dose Combination (FDC) ye-Highly Active Antiretroviral Therapy (HAART) Tenofovir / Emtricitabine ne-Efavirenz (TDF / FTC / EFV) emandleni okuzala abesilisa futhi iphinde ihlole amandla okuvikela we-flavonoid ephilayo, I-Naringenin ku-perturbations ye-testicular. Ucwaningo lwalukhuthazwa imibuzo emibili yocwaningo; (1) yiziphi impembezo zokwelashwa kwe-antiretroviral emidlalweni yakamuva eyamukelwa yilabo abadala abadala i-FDC, i-TDF / FTC / EFV kuma-testes? (2) Iyini indima ye-Naringenin, i-antioxidant enamandla ekunciphiseni ukuphazamiseka kwe-testicular okubangelwa i-HAART? Ucwaningo oluphambili lubonisa imiphumela emibi yesizukulwane esidala se-FDC ye-HAART emkhakheni wezinja kanye ne-histomorphometry yezivivinyo ezilandela ukusetshenziswa kwesikhathi eside. Ucwaningo lubhekisela kokubili ukusetshenziswa kwesikhathi eside nesikhathi esifushane semishanguzo ye-antiretroviral njengoba kwenzeka ngaphambi kokushiswa kwe-prophylaxis (PrEP) nokuthunyelwa kwe-post exposure prophylaxis Ucwaningo aluhlolisi kuphela impembelelo kwi-histomorphometry kanye nekhwalithi yesilisa kepha futhi nangokwazi ukuzala.

Siphikisa ukuthi imiphumela emibi ilandelwa kokubili ukusetshenziswa kwesikhathi esifushane nesikhathi eside. Ukufanekisa lesi sizathu, isifundo senziwa ngezigaba ezimbili ezihlukene. Isigaba sokuqala sicubungula ubude be-PEP noma i-PrEP futhi bugcine izinsuku ezingu-28. Isigaba sesibili sagcina amasonto ayishumi ukuze athathe zonke izigaba ze-spermatogenesis kuma rats. Kulesi sigaba sokuhlolwa, izibonelo zezilwane, amantombazane amadala aseSprague Dawley abuye avulekele kwabesifazane abakhulayo abalandela ukwelashwa. Ngakho-ke isifundo sithuthukisa ukuqonda kwethu indlela yokulimala kwe-HACT-induced testicular. Sasebenzisa umthamo wokwelashwa we-TDF / FTC / EFV olungiselelwe isisindo sezilwane esilinganisweni sezilwane ezingu-48 ngezikhathi ezithile ezihlukaniswe ngamaqembu alinganayo angu-6 ngamunye onokwelashwa okuhlukile kanje; Iqembu A:

Ukulawula (Amanzi alayishiwe); Iqembu B: I-HAART (TDF / FTC / EFV), Iqembu C: I-Naringenin, 40 mg / kg; Iqembu D: Naringenin, 80 mg / kg; Iqembu E: HAART + Naringenin, 40 mg / kg; Iqembu F: HAART + Naringenin, 80 mg / kg. Ekupheleni kwesigaba ngasinye sesilingo, izivivinyo zavuna futhi zahlushwa i-histomorphometry kanye nokuhlaziywa kwezingqalasizinda kulandelwa izifundo zesimo sokuhlola ukuvunguza kwemifino kanye nemithi ephelele ye-epithelium yegciwane, i-lumen ne-intersitium. I-epididymis e-caudal yahlolwa nge-paramen parameters kanye nokwahlukana kwe-DNA (i-mtDNA) ye-sperm.

Imingcele ye-biochemical efana nama-serum ama-hormone wokuzala (i-Luteinizing hormone ne-Testosterone) nomsebenzi wokuvikela i-antioxidant enzyme. Inkulumo ye-testicular 3-beta hydroxysteroid dehydrogenase (3β HSD) inqume ukuhlola izifundo ze-immunohistochemical.

Okutholakele kwalolu cwaningo oluphambene nezinkolelo zangaphambili kubonisa ukuthi imiphumela esheshayo emva kokusebenzisa isikhathi esifushane kwe-HAART yinto ehlukumeza kakhulu ukusetshenziswa kwesikhathi eside. Lokhu kuboniswa ukuhlaselwa okuphawulekayo ekubaleni kwesidoda ($p<0.001$) kanye nezidakamizwa ezine-morphology evamile ($p<0.001$) uma kuqhathaniswa ne ($p<0.01$) esikhathini eside. Ukuhlaziywa kwe-Histomorphometric kwabuye kubonisa ukuthi i-tubul ne-shrunken isetshenziswe kakhulu ngokulandela isikhathi esifushane. Lokhu kwahlobaniswa nokwanda kwendlela yokuhlukaniswa kwe-mtDNA nokwanda kwamademoni e-group B uma kuqhathaniswa nokulawula ($p<0.05$). Ucwaningo lubonisa indima yokuvikela yeNaringenin. Lokhu kwakuvezwe ngokwengeziwe esifundweni esifushane. Ukungahleleki kwemvelo kwaguqulwa emaqenjini E no-F abonisa imingcele ye-semen engcono esikhathini esifushane kunesikhathi eside. Ngokuqondene nokubalwa kwesidoda kwakukhona ukuthuthukiswa kwezigaba ezimbili ngokuhlanganiswa kwe-HAART ne-Naringenin kodwa ukubaluleka kwaqoshwa kuphela esikhathini esifushane ($p<0.01$). Kodwa i-motility yayithuthukisiwe kangcono ngokuhamba kwesikhathi ($p <0.001$). Izinga le-serum testosterone lashintshwa kuzo zombili izigaba. Inkulumo ye-3β HSD yancishiswa

ngokusetshenziswa kwe-HART kodwa uNaringenin wakwazi ukubuyisela inkulumo kuye eduze evamile. Imiphumela yonke yalezi zinguquko yabonakala ngesikhathi sokukhulelwa okwehliswa eqenjini B uma kuqhathaniswa nawo wonke amanye amaqembu.

Lolu cwaningo luqinisekisa ukuthi ukwelashwa kwe-antiretroviral esebenzayo kunemiphumela emibi kakhulu kwi-microanatomy ye-testicular, kanye nomsebenzi. Lezi zingaphazamisa i-steroidogenesis futhi ekugcineni i-spermatogenesis, ngaleyo ndlela iphazamisa ukuzala kodwa uNaringenin uthembisa ukuba yi-adjuvant engase ibe yinkimbinkimbi ikakhulukazi emitholampilo

1. CHAPTER ONE

INTRODUCTION

1.0. Background

The testis is the cardinal organ of reproduction and endocrinal functions in males. An optimised reproductive functioning in males is dependent on an intact testicular microanatomy. However, it is highly susceptible to damage due to its highly proliferative nature (Shaha et al., 2010). Mammalian spermatogenesis is complex because the process that allows minimal deviations from the normal homeostatic milieu of structures involved. The testes are exposed to many environmental toxins that threaten the precise ambience required for its functions (Tomova and Carroll, 2018). These include radiations, chemicals, diet, infections, and drugs (Hao et al., 2016, Ma et al., 2018, Kong et al., 2014, Li et al., 2016, Marettová et al., 2015, Awodele et al., 2018). Popular explanations for these injuries are an alteration in the microanatomy of the testes ranging from changes in vascular endothelial cells and smooth muscle cells, atrophy of the seminiferous tubules, cytoplasmic reorganisation and disruption of Sertoli-Sertoli tight junctions (Marettová et al., 2015). Other hypotheses regarding testicular insult include hormonal perturbations (Wunder et al., 2008, Ma et al., 2007) and free radical injury (Anim et al., 2005, Ma et al., 2018). Despite the extensive research on male factor infertility (MFI), many cases are still classified as idiopathic which indicates that the exact mechanism of injury is yet to be deciphered.

Many of the identified toxic agents, particularly drugs, are indispensable due to the lack of alternatives. Antiretroviral (ARV) drugs are a good example of this. The drugs have succeeded in promoting the health and well-being of people living with Human Immune Deficiency Virus (HIV)/ Acquired Immunodeficiency Syndrome (AIDS) (PLWHA)

(Nakagawa et al., 2012, WHO, 2016). However, they have had to live with the attendant side effects of these drugs ranging from minor dermatological to life-threatening anaphylactic reactions. An impairment of male sexual and reproductive performance has been identified but neglected.

Similar to other medical conditions with unknown aetiology, phytonutrients are being considered to possess beneficial potentials in restoring testicular functions. A major challenge contributing to the paucity of data in this regard is the ethical dilemma researchers face in obtaining testicular samples from patients (Ramstein et al., 2017). Fortunately, in spite of the interspecies variations, the rodent testis has been found to be similar to that of humans in some ways making it a good model for testicular studies (Working, 1988).

1.1. Anatomy and Physiology of Reproduction in Males

The male reproductive system comprise of several structures in reproductive, nervous and endocrine systems simply termed the hypothalamic-pituitary-testis axis. Production of the male reproductive gamete, which is the functional unit of the male reproductive system, takes place within the testes and it lasts approximately 3 months. This process is preceded by hormonal interactions in the pituitary and hypothalamus. The hormones travel through the bloodstream to influence activities within the gonads.

1.1.1. Anatomy of the rat testis

The testes in rats are paired almond-shaped organs located in the pelvis underneath the hind limbs. They are particularly larger when compared to the body size of the rats. This fact is attributed to the mating habits that are more polygamous (Kenagy and Trombulak, 1986) and to the high copulatory frequency and sperm production (Kenagy and Trombulak, 1986, Gomendio and Roldan, 1991). The testes are contained in two separate membranes called

scrotal sacs. In a young rat, they descend between the ages of 4-6 weeks. Throughout the rat's life, the testes are able to move up into the rat's abdominal cavity because it has an open inguinal canal. Spermatogenesis occurs in the epithelial lining of the seminiferous tubes found within the testes. Further description of the rat testis is similar to that of humans as described below in figure 1.

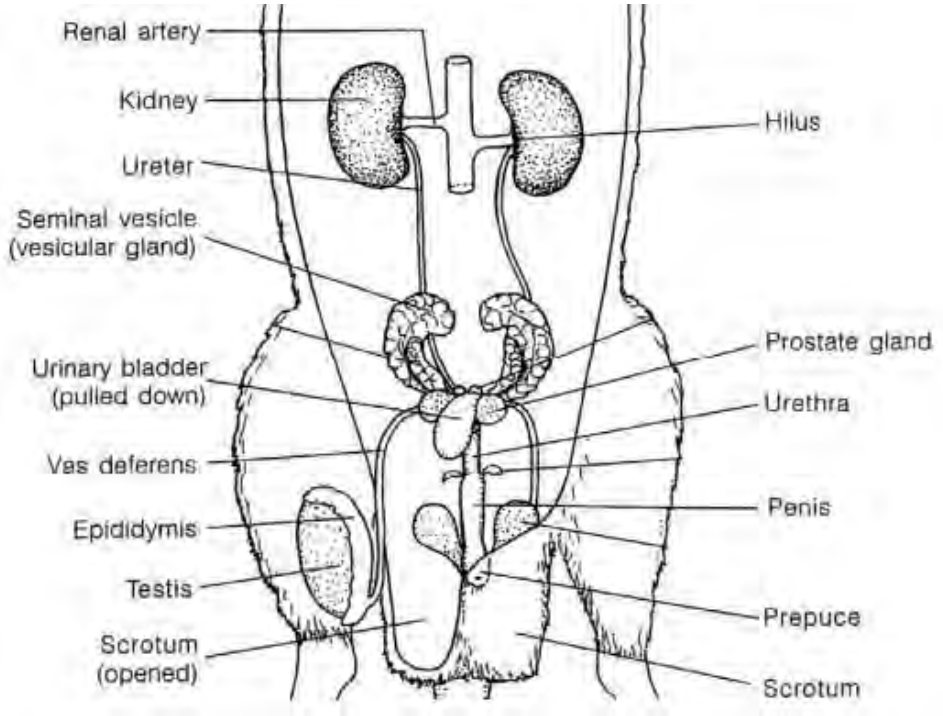

Figure 1: The Reproductive Structures in Rats (Jones, 2018).

1.1.2. Gross anatomy of the human testis

The testis is the male gland known to be important for both reproductive (exocrine) and endocrine functions. Initially, it begins as an undifferentiated gonad in the retroperitoneal area. As the foetus develops, the functioning testis produces the male hormone testosterone

to allow development of male genitalia. Within the last 3 months of gestation, the testis must course its way down from its original retroperitoneal position to its final destination in the scrotum. During its journey, it must pass through the peritoneum, abdominal wall enroute the inguinal canal, and then into the scrotal pouch.

The testis is a paired, ovoid male reproductive organ that sits in the scrotum. It is separated from each other by a scrotal septum. Described as being shaped and sized like a large olive or small plum, the average volume of the adult testis is approximately 25ml. Typically, it measures 3.5-5 cm in length, 2.5-3 cm in both width and 3 cm in depth (anteroposterior diameter).

The testis sits obliquely with its long axis mostly vertical with a slight anterior and lateral slant to the superior pole. Superiorly, it is suspended by the spermatic cord, with the left testis often sitting lower than the right testis. Inferiorly, the testis is anchored to the scrotum by the scrotal ligament, a remnant of the gubernaculum. The tunica vaginalis testis (a remnant of the processus vaginalis) envelopes the testis in a double layer, except at the superior and posterior borders, where the spermatic cord and epididymis attach to the testes.

The visceral layer of the tunica vaginalis testis is closely applied to the testis, epididymis, and ductus deferens. On the posterolateral surface of the testis, the layer invests a slit-like recess between the body of the epididymis and the testis that is called the sinus of epididymis (Moore K, 2006).

The parietal layer of tunica vaginalis which is adjacent to the internal spermatic fascia, is more extensive. It extends superiorly into the distal part of the spermatic cord. Deep to the tunica vaginalis, the tunica albuginea is a tough fibrous outer covering of the testis. On the

posterior surface, it is reflected inwardly to form an incomplete vertical septum called the mediastinum testis.

The mediastinum testis extends from the superior portion to near the inferior area of the gland. It narrows in width as it travels inferiorly. Anteriorly and laterally, numerous imperfect septa are given off, which radiate to the glands surface and are attached to the tunica albuginea. This structure divides the interior of the testis into numerous, cone-shaped spaces that have a wide base at the gland's surface and became narrow as they converge to the mediastinum. In the spaces, the numerous lobules of glandular structures (the minute but long and highly coiled seminiferous tubules) are housed. The mediastinum supports the ducts and vessels as they pass to and from the glandular substance. The seminiferous tubules are lined with germ cells that produce sperm and nutrient fluid. These tubules empty their contents into a network of anastomosing ducts, which ultimately empties into the epididymis.

The epididymis is a comma-shaped, elongated structure composed of a single, fine tubular structure estimated up to 6 meters (approximately 20 feet) in length. This tube is highly convoluted and tightly compressed (average size of approximately 5 cm) to the point of appearing solid. Located at the posterior border of the testis, it is composed of three parts, namely: the head (caput), body (corpora), and tail (cauda). The epididymal head overhangs the upper pole of the testis. It receives the seminal fluid from the ducts of the testis (which pierce the upper portion of the mediastinum). It then allows the passage of the sperm into the distal portion of the epididymis. Because of its length, the epididymal duct allows space for storage and maturation of sperm. Progressively tapering in width, the narrow tail continues as the ductus deferens (Daniel, 2016).

The blood supply to both testes is primarily from the testicular arteries, which arise from the anterolateral aspect of the abdominal aorta just inferior to the renal arteries. They travel retroperitoneally, cross over the ureters and the inferior parts of the external iliac arteries and pass through the deep inguinal ring into the inguinal canal. It then becomes one of the components of the spermatic cord. The testicular artery enters the testis through the posterior midportion. The testicular artery or one of its branches anastomoses with the artery of the ductus deferens.

Venous drainage from the testis and epididymis form a network of 8-12 veins, called the pampiniform venous plexus, lying anterior to the ductus deferens. It surrounds the testicular artery in the spermatic cord. The veins converge superiorly forming a testicular vein, after passing through the deep inguinal ring. The right testicular vein enters the inferior vena cava, while the left testicular vein drains into the left renal vein. Lymphatic drainage of the testis follows the testicular vessels (in the spermatic cord) to the right and left lumbar (caval/ aortic) and preaortic lymph nodes at the second lumbar level.

Autonomic innervations of the testis arise as the testicular plexus of nerves on the testicular artery, which contains vagal parasympathetic and visceral afferent fibres and sympathetic fibres from the T7 segment of the spinal cord.

1.1.3. Microscopic Anatomy of the Testis

The testis is composed of lobules of glandular tubules. These tubules are highly convoluted structures held together by loose connective tissue with interspersed groups of “interstitial cells,” which contain Leydig cells. The individual tubule consists of a basement membrane

formed by laminated connective tissue with numerous elastic fibres. There exists flattened cells between the layers and covered by an external layer of flattened epithelioid cells. Within the basement membrane are epithelial cells arranged in several irregular layers but may be separated into germ cells at the periphery and varihumanous cells of spermatogenesis up to mature sperm cells as they advance toward the lumen. Also, interspersed in the layer are Sertoli cells, which project inward from the basement to the lumen and provide support to the developing sperm cells.

Testicular histology magnified 500 times. Leydig cells reside in the interstitium. Spermatogonia and Sertoli cells lie on the basement membrane of the seminiferous tubules. Germ cells interdigitate with the Sertoli cells and undergo ordered maturation, migrating towards the lumen as they mature. In the apices of the lobules, the tubules are less convoluted. They converge into 20-30 larger, straight ducts (tubuli recti). These ducts merge into anastomosing tubes in the fibrous stroma, lined with flattened epithelium (rete testis). The tubes terminate into approximately 15 ducts that are initially straight in their course.

After piercing the tunica albuginea at the superior mediastinum, the tubes enlarge and become increasingly convoluted. These convolutions are held together by fine areolar tissue and bands of fibrous tissue. They form a series of conical masses, the conic vasculosi. The series forms the head of the epididymis. The ducts are thicker and lined by ciliated columnar epithelium. Below this epithelium is muscular tissue arranged in a mostly circular fashion. As the tail of the epididymis merges with the ductus deferens, the microscopic anatomy demonstrates a thickened duct with an increased muscular material, increased in diameter, and still lined with ciliated columnar epithelium.

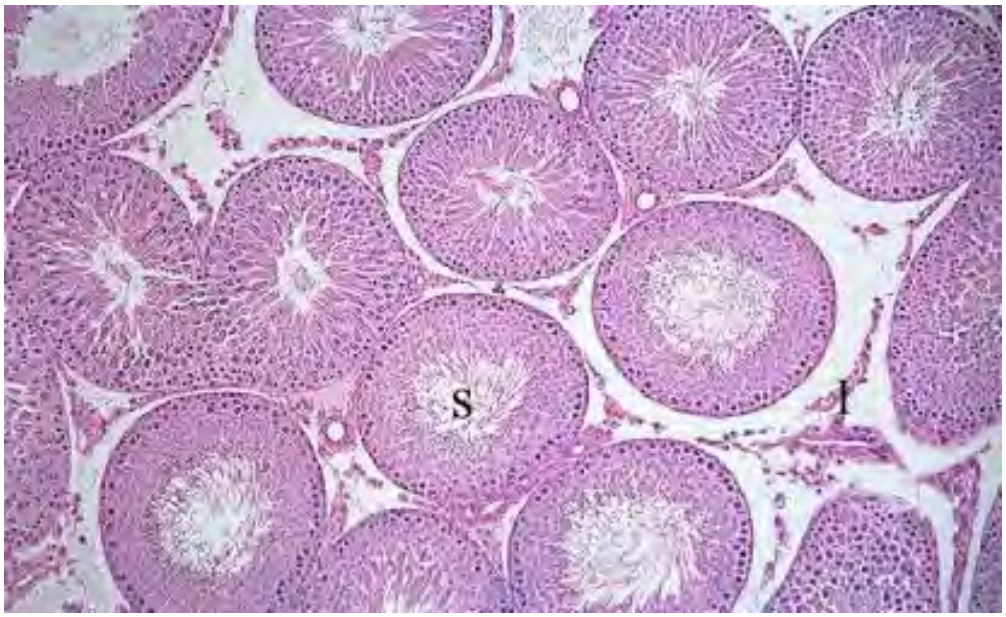

Figure 2(a): Cross section of the Haematoxylin and Eosin stained rat testis showing the microanatomy. **S** represents seminiferous tubules and **I** represents the interstitium (Source: http://www.columbia.edu/cu/biology/courses/w2501 accessed 08/07/2018)

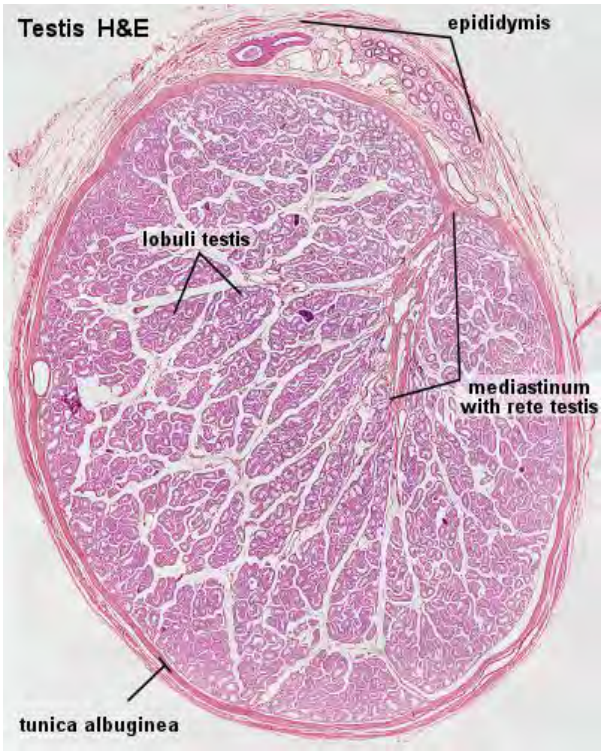

Figure 2(b): Sagittal section of the Haematoxylin and Eosin stained rat testis showing the microanatomy (Source: www.embryology.med.unsw.edu.au/embryology/index.php?title=file:Testis_histology.jpg&mobileaction=toggle_view_mobile accessed 08/07/2018)

1.1.4. Comparative testicular histology in man and rodent

The spermatogenic cycles of man and animals have several similar characteristics with every stage being precise in timing, and maturity (Clermont, 1972). There are about five to six generations of germ in any given area of the germinal epithelium with the seminiferous tubule. These cell populations form associations of definite and fixed configuration (Leblond and Clermont, 1952). During any stage of the cellular maturation in the seminiferous epithelium, one or two generations of spermatids would be allied with one or two cohorts of spermatocytes and spermatogonia. Each of this cell association cytologically forms a stage of spermatogenesis. It ranges from specie to specie. There are 6 stages in humans while the

rats have 14 (Leblond and Clermont, 1952, Heller, 1964). The orderly arrangement of the spermatogonic series makes up the cycle of the germinal epithelium that appears sequentially over time in the same area of the seminiferous epithelium. The duration of time for a cycle corresponds to the period from the disappearance of one particular stage to its reappearance in a given area of the seminiferous tubule which ranges from 8.6 days in the mouse to 16 days in humans. The length of time for the spermatogonic process to be completed, in other words for a given stem cell to produce mature sperm cell corresponds to the length of approximately 4 to 4.5 cycles in laboratory animals and humans (ranging from 35 days in the mouse to 74 days in humans).

A cross-section of a mammalian testis displays numerous cross-sections of the seminiferous tubules (Figure 2). It has been revealed that "In most mammals, the stages of spermatogenesis appear not only sequentially in time, but also sequentially in space (along the length of the seminiferous tubule). This wave of the seminiferous epithelium has been described for numerous model species" (Perey et al., 1961). The unpredicted consequence of this wave is that each seminiferous tubule has only a single spermatogenic stage when viewed in cross-section. This makes it easier to quantify the toxic effects of gonadotoxic agents in the animal testis. However, this is not the case in humans as the arrangement of germ cells in the seminiferous epithelium are not as orderly when the tubule is viewed in whole mount. Specific cellular associations appear in irregular zones throughout the seminiferous epithelium. Also there are numerous heterogeneous stages made up of unexpected presence or absence of germ cells in typical cell associations (Heller, 1964). Consequently, a cross-section through a tubule reveal an array of cells of diverse stages. Later, some researchers who utilized morphological and morphometric methods to study human spermatogenesis (Schulze and Rehder, 1984) reported that increasingly mature spermatocyte populations are

arranged on helices that are contracted conically towards the lumen of the seminiferous tubule. They emphasised that this arrangement could be a natural outcome of the markedly lower rate of spermatogonial division in humans in comparison with other mammals. They, therefore, concluded that "quantitation of toxic effects on spermatogenesis is a much more difficult task in humans, and direct comparison to animal models may not always be possible or warranted." (Schulze and Rehder, 1984).

Determination of spermatocyte and spermatid numbers are alternative methods that have been described for the determination of sperm production rates from testicular histology in humans (Berndston, 1977, Steinberger and Tjioe, 1968). These methods can also be used to assess reproductive toxicity if testicular samples are available. Due to the difficulties in obtaining testicular biopsies of humans, assessment of reproductive toxicity is usually limited to semen analysis which consists of sperm count, determination of the percentage of progressively motile sperms, the percentage of sperms with abnormal morphology and the volume as well as the consistency of the semen over a short period.

1.2. Spermatogenesis

Spermatogenesis is the process of gradual transformation of germ cells into spermatozoa. It occurs mainly within the seminiferous tubules of the testes and can be divided into three phases, each of which is associated with a different type of germ cell:

- Proliferative phase: spermatogonia → spermatocytes
- Meiotic phase: spermatocytes → spermatids
- Differentiation phase (also known as spermiogenesis): spermatids → spermatozoa

Unlike the female production of gametes which occurs entirely before birth and gamete maturation occurring in a pulsatile fashion after puberty, males produce gametes continuously from puberty all through their reproductive lives and the release of the gametes is constant.

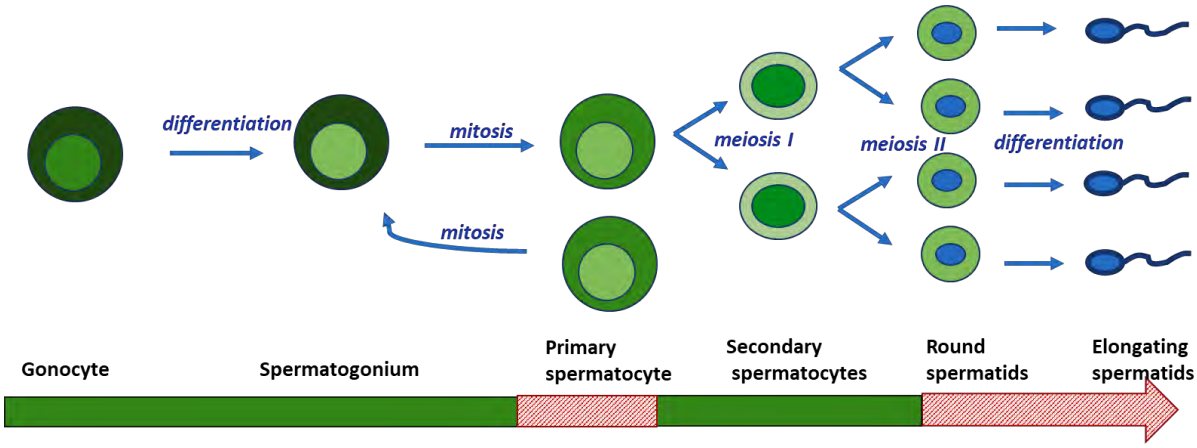

Figure 3: Schematic illustration of the stages of spermatogenesis. The direction of the arrow indicates the successive stages of spermatogenesis. The green parts of the arrow indicate active transcription, the red parts represent point of transcriptional arrest and transcriptional cessation.

The seminiferous tubules are the site of spermatogenesis. The two main cell types within the tubules involved in spermatogenesis are the germ cells, which develop into sperms and somatic cells known as Sertoli cells, which nurture the germ cells throughout the development process. As the germ cells progress through the various stages of development, they move slowly from the basement membrane of the tubules through the tight junctions between the Sertoli cells into the tubular lumen.

1.2.1. Stages of spermatogenesis

1.2.1.1. Proliferation phase

Stem or A spermatogonia located in the basal region of the tubular epithelium undergo mitosis. The progeny of these divisions maintain their own numbers as well as giving rise to several interconnected B spermatogonia (the number of these arising from a single A spermatogonia is species dependent). B spermatogonia divide to give rise to primary (1°) spermatocytes. All descendants of a B spermatogonium remain connected by cytoplasmic bridges, forming a syncytium - like cell clone which undergoes synchronous development.

1.2.1.2. Meiotic phase

Each 1° spermatocyte divides to give rise to two short-lived secondary (2°) spermatocytes, which in turn give rise to two spermatids each. The spermatids contain a haploid number of chromosomes (half the number of a somatic cell). Primary spermatocytes are the largest cells in the spermatogenic series and are located approximately midway within the seminiferous epithelium. The process of meiosis occurs over a long period, with prophase of the first meiotic division taking up to three weeks (Hess 1999; Cunningham, 2007).

1.2.1.3. Differentiation phase

The differentiation phase is also known as spermiogenesis. During this phase spermatids undergo transformation into spermatozoa. Many changes occur within the cells, the three major changes are:

i) formation of the acrosome that covers the cranial part of the head. The acrosome will contain hydrolytic enzymes to allow fusion of sperm and egg for fertilization.

ii) condensation of nuclear chromatin in the head to form a dark-staining structure

iii) growth of the tail opposite the acrosome, and loss of excess cytoplasmic material which is shed as a residual body. The body is phagocytosed by the Sertoli cells. The morphological changes occurring during this process can be seen when sections of different seminiferous tubules are examined.

1.2.2. Spermatogenic cycle and wave

A spermatogenic cycle represents the time required for one cell to go through all the stages of spermatogenesis. Since the spermatogenic cycle lasts a certain length of time and each step occupies a specified duration, a finite number of cellular association results. This means that specific groupings of cells in different steps of spermatogenesis will coexist. There usually exist an overlapping of different generations presents within the germinal epithelium at a particular point in time. The series of changes between two successive appearances of the same cellular associations make up one cycle of the seminiferous epithelium. One cellular association is a stage of the seminiferous cycle. Stages of the cycle may be recognized by the appearance of the acrosome, meiotic divisions, and the shape of the spermatid nucleus, the release of the spermatozoa or a combination of all characteristics (Clermont, 1972).

There exist an organized progression of the different stages of spermatogenesis along the tubule. This phenomenon is known as the spermatogenic wave. It is in actual fact the changing cellular associations in space instead of time. This progression of stages makes certain the continual production of spermatozoa at any specific time (Hogarth and Griswold, 2010). The cycle is useful in quantitating the effects of drugs, hormones, nutritional factors, etc., on the different steps of spermatogenesis. There are specie differences in the

number of cellular associations in a cycle of the epithelium depending on the number of spermatogonial divisions in that specie and the number of distinguishable stages of spermatid development in that specie (Perey et al., 1961).

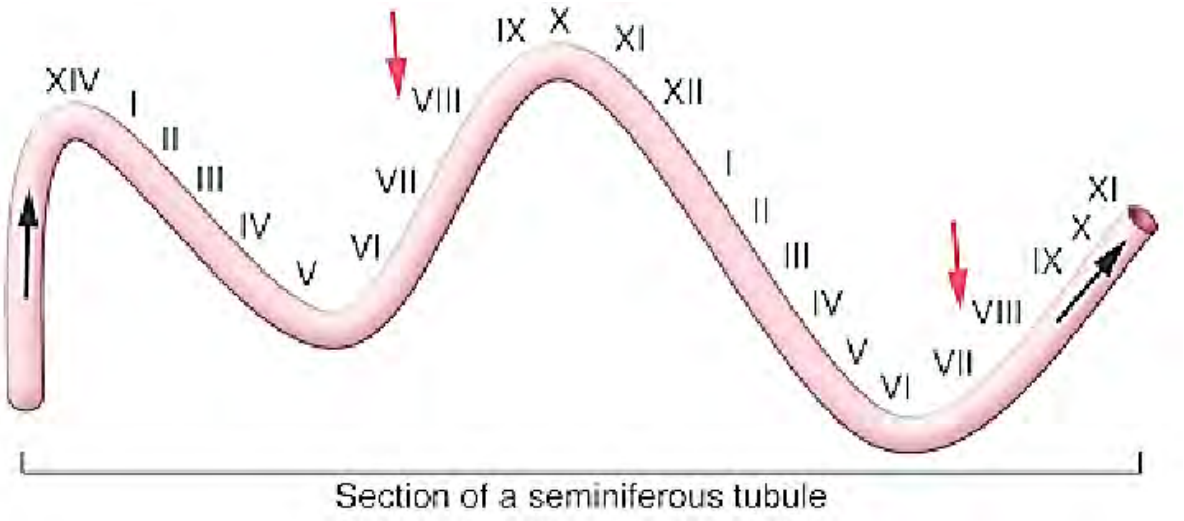

Figure 4: Depiction of the spermatogenic wave, the organized progression of the different stages of spermatogenesis along the tubule. The point of meiotic initiation (red arrows) moves in the direction of the black arrows leading to continual and asynchronus release of spematozoa (Hogarth and Griswold, 2010).

1.2.3. Interspecies spermatogenetic differences

The human male is of relatively low fertility compared to most animal species. An estimated 15 to 20% of all American couples are infertile (Gray, 2000), less than one-third of all conceptions result in a live birth and some 20% to 30% of all developmental defects have genetic origins (Swartz MH, 2006). Between 10 and 20 chemicals or drugs have been directly demonstrated to adversely affect human male reproduction (Gray, 2000). Substantial

information concerning reproductive toxicants has been collected using animal models. Thus, the scientific process for the evaluation of human reproductive risk from drug and chemicals includes extrapolation from data obtained using these models.

Spermatogenesis in rats occurs in the seminiferous tubules, consisting of approximately 10 - 20 in each rat testis. During spermatogenesis, the primitive, diploid, stem cell spermatogonia give rise to highly differentiated, haploid spermatozoa. The process involves several mitotic divisions of the spermatogonia, which eventually results in the production of the spermatocyte. The spermatocyte is the cell which undertakes the course of meiosis beginning with duplication of its DNA during preleptotene, pairing and condensing of the chromosomes during pachytene and finally culminating in two reductive divisions to produce the haploid spermatid. The spermatid starts as a round cell then rapidly undergoes a series of complex morphological changes. The nuclear DNA becomes highly condensed and elongated into a head region which is covered by a glycoprotein acrosome coat while the cytoplasm develops into a whip-like tail flagellum and tightly-packed mitochondria. The successive morphological steps in the differentiation of the spermatid (19 steps of spermiogenesis) provide the basis for the identification of the stages of the spermatogenic cycle in the rat.

In a cross-section of a seminiferous tubule, the germ cells are arranged in discrete layers. Spermatogonia lie on the basal lamina, spermatocytes are arranged above them and then one or two layers of spermatids above them. In any given normal tubule, four generations of cells develop concurrently and in precise synchrony with each other. As each generation develops, it moves up through the epithelium, continuously supported by Sertoli cells until the matured sperms are released into the tubular lumen in a process called spermiation. The synchrony of the development between the 4 cells is such that each successive stage of development of

the spermatogonium is found with its characteristic spermatocyte and spermatids. The synchronous development of the 4 in the repetitive appearance of specific cell associations are referred to as stages of the spermatogenic cycle. Fourteen of such cell associations occur in the rat and are referred to as stages I-XIV of the spermatogenic cycle. The main known effects of testosterone that supports spermatogenesis are to stimulate seminiferous tubule fluid production, regulate the release of the mature spermatids from the Sertoli cell and to support the pachytene spermatocytes and later germ cell types through stage VII of the spermatogenic cycle. This spermatogenic support appears to be mediated by the secretion of several specific proteins from the Sertoli, peritubular and germ cells whose secretion is dependent on testosterone as well as a full complement of germ cells. Selective depletion of any of the different populations of cells from these stages will differentially alter the secretion of each of the androgen-regulated proteins.

1.2.4. The hypothalamus, pituitary gland and hormonal control of spermatogenesis

The hypothalamus is a small almond-sized region of the brain located directly above the brainstem. It is a collection of nuclei that performs a variety of functions mainly homeostatic and hormonal. It controls body temperature, blood pressure, and caloric intake as well as the expenditure at a fairly constant level. One of the ways in which the hypothalamus can restore homeostasis and influence behaviour is through the control of hormone release from the pituitary gland. It does this by releasing gonadotropin-releasing hormone (GnRH). The hypothalamus is anatomically and functionally related to the pituitary gland (hypophysis), a bean-sized organ lying within the sella turcica of the sphenoid bone of the skull. The pituitary gland is suspended from it by a stem called the infundibulum, a bridge of capillaries that

connects the hypothalamus to the anterior pituitary. It is made up of two lobes- the anterior and the posterior.

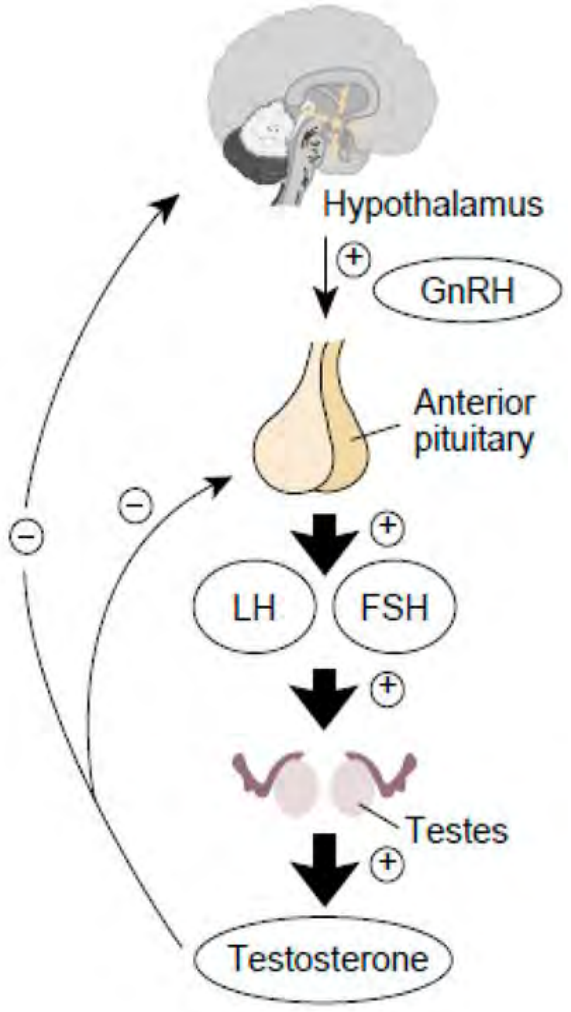

Figure 5: Schematic representation of the hypothalamic–pituitary–testicular (HPT) axes (Kong et al., 2014).

The posterior stores two hormones produced by the hypothalamus (oxytocin and antidiuretic hormone). The anterior is responsible for the secretion of hormones under the influence of releasing hormones from the hypothalamus. The hypophyseal portal system allows hypothalamic hormones to be transported to the anterior pituitary without first entering the systemic circulation.

Spermatogenesis is controlled by a complex feedback mechanism involving the hypothalamus, anterior pituitary, and testes. Gonadotrophic releasing hormone (GnRH) is released by the hypothalamus in a pulsatile manner and travels through portal vessels to the anterior pituitary, where it acts as the gonadotrophic cells. These cells respond to the stimulation by producing either follicle stimulating hormone (FSH) or luteinizing hormone (LH) depending on the pattern of GnRH secretion.

LH and FSH travel in the bloodstream to the testes, where LH acts on the Leydig cells to stimulate conversion of steroids to testosterone and other androgens. This process in turn contribute to the stimulation of Sertoli cells. FSH acts on the receptors of Sertoli cells and, in combination with testosterone, stimulates many functions, including synthesis and secretion of oestrogen, inhibin and many other products, meiosis, spermatocyte maturation and Leydig cell function. Inhibin, testosterone and oestrogen feedback negatively on the anterior pituitary and hypothalamus to suppress secretion of gonadotrophic hormones (Figure 2).

The disruption of the balanced system may cause disorders of reproduction. The lack of appropriate cyclical release of GnRH by the hypothalamus leads to loss of normal pulsatile LH release by the pituitary gland. This results in impaired testosterone and sperm production in men. The cause of disturbed cyclic release of GnRH is unclear, though hyperprolactinemia, elevated endorphins, and high levels of GnRH and LH caused an underlying medical condition have been implicated.

1.3. Fertility issues in males

Reproduction is the result of an interaction between the structural and functional property of an organism. A successful reproduction is thus dependent on the efficiency of the physiology of the reproductive system which is also largely determined by a normal anatomy of the organism. It, therefore, means that a structural defect in the reproductive system of an organism may result in subfertility or infertility when severe. Infertility is "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild et al., 2009). Male infertility, however, refers to a male's inability to cause pregnancy in a fertile female. In men, it accounts for 40–50% of infertility (Hirsh, 2003, Brugh and Lipshultz, 2004) and approximately 7% of all men are affected (Lotti and Maggi, 2014). A male infertility factor is identified in about 50% of these cases and is solely responsible in 20% of couples. Majority of male reproductive problems have an underlying anatomic basis.

1.3.1. Anatomical basis of male infertility

For the purpose of this write-up, the causes of male infertility will be categorized as pre-testicular, intra-testicular and post-testicular causes.

1.3.1.1. Pre-testicular causes

These are a group of rare endocrine conditions in the extra-gonadal system resulting in male factor infertility, such as those originating in the hypothalamus, pituitary, or adrenals, and have an adverse effect on spermatogenesis (Robertson et al., 1999). It is a less common aetiology than other causes of male infertility that result in deficient androgen secretion and

subsequently impaired spermatogenesis. It is termed hypogonadotropic hypogonadism, secondary or central hypogonadism. There are two main types,

- Primary hypogonadism hypogonadotropic: this is a genetic disorder that prevents onset or completion of puberty. All hormonal levels are diminished, the hormones of the pituitary and testicles do not have the required levels of proper testicular function. The underlying cause of hypogonadotropic hypogonadism is a failure in the correct action of the hypothalamic hormone GnRH. This failure in GnRH activity can either be due to the absence of the GnRH releasing neurones inside the hypothalamus due to a failure of LHRH cells to migrate into the brain (Schwanzel-Fukuda et al., 1989) or the inability of the hypothalamus to release GnRH in the correct pulsatile manner to ensure LH and FSH release from the pituitary (Mitchell et al., 2011). A number of gene defects have been attributed to this condition (Layman et al., 2013, Valdes-Socin et al., 2014, Melmed, 2015). A prevalence rate of 1: 10,000-30,000 males have been reported (Chauvel, 1973, Laitinen et al., 2011).
- Secondary hypogonadotropic hypogonadism, also known as acquired or syndromic hypogonadotropic hypogonadism, is commoner than primary hypogonadotropic hypogonadism. It is responsible for most cases of the condition. It has many causes ranging from pituitary and hypothalamic tumours, pituitary apoplexy, head trauma, ingestion of certain drugs including abusive alcohol or illicit drug intake, hemochromatosis, systemic disorders such as chronic illnesses, pituitary/brain radiation, exhausting exercise, nutritional deficiencies to obesity (Fraietta et al., 2013).

Other causes of pre-testicular infertility include coital disorders such as erectile dysfunction and ejaculatory maladies. These are difficulties experienced by an individual or a couple during any stage of a normal sexual activity, including physical pleasure, desire, preference, arousal or orgasm. They are very rare causes of infertility. Anejaculation, retrograde ejaculation and orgasmic dysfunction are examples of conditions that can give rise to coital disorders. They are associated with retroperitoneal lymph-node dissection (Dimitropoulos et al., 2015), spinal cord injury (Brackett, 1999), bladder neck surgery (Hedlund and Ek, 1985, Kochakarn and Lertsithichai, 2003) and multiple sclerosis (Colpi et al., 2004). Anejaculation is the complete absence of an antegrade or retrograde ejaculation. It is caused by a failure of emission of semen from the seminal vesicles, the prostate, and the ejaculatory ducts into the urethra while retrograde ejaculation is the total or sometimes partial absence of an antegrade ejaculation because semen passes backwards through the bladder neck into the bladder. Anorgasmia is an orgasmic dysfunction characterized by the inability to reach orgasm and result in anejaculation (Colpi et al., 2004).

1.3.1.2. Intra-testicular causes

The testis is an organ saddled with two main functions- androgen synthesis (steroidogenesis) and spermatozoa production spermatogenesis. Perturbations in either or both function for any reason can lead to infertility. Intratesticular cause of infertility refers to factors that affect normal sperm production by the testicle. The testes produce sperm of low quantity and/or poor quality despite adequate hormonal support from the hypothalamus and pituitary gland. This can be due either to a disorder of steroidogenesis or spermatogenesis.

- Disorders of steroidogenesis

Hypergonadotropic hypogonadism also known as primary or peripheral/gonadal hypogonadism, is a condition which is characterized by hypogonadism due to an impaired response of the gonads to the gonadotropins, and in turn a lack of sex steroid (testosterone) production and elevated gonadotropin levels. The disorder may be congenital or acquired (Katz et al., 2011). It causes dysfunctional testes presenting as oligospermia or azoospermia. This is also known as primary testicular failure.

Congenital causes include:

i. Chromosomal abnormalities (resulting in gonadal dysgenesis) - Turner's syndrome, Klinefelter's syndrome, Swyer's syndrome, XX gonadal dysgenesis, and mosaicism.
ii. Defects in the enzymes involved in the gonadal biosynthesis of the sex hormones - 17α-hydroxylase deficiency, 17, 20-lyase deficiency, 17β-hydroxysteroid dehydrogenase III deficiency, and lipoid congenital adrenal hyperplasia.
iii. Gonadotropin resistance (e.g., due to inactivating mutations in the gonadotropin receptors) - Leydig cell hypoplasia (or insensitivity to LH) in males, and LH and FSH resistance due to mutations in the GNAS gene (termed pseudohypoparathyroidism type 1A).

Acquired causes are due to damage to the testes include vanishing/anorchia, orchitis, trauma, surgery, autoimmunity, chemotherapy, radiation, infections, toxins (capable of endocrine disruption), and drugs such as antiandrogens, opioids, alcohol (Luzzatto, 2007, Katz et al., 2011).

Disorders of spermatogenesis

- Germ cell aplasia also known as Sertoli cell-only syndrome, is a rare histologic diagnosis whereby the seminiferous tubules are completely devoid of germ cells and are lined only with Sertoli cells with minimal or no fibrosis or hyalinization (Matsumoto and Bremner, 2011). The disorder is characterized by infertility, normal androgenization, normal to moderately small testes, azoospermia, normal testosterone and LH levels, and selectively elevated FSH levels. The high level of FSH suggests severe seminiferous tubule dysfunction (Whorton et al., 1979). Seminal plasma protein TEX101 was proposed for differentiation of Sertoli cell-only syndrome from maturation arrest and hypospermatogenesis (Drabovich et al., 2013, Korbakis et al., 2017).

The cause of Sertoli cell-only syndrome is not known, though it is thought to result from the congenital absence of germ cells due to a failure of gonocyte migration. A Sertoli cell–only histology may be associated with microdeletions in the long arm of the Y chromosome in the AZF regions (Kong et al., 2014). Severe germ cell damage and loss occurring with Klinefelter syndrome, mumps orchitis, cryptorchidism, ionizing radiation, or alkylating agents may result in seminiferous tubules lined only with Sertoli cells. However, in these cases of acquired Sertoli cell–only syndrome, there is usually extensive seminiferous tubule sclerosis or hyalinization, and the testes are usually smaller. Infertility is irreversible in congenital Sertoli cell–only syndrome, but it may be reversible with time in some cases of an acquired Sertoli cell-only syndrome (Matsumoto and Bremner, 2016).

1.3.1.3. Post-testicular causes

These are conditions that affect the ability of the sperm to travel from the testes where they are produced to leave the body in the ejaculate. Examples include:

- Complications from a hernia repair,

- Absent vas deferens,
- Ejaculatory duct abnormalities
- Vasectomy
- Genital tract infection
- Genetic factors such as the congenital bilateral absence of vas deferens (CBAVD) in cystic fibrosis,
- Retrograde ejaculation,
- Hypospadias and
- Erectile dysfunction.

1.4. Human Immunodeficiency Virus (HIV)/ Acquired Immune Deficiency Syndrome (AIDS)

Human Immunodeficiency Virus Infection and Acquired Immune Deficiency Syndrome (HIV/AIDS) is a spectrum of illnesses caused by infection with the Human Immunodeficiency Virus through unprotected sex, contaminated blood transfusions, hypodermic needles, and from mother to child during pregnancy, delivery, or breastfeeding (Rom and Markowitz, 2007). In 2016, an estimated 36.7 million people were living with HIV and it led to 1 million deaths (UNAIDS, 2017a). Since the early 1980s when AIDS was identified until 2017, the disease has caused an estimated 35 million deaths worldwide (UNAIDS, 2016) HIV/AIDS is considered a pandemic that originated from west-central Africa during the late 19th or early 20th century (Sharp and Hahn, 2011). AIDS was first recognized by the United States Centre for Disease Control and Prevention (CDC) in 1981 and its cause—HIV infection—was identified in the early part of the decade (Gallo, 2006).

HIV/AIDS has impacted greatly on society, as a disease, a source of discrimination as well as a huge economic burden both on the individuals and the government (Jain et al., 2018, Ismail et al., 2017b, Poudel et al., 2017). The disease has become subject to many controversies involving religions, such as the Catholic Church's position not to support condom use as prevention (Carey, 2018). It has attracted global medical and political attention as well as significant funding since it was discovered (Piot et al., 2015, McKinney and Marconi, 2016).

1.4.1. HIV/AIDS Pathogenesis

The disease is characterized by three clinical stages. The initial period following infection with the HIV is called acute retroviral syndrome (Kirton, 2010, AIDS Education and Training Centers National Coordinating Resource Center (AETC NCRC)", 2013, World Health Organization, 2007), The stage is characterized by an influenza-like or a mononucleosis-like illness within 2–4 weeks of exposure in many individuals while others have no significant symptoms. Symptoms occur in 40–90% of cases and most commonly include fever, large tender lymph nodes, throat inflammation, a rash, headache, tiredness, and/or sores of the mouth and genitals. Due to their nonspecific character, these symptoms are not often recognized as signs of HIV infection. The second stage following the initial symptoms is followed by a stage called clinical latency, or chronic HIV (Rom and Markowitz, 2007). Without treatment, this second stage can last from about 3 years (Evian, 2007) to an average of about eight years) (Elliott et al., 2012). While typically there are few or no symptoms at first, near the end of this stage many people experience fever, weight loss, gastrointestinal problems and muscle pains. Between 50 and 70% of people also develop persistent

generalized lymphadenopathy, characterized by unexplained, non-painful enlargement of more than one group of lymph nodes (Tenner-Racz et al., 1988).

The last stage is the development of Acquired immunodeficiency syndrome (AIDS) is defined in terms of either a CD4+ T cell count below 200 cells per μL or the occurrence of specific diseases in association with an HIV infection (Smurzynski et al., 2010). In the absence of specific treatment, around half of people infected with HIV develop AIDS within ten years. The most common initial conditions that alert to the presence of AIDS are pneumocystis pneumonia (40%), cachexia in the form of HIV wasting syndrome (20%), and oesophageal candidiasis (World Health Organization, 2007). Other common signs include recurrent respiratory tract infections (Smurzynski et al., 2010). Opportunistic infections that are normally controlled by the immune system (Holmes et al., 2003) affect almost all organ system at this stage of the disease (Chu and Selwyn, 2011).

1.4.2. Drug Treatment of HIV/AIDS

Antiretroviral (ARV) drugs are medications for the treatment of infection caused by retroviruses, such as HIV. The World Health Organization (WHO) has recommended a combination of ARV in the form of antiretroviral therapy (ART) for people starting HIV treatment (WHO, 2016). ART involving a combination of three or more ARV drugs for the lifelong treatment of persons living with HIV is known as Highly Active Antiretroviral Therapy (HAART) (WHO, 2016).

1.4.2.1. Highly Active Antiretroviral Therapy (HAART)

The standard treatment consists of a combination of at least three drugs often HAART that is used mainly to suppress HIV replication. These drugs belong to different classes of antiretroviral agents that act on different stages of the HIV life-cycle. The advent of HAART in the management of HIV witnessed a significant reduction in the morbidity and mortality rates (UNAIDS, 2017b, UNAIDS, 2013, Watkins and Treisman, 2015). Significant advances in ART have been made since the introduction of zidovudine (AZT) in 1987. The mortality among patients with AIDS was nearly halved in the HAART era (Mocroft et al., 2003). (See Figure 3).

Today people living with HIV and AIDS (PLWHAs) have a life expectancy of more than 60 years and can go about their normal daily activities with little or no fear of stigmatization and discrimination. This is shown in Figure 3. As HIV management with ART has become more aggressive, survival has improved. Life expectancy with HIV infection is not yet equivalent to that in uninfected people. In spite of this, HAART has been accompanied with new challenges especially as regards toxicity (Bujan et al., 2007, Semprini et al., 2013, 2015). Table 1 shows the different classes of ARV drugs and their side effects.

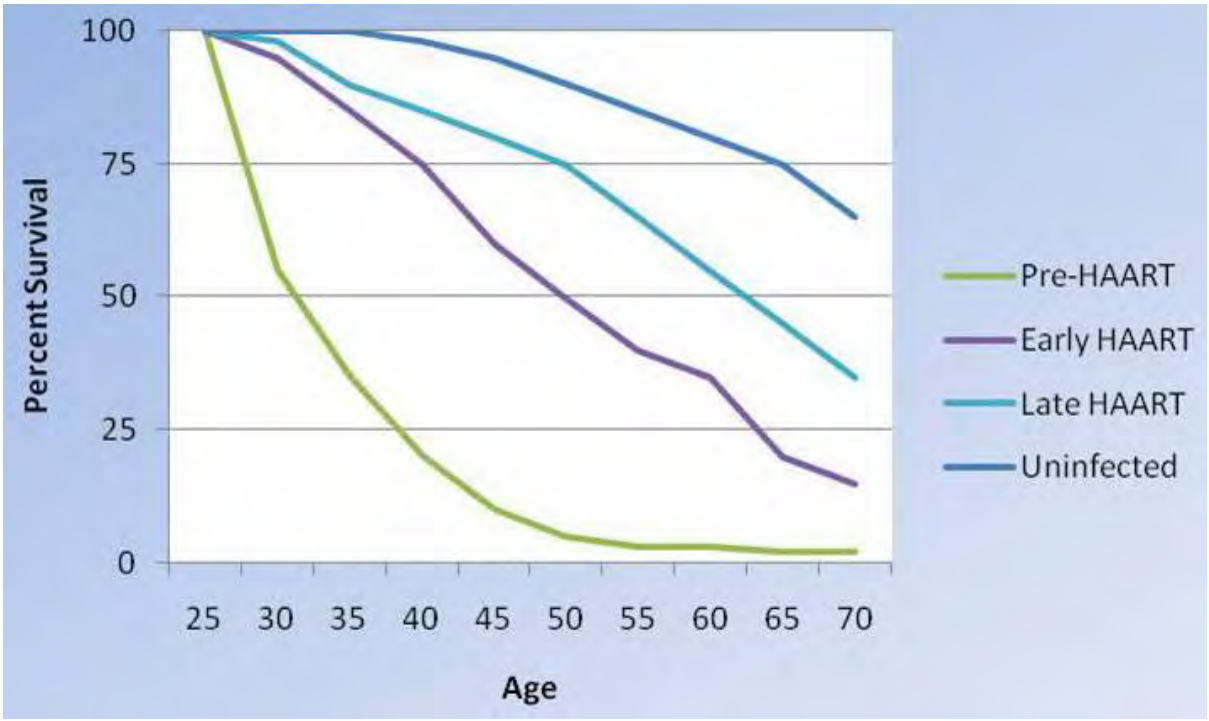

Figure 6: Changes in the survival of PLWHA in the Pre-HAART, Early HAART, and Late HAART era

Source: https://emedicine.medscape.com/article/1533218-overview accessed 19/04/2018

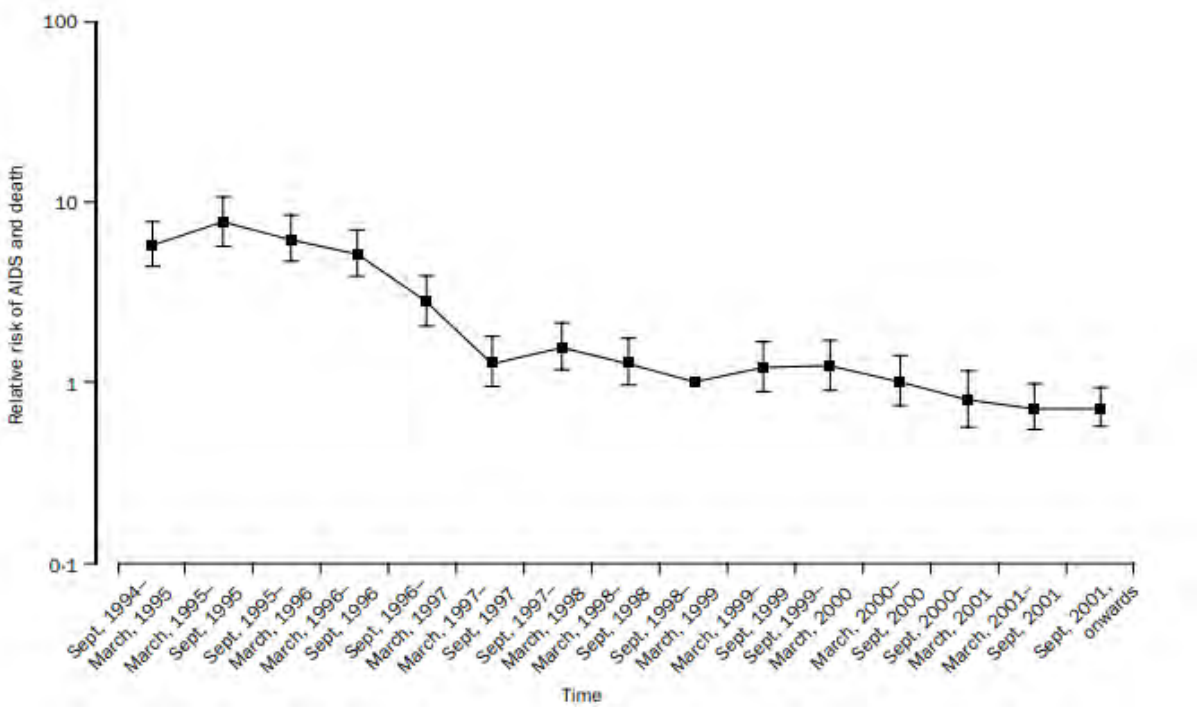

Figure 7: Adjusted relative risk of AIDS or death in PLWHA over time (Mocroft et al., 2003).

1.4.2.2. Pharmacologic drug classes of ARDs

Pharmacologic drug classes of ARDs include:

- Nucleoside reverse transcriptase inhibitors (NRTIs)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Protease inhibitors (PIs)
- Integrase inhibitors (INSTIs)
- Chemokine receptor antagonists (CCR5 antagonists)

The use of these agents is tailored to the individual patient and is essentially dictated by their ease or complexity of use, side-effect profile, efficacy based on clinical evidence, practice

guidelines, and clinician preference. Other factors such as Resistance, adverse effects, pregnancy, and coinfection with hepatitis B virus, or hepatitis C virus are also important considerations before the choice of drug combination is made. The table 1 summarises the Federal Drug Agency (FDA)-Approved antivirals and regimens

Table 1: Pharmacologic drug classes of ARD

	Class	Examples	Side effects
1	Nucleoside reverse transcriptase inhibitors (NRTIs)	Abacavir, Stavudine didanosine, emtricitabine, lamivudine, Tenofovir, zidovudine	Peripheral neuropathy, lipoatrophy, pancreatitis, hepatitis, lactic acidosis, mitochondrial toxicity, nausea, vomiting, haematological toxicities (Zidovudine)
2	Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Efavirenz, Etravirine, Neirapine, Rilpivirine	Rash, teratogenicity, enzyme induction, fever, nausea, diarrhoea, hepatotoxicity. CNS abnormalities e.g. somnolence, vivid dreams, confusion, visual hallucinations (efavirenz)
3	Protease inhibitors (PIs)	Atazanavir, Fosamprenavir, Darunavir,	Lipodystrophy, gastrointestinal intolerance, hyperglycaemia, lipid abnormalities,

		Indinavir, Lopinavir/ritonavir, Ritonavir,	
4	Integrase inhibitors (INSTIs)	Raltegravir, Dolutegravir, Elvitegravir	Diarrhoea, nausea, fatigue, headache, insomnia, skin reactions
5	Chemokine receptor antagonists (CCR5 antagonists)	Maraviroc, Enfuvirtide	Diarrhoea, nausea, hepatitis, upper respiratory tract infection, fatigue, dizziness, headache, joint and muscle pain

Initial regimens use first-line drugs that have a high efficacy and low side-effect profile. The WHO preferred First-line ART for adults should consist of two nucleoside reverse transcriptase inhibitors (NRTIs) plus a non-nucleoside reverse transcriptase inhibitor (NNRTI) or an integrase inhibitor (INSTI) (WHO, 2016).

1.4.3. Reproductive toxicities of HAART

The effects of HIV treatment on several organ systems in the body have been examined by many researchers. Adverse effects have been reported with the use of all ARV drugs and are among the most common reasons cited for switching or discontinuing therapy and for medication non-adherence. These have compromised the benefits of ART. The adverse

effects range from those that are associated with minor discomfort to those that are life threatening such as insulin resistance, lipodystrophy syndrome, gastrointestinal symptoms and bleeding dyscrasias (Schambelan et al., 2002, Hagmann et al., 2003). As a consequence, the side effects of ARVs on fertility especially in a growing population have become a significant cause for concern.

Testicular toxicity has been a major albatross of HAART especially with the Nucleoside reverse transcriptase inhibitors (NRTIs) as it affects and limits the fertility of young men (Lewis W, 2003, van Leeuwen E, 2008, Azu et al., 2014). Tenofovir has been reported to cause renal proximal tubular ultrastructural defects and depleted mtDNA in that specialized tissue (Kohler et al., 2009, White et al., 2001), suggested that the proportion of patients with mitochondrial DNA (mtDNA) deletions was significantly greater in those patients who had taken HAART for more than 12 months compared with those who had taken HAART for less than 12 months. In addition, there are some case base reports of Acute Liver Toxicity due to Efavirenz/Emtricitabine/Tenofovir combination (Patil et al., 2015, Echenique and Rich, 2013, Sperm morphology has been regarded as the hallmark of successful fertilization (Franken et al., 2000). Spermatogenesis and androgen production are under the hormonal regulation from the hypothalamus, anterior pituitary, and the testes. Some ARV has been identified as disruptors of gonadal function by causing lower testosterone levels, oligospermia, azoospermia and gynaecomastia (Sellmeyer and Grunfeld, 1996) thereby leading to reproductive failures.

The seminiferous tubules have been shown to be particularly sensitive to the effects of HAART thereby rendering patients oligospermic and even azoospermic (Wallace et al., 2005). Several studies have discovered that HAART is particularly injurious to the testis as

autopsy studies done on individuals who died of Acquired Immunodeficiency Syndrome (AIDS) revealed testicular histomorphologic abnormalities reduced spermatogenesis as well as testicular atrophy in more than 95% of cases (Da Silva M, 1990). In 2011, Nicopoullos and his colleagues postulated that HAART significantly decreases total sperm count, progressive motility, and increases the number of abnormal forms (Nicopoullos et al., 2011). The spermatozoa have been shown to contain numerous mitochondria necessary to provide the energy required for progressive motility (van Leeuwen E, 2008). Some authors have hinged the decrease in progressive sperm motility on the nexus between HAART induced toxicity and mitochondrial damage (Soriano et al., 2008, Kohler et al., 2009, Patil et al., 2015) links hepatotoxicity with mitochondrial DNA damage also stating that NRTIs inhibit the mitochondrial polymerase γ (poly-γ hypothesis). In addition, HAART induced toxicity has been reported to cause deleterious histopathological changes in the testes causing tubular atrophy with altered morphometric indices (Azu et al., 2014). This brings to the fore a looming challenge of infertility among men on HAART which may militate against their ability to father their own biological children. The above mentioned toxicities associated with the different class of antiretroviral drugs have been further summarised in table 2.

The mechanism by which HAART induces organ toxicity is due to a cascade of events involving the oxidant-antioxidant pathway (Azu et al., 2014, Babatunde et al., 2015). Earlier studies have reported that increased activities of reactive oxygen species (ROS) overwhelm tissue`s total antioxidant status leading to oxidative stress (Aitken, 2008). Oxidative stress leads to increased lipid peroxidation indicated by malondialdehyde (MDA) markers, deleterious to the unsaturated fatty acid-rich membrane of the spermatozoon (Oremosu and Akang, 2015).

Consequent on the fact that HIV is most prevalent among individuals of reproductive age with about one-third of them desiring to have their own biological children, consideration of reproductive desires has become clinically important in the management of PLWHAs (Kushnir and Lewis, 2011). This agrees with the findings of a study in Tanzania among HIV positive men and women. The study reported a significant number of HIV positive men mostly those who had never had children desired to have their own biological children (Mmbaga et al., 2013).

Currently, the most specialized means of managing infertility is through assisted reproduction techniques especially in vitro fertilization (IVF) (Agarwal and Allamaneni, 2005, Buzadzic et al., 2015, Practice Committee of the American Society for Reproductive Medicine, 2006). The financial implication of using this method is alarming and beyond the reach of most middle class citizens (Okonofua and Obi, 2009, Tjon-Kon-Fat et al., 2015). More so, IVF is vitiated by the low success rates in most fertility centres (Crawford et al., 2016, Smith et al., 2015), necessitating the quest for a new therapeutic approach for improvement in fertilization.

Table 2: Summary of testicular effects of Highly Active Antiretroviral Therapy (HAART) (adapted from Azu et al., 2012; Azu, 2012)

Treatment	Effects	Experimental Type	Duration	Reference
NNRTI: nevirapine	Degeneration of seminiferous tubules, necrosis of spermatids, and defoliation of spermatocytes, reduction in sperm motility and viability	Animal	4 weeks	(Adaramoye et al., 2012)
Zidolam (AZT+3TC)	Reduced sperm motility and count and viability, reduced testosterone	Animal	21 days	(Osonuga et al., 2010)
Zidovudine + lamivudine	Increase in free testosterone	Human prospective randomized trial	7 days	(Dubé et al., 2007)

HAART	Impairment in testosterone levels	Human, cross-sectional study	6 months	(Rochira et al., 2015, Rochira et al., 2011)
HAART	Levels of testosterone, 17-estradiol, 17-estradiol/testosterone ratio, FSH, LH, and prolactin were not significantly different in patients who reported sexual dysfunction	A prospective study in human	4 y (1998–2001)	(Collazos et al., 2002)
PIs: saquinavir	Semen alteration decreased sperm motility, and negatively affects mechanisms that are essential to fertilization of an oocyte, such as the acrosome reaction	In vitro evidence (trial)	…	(Ahmad et al., 2011)

HAART	Semen alteration, reductions in ejaculate volume, increased rates of abnormal sperm morphology, and decreased sperm motility	In vitro evidence(Trial), clinical evidence (trial)	…	(Nicopoullos et al., 2010, van Leeuwen E, 2008, Kehl et al., 2011)
NNRTI-containing regimen: nevirapine and PI	Associated with a higher percentage of progressively motile spermatozoa, vitality	A Cross-sectional study in human	Semen samples	(Lambert-Niclot et al., 2012)
HAART	A decline in sperm concentration, total count, progressive motility, and normal morphology	…	…	(Nicopoullos et al., 2004)
HAART	A decrease of the rapidly progressive motile spermatozoa and an increase of less rapidly progressive spermatozoa	…	…	(Dulioust et al., 2002)

HAART - Zidovudine, Lamivudine, and Nevirapine	The significant decline in sperm motility, decline in epithelial height closely mirrored by extensive reticulin framework and positive PAS cells.	Animal	56 days	(Ogedengbe et al., 2016)
HAART- Lamivudine, Stavudine, and Nevirapine)	extensive atrophy of seminiferous tubules with loss of cellular components depleted spermatogenic cell series	Animal	56 days	(Jegede et al., 2017)
HAART- Tenofovir, Emtricitabine, Efavirenz	A decrease in sperm count, increase in spermatozoa with normal morphology reduction in progressive sperm motility. Fragmentations increased in tail lengths of sperm DNA. Reduction in the area and diameter of the seminiferous tubules	Animal	4 weeks	(Adana et al., 2018)

HAART - Zidovudine, Lamivudine, and Nevirapine	Tubular necrosis with detached spermatogenic cells from the basal lamina reduced expression of androgen receptor in the seminiferous tubule	Animal	8 weeks	(Ismail et al., 2017a)
Nucleoside-containing antiretrovirals	Do not induce sperm damage but may produce improvements in semen quality	Longitudinal study over	12 weeks	(Robbins et al., 2001)Robbins et al, 2001 (Robbins et al., 2001)

Abbreviations: 3TC, Lamivudine; AZT/ZDV, Zidovudine; BEST, BID Efficacy and Safety Trial; d4T, Stavudine; ddl, Didanosine; FSH, follicle-stimulating hormone; IDV, Indinavir; LH, luteinizing hormone; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside analogue reverse transcriptase inhibitor; PI, protease inhibitor; RTV, Ritonavir.

1.5. Reproductive Uses of medicinal herbs and natural antioxidants

An increasing attention is being paid to traditional medicines, as they have been proved preventive and ameliorative in a variety of diseases. Several epidemiological studies have conclusively shown that consumption of fruits and vegetables are linked to significant reduction in the risk of developing major chronic diseases (Butt et al., 2015, Appel et al., 1997, Rizzo and Baroni, 2018). This has been attributed to the presences of phytochemicals, the bioactive compounds present in plant-derived foods. It is further proven by the dependence of an estimated 3.2 billion people (64%) of the whole world population on traditional medicines (Khan et al., 2017, Sofowora et al., 2013 Studies have shown that almost 80% of Africans use and rely on medicinal plants for their health care needs (Goggin et al., 2009). Many South Africans regard medicinal plants as a safe, cheaper and desirable alternative to treating their health problems (McKean et al., 2018). The use of medicinal plants most especially in Africa for the treatment of diseases is gaining more popularity in the 21st century (Kayode and Kayode, 2011, Mahlangeni et al., 2014). This is consequent on their various biologically active phytochemicals including flavonoids (Krishnaiah et al., 2015).

1.5.1. Commonly used medicinal herbs for reproductive health issues

The use of herbal therapies for the management of reproductive health issues is quite popular in many indigenous communities around the world. Even though infertility is blamed on the female partner in many indigenous settings, some herbs have been recognized for their beneficial properties in male reproductive functions (Table 3)

Table 3: Commonly used Medicinal Herbs for Reproductive Health Issues

Herb/ Plant	Active component	effect	Proposed Mechanism of action	Reference
Ferula assa-foetida L.	volatile oil, resin, and gum, The resinous constituents include asaresinol ferulate, free ferulic and umbellic acids	aphrodisiac activity, increased penile erections/hour, augmentation of sperm counts, Improvements in sperm motility and microstructure.	The endothelial release of nitric oxide which stimulates the synthesis of cyclic guanosine monophosphate in the penile corpus cavernosum. A contributing role for an effect upon prostacyclin synthesis was suggested	(Kassis et al., 2009)
Nigella sativa (black seeds)	thymoquinone, dithymquinone, thymohydroquinone and thymol	Increased spermatogenesis	Increased androgen levels	(Mohammad et al., 2009)
Withania somnifera (Ashwagandha)	alkaloids, ergostane steroids, amino acids, and neurotransmitters	Increase semen volume, sperm count, and motility	increased testosterone and LH	(Ambiye et al., 2013)

Panax Ginseng	Ginsenosides, a class of natural product steroid glycosides and triterpene saponins	Increase sperm count and motility	DNA and protein syntheses in rat testes, activation of testicular cAMP-responsive element modulator, agonists of steroid hormone receptors	(Seock-Yeon et al., 2004, Salvati et al., 1996, Yamamoto et al., 1977, Park et al., 2007)
Eurycoma longifolia (Jack)	quassinoids, canthine-6-one alkaloids, triterpenes, squalene derivatives, carboline alkaloids	Increase in semen volume, sperm count, and normal forms	increase the testosterone levels in rat Leydig cells, antioxidant activity	(Tambi and Imran, 2010, Low et al., 2013)
Astragalus mongholicus	Isoflavonoids (formononetin, ononin, calycosin, and its glycoside), saponins	Increase sperm motility	protects against ROS toxicity through inhibiting the protein dephosphorylation	(Hong et al., 1992)

1.6. Naringenin

Naringenin is a bitter, colourless (Nihon nogei, 1992, Abe, 2008) flavanone, a type of flavonoid. It is the predominant flavanone in grapefruit (Felgines et al., 2000) and is found in a variety of fruits and herbs (Yanez et al., 2007).

1.6.1. Chemical structure of Naringenin

Naringenin has the skeleton structure of a flavanone with three hydroxyl groups at the 4', 5, and 7 carbons. It may be found both in the aglycol form, Naringenin, and in its glycosidic form, naringin, which has the addition of the disaccharide neohesperidose attached via a glycosidic linkage at carbon 7. Like the majority of flavanones, Naringenin has a single chiral centre at carbon 2, resulting in enantiomeric forms of the compound (Yanez et al., 2007). The enantiomers are found in varying ratios in natural sources (Yáñez et al., 2008). Racemization of S(-)-Naringenin has been shown to occur fairly quickly (Krause and Galensa, 1991a). Separation and analysis of the enantiomers have been explored for over 2 decades (Yanez et al., 2007) primarily via high-performance liquid chromatography on polysaccharide-derived chiral stationary phases (Krause and Galensa, 1991a, Krause and Galensa, 1991b, Gaggeri et al., 2011). There is evidence to suggest the stereospecific pharmacokinetics and pharmacodynamics profiles explaining the wide variety in Naringenin's reported bioactivity (Yáñez et al., 2008).

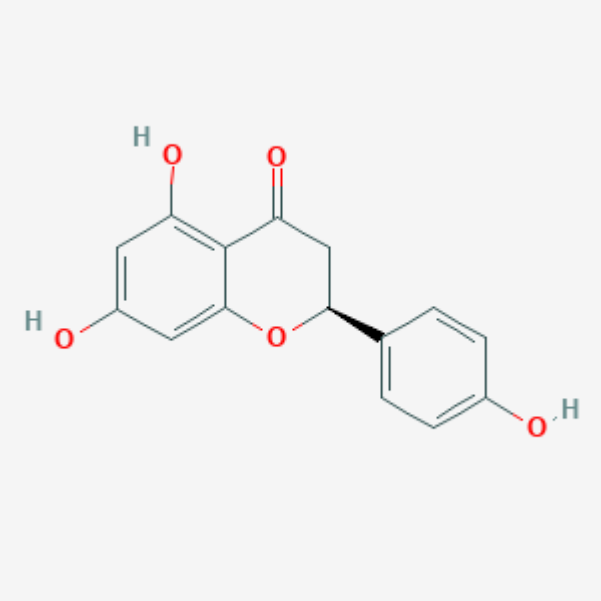

Figure 8: Chemical structure of Naringenin. Source: National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov/compound/naringenin#section=Top)

1.6.2. Biological activities and potential beneficial role of Naringenin in male Reproduction

Naringenin is a flavanone that is considered to have a bioactive effect on human health in a wide range of beneficial ways.

i. **Estrogen activity modulator**: while some research has shown that Naringenin exhibits mild anti-estrogenic activity, (Ruh et al., 1995) some have shown that it is able to act as a competitive antagonist in the presence of a potent agonist like E2 or genistein. (Guo, 2004). It is therefore suggested that Naringenin has a double directional adjusting function of both estrogenic and antioestrogenic activities

(Diamanti-Kandarakis et al., 2009). This is what is required in the regulation of male reproduction as oestrogen is present in the fluid that accompanies sperm through the male reproductive tract. Researchers found that oestrogen plays a role in the male tract (Roser, 2001). It makes sperm more concentrated in semen and probably boosts sperm count in the ejaculate. Based on this, the small amount of oestrogen normally made in men is likely important for normal fertility (Eddy et al., 1996). However, it is well known that exposure to an excessive amount of oestrogen during foetal development or later in life can lead to significant problems (Newbold, 2004, Hileman, 1994, Sharpe and Skakkebaek, 1993, Adeel et al., 2017, Cederroth et al., 2010, Assinder et al., 2007).

ii. **Anti-inflammatory properties**: Various studies have demonstrated the anti-inflammatory properties of Naringenin (Bodet et al., 2008, Wu et al., 2016, Maatouk et al., 2016). Naringenin is a potent inhibitor of the pro-inflammatory cytokine response induced by lipopolysaccharide in both macrophages and in whole blood. Naringenin markedly inhibited the phosphorylation on serines 63 and 73 of Jun proto-oncogene-encoded AP-1 transcription factor in lipopolysaccharide-stimulated macrophages (Bodet et al., 2008). The anti-inflammatory potential of Naringenin has been shown to be comparable to that of indomethacin, a non-steroidal anti-inflammatory agent (Fan et al., 2017). Naringenin is now being actively investigated as a possible candidate to protect against inflammatory injuries. To account for the diverse effects of Naringenin, it has been proposed that its biological activities involve downregulation of the expression of proinflammatory markers, including iNOS and COX-2, by reducing the activities of NF-κB (Hämäläinen et al., 2007).

Inflammation of the reproductive tract due to ascending infections caused by sexually transmitted bacteria or urinary tract pathogens are significant causes of male factor infertility (Schuppe et al., 2017, Fijak et al., 2018). They represent the most frequent aetiology of epididymo-orchitis. Growing shreds of evidence reveal that environmental factors such as exposure to toxicants, the parents' and individual's diet, and even infectious and inflammatory events in the male reproductive tract may influence epigenetic reprogramming. These can induce damage on the germline chromatin, resulting in negative consequences for fertility and health (Schagdarsurengin et al., 2016).

iii. **Antioxidant activity**: Both clinical and animal trials have emphasized the antioxidant role of Naringenin in the body (Cavia-Saiz et al., 2010). This has been demonstrated in several organ systems in the body including the brain, (Hegazy et al., 2016) kidney (Renugadevi and Prabu, 2009, Hermenean et al., 2013), liver (Arul and Subramanian, 2013, Goldwasser et al., 2010), blood (Lee et al., 2002) and testes (Roy et al., 2013, Sahin et al., 2017).

Many identifiable causes of subfertility in men (such as varicocele) and idiopathic cases have been linked to disruption of oxidant-antioxidant balance in the testes (Gharagozloo et al., 2016, Wright et al., 2014, Ma et al., 2017).

iv. **DNA protection and repair**: Observations from various researchers reveal its ability to repair DNA (Oršolić et al., 2011, Adana et al., 2018). The hydroxyl damage observed in a DNA molecule was found to be significantly reduced when cells were exposed to Naringenin for 24 hours (National Center for Biotechnology Information) (National Center for Biotechnology Information). In an in vitro study on plasmids,

Naringenin and another flavonoid rutin were able to protect against UV-B-induced DNA damage (Kootstra, 1994).

The Mitochondrion is an intracellular organelle in control of energy metabolism in eukaryotic cells. In each cell, there are 100-1,000 mitochondria and about two to ten mitochondrial Deoxyribonucleic Acid (mtDNA) per mitochondrion (Medina et al., 1994). The movement of sperm requires a large amount of ATP to drive the flagellar apparatus, thus a defect in the mitochondrial respiratory function will lead to a decline in its motility and consequently fertilizing potential (Sampson et al., 2001, Kumar and Sangeetha, 2009). Sperm mtDNA damage has been linked to oxidative stress, a popular explanation in the pathogenesis of many idiopathic seminal aberrations (Andrews et al., 1999).

v. Other health benefits of Naringenin

Naringenin has been shown to affects peroxisome proliferator-activated receptor (PPAR) alpha and PPAR gamma signalling by enhancing insulin-stimulated tyrosine phosphorylation and insulin tissue sensitivity (Kuiper et al., 1998, Kannappan and Anuradha, 2010, Goldwasser et al., 2010). Flavonoids mitigate free radical/oxidative stress induced tissue damage (Chen et al., 2016, Ben Salem et al., 2015). Naringenin has also been shown to have antiviral activities which make it a desirable adjuvant treatment in patients with HIV- hepatitis co-infection. It reduces hepatitis C virus production by infected liver cells in cell culture by inhibiting the secretion of very-low-density lipoprotein by the cells (Nahmias et al., 2008). Reports of antiviral effects

on polioviruses HSV-1 and HSV-2 have also been made (Mucsi and Pragai, 1985, Lyu et al., 2005).

1.7. Statement of Problem

Since 1983 the pandemic-HIV has caused untimely deaths in over 25 million persons (Chandra et al., 2016). Currently, an estimated thirty-seven million persons in the world are affected by the Human Immunodeficiency Virus (Platt et al., 2016) and South Africa bears the largest burden (Kang'ethe, 2015). About 11.2% (6.3 million) of South Africans are living with HIV and AIDS (Kharsany and Karim, 2016). HAART has greatly improved the lifespan of PLWHAs with a life expectancy of 60.6 years for males and 64.3 for females (UNAIDS, 2016). It is proposed that by 2020, 90% of individuals diagnosed with HIV will be on HAART (Granich et al., 2015, (UNAIDS, 2017). Unfortunately, HAART has been associated with deleterious organ toxicity levels especially as it affects the gonads (Bujan L, 2007, van Leeuwen E, 2008, Semprini AE, 2013, Azu et al., 2014). Basu (2007) reports that most PLWHAs are in their reproductive age. In South Africa, an estimated 16.6% of those aged 15-49 years are HIV positive and about 48.91% are males (Basu, 2007). The consequence, therefore, is that most of the HIV positive men will never have the opportunity to father their own biological children. This vitiates the whole essence of the therapy as it deprives patients of the benefit of a good reproductive health. Therefore, it is of high importance that a new therapy is developed to attenuate these gonado-toxic effects of HAART.

Furthermore, sequel to the fact that the success rate of the most specialized form of treating infertility, in vitro fertilization is still below 50% (Buzadzic et al., 2015) and intracytoplasmic sperm injection (ICSI) are too expensive and beyond the reach of the middle class (Agarwal and Allamaneni, 2005), it is only pragmatic that an effective therapy affordable and easily

accessible be developed. In spite of the wide application of traditional medicine among South Africans (Mahlangeni NT, 2014, Maroyi, 2017), there remains paucity of literature on an effective and easily accessible phytochemical therapy targeted at protecting the fertility of PLWHAs.

1.8. Research questions

i. What are the impacts of the recently approved first line antiretroviral therapy for adults FDC, TDF/FTC/EFV on the testes?
ii. What are the ultrastructural changes that accompany the use of FDC, TDF/FTC/EFV
iii. What is the effect of FDC, TDF/FTC/EFV on the expression pattern of 3 beta-hydroxysteroid dehydrogenase?
iv. What is the role of Naringenin in alleviating testicular perturbations induced by HAART?
v. What is the effect of FDC, TDF/FTC/EFV on the reproductive capability?

1.9. Aims and objectivess

1.9.1. Overall aim

The overall aim of this study is to determine the possible beneficial effects of Naringenin on HAART induced testicular-toxicity.

1.9.2. Specific objectives

i. To determine the effects of Naringenin on HAART-induced cytoarchitectural changes in the testis: Histomorphometric indices including the volume of germ and Leydig cells.
ii. To investigate the effects of Naringenin on HAART-induced Sperm mtDNA fragmentations and progressive sperm motility.
iii. To access the effects of HAART and Naringenin on fertility index including the fetal number.

iv. To determine the effects of Naringenin on HAART-induced testicular oxidative stress using intratesticular antioxidant enzyme activity: Catalase and Glutathione peroxidase (GSH).

v. To determine the reproductive hormonal changes (Testosterone, Luteinizing hormone (LH), Follicle Stimulating Hormone (FSH) following the co-administration HAART and Naringenin.

1.10. Potential benefits

Available evidence indicates that by the year 2020 over 90% of PLWHAs will be on HAART (UNAIDS, 2014). Most of these persons will be in their reproductive age and will want to have their own biological children. Unfortunately, the use of HAART induces infertility. Ameliorating this deleterious effects of HAART will be a remarkable advancement in the management and treatment of HIV.

Currently, the most specialized form of treatment of infertility has been through assisted reproductive techniques especially in vitro fertilization. However, availability and access to these techniques have been hindered by the relatively high cost. In addition, failure rates have been high even when the male appears to have normal spermiograms. This has generated interest in studies of subcellular changes (mtDNA) in sperm cells in order to reduce the failure rates and congenital anomalies. Therefore, the need for developing new therapeutic measures for the treatment of subcellular levels of infertility is undebatable topical. More so, reducing mtDNA fragmentation in spermatozoa may just be the glimmer needed for the management and treatment of HAART related toxicities.

1.11. Methodology

1.11.1. Materials

Sixty (60) adult male and 18 female Sprague-Dawley (SD) rats, weighing 200–220 g, were obtained from the animal house of the Biomedical Resource Unit, University of KwaZulu-Natal, South Africa. They were housed in standard cages under controlled environmental conditions (25^{o}C and a 12-h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water.

A fixed-dose combination (FDC) containing efavirenz (EFV) 600 mg, emtricitabine (FTC) 200 mg and Tenofovir (TDF) 300 mg was administered daily (Adult antiretroviral therapy guidelines 2014). The therapeutic dose was adjusted for animal weight using the human therapeutic dose equivalent for a rat model. The drug was procured from Pharmacare Ltd, Port Elizabeth, South Africa. Naringenin was procured from Sigma Aldrich Avergelee.

1.11.2. Experimental Design

The study was carried out in 2 phases- phase I and II.

Phase I

Phase I was conducted over a period of 4 weeks to determine the feasibility of the doses for the Naringenin as proposed by (Raza et al., 2013). Twenty four male Sprague Dawley (SD) rats were randomly divided into 6 equal number groups as illustrated in figure 9, each group with a different treatment protocol viz;

- Group A: Control (Distilled water)
- Group B: HAART (a fixed dose combination of Tenofovir, Emtricitabine, and Efavirenz)
- Group C: Naringenin, 40 mg/kg for 21 days (Raza et al., 2013)
- Group D: Naringenin, 80 mg/kg for 21 days (Raza, 2013)
- Group E: HAART + Naringenin, 40 mg/kg
- Group F: HAART + Naringenin, 80 mg/kg

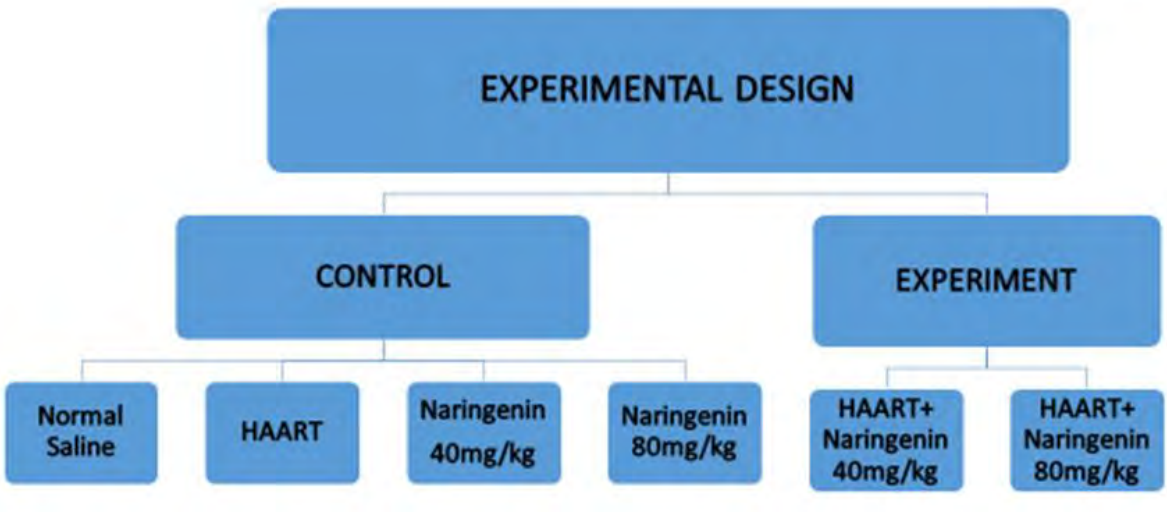

Figure 9: Experimental design

At end of the experiment, the animals were euthanized and the following parameters studied:

i. Semen analysis (sperm count, sperm motility, and sperm morphology)
ii. MDA, SOD, Catalase, and GSH
iii. Histology of the testis
iv. Immunohistochemical studies of 3β-Hydroxysteroid dehydrogenase (3β-HSD)
v. Electron microscopic studies
vi. Fertility index

Phase II

The results from the phase I study was used to determine the dosage for the main experiment which lasted 10 weeks. Thirty (30) adult male SD rats were randomly divided into 6 equal number groups as was done in the phase I. All administrations were through the oral route and was done between 8:00 a.m. and 10:00 a.m

1.11.3. Weight Determination

Animals were weighed daily. Weights were taken in the morning between 7:30 and 9:30 prior to administrations.

1.11.4. Determination of Fertility Index

Three female animals were introduced to three males at the end of the 10 weeks of treatment in phase II. Animals in each group were kept with a female animal for copulation to take

place. The morning on which sperm is found in the vagina lavage was designated as gestational day zero (gd 0) (Hafez and Hafez, 2013). Female animals were studied at day 20 of pregnancy. The number of pregnancies and subsequently the number and size of litters per group were noted. Fertility index (FI) was calculated as follows:

FI= (number of pregnancies)/(number of mated females) x 100

1.11.5. Animal Sacrifice and Collection of Samples

Five days after mating each male SD rat was euthanized and blood samples were collected via transcardial puncture. Five millilitres (5ml) of blood sample was obtained from each rat and divided into two: One half in a plain bottle and the other half in an ethylenediaminetetraacetic acid (EDTA) bottle. Plasma and serum were obtained by centrifugation at 3000 rpm for 20 min (g = 9.78 m/s2). The testes were excised and fixed in either 4% paraformaldehyde, glutaraldehyde or Bouin`s fluid for immunohistochemistry, electron microscopic and histological analyses respectively. The cauda epididymis was assayed for sperm count, sperm motility, and sperm morphology.

1.11.6 Histomorphological Study

For histopathological examination, testes tissues were subjected to systematic serial sectioning at 4µm intervals using a microtome (Microm HM 315, Germany). Samples were then processed using a graded ethanol series and embedded in paraffin (see appendix II). The paraffin sections were cut into 4µm-thick slices and stained with hematoxylin and eosin (H & E) for histological examination (Sukumaran and Raveendranath, 2012). The sections were

viewed and photographed using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Optical Co. Ltd., Japan).

1.11.6 Ultrastructural Study

Transmission electron microscope (TEM) was used to further provide a more detailed morphologic and compositional information on samples (most especially Sertoli-Sertoli cell junctions). This was done after the tissues were fixed overnight at 4^{o}C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and transferred to 0.1 M phosphate buffer (pH 7.2). The cells were postfixed in 1% osmium tetroxide in S-collidine, dehydrated in graded ethanols, transferred to propylene oxide, and embedded in Epon 812. Semi-thin sections were obtained stained with 1% methylene blue. The samples were further sectioned into ultrathin slices (75nm thick) on the cutting edge of glass knives using Leica EM UC7 Ultramicrotome (Austria), mounted on copper grids and contrasted with uranyl acetate and lead citrate (Bressenot et al., 2009) and observed on a transmission electron microscope 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.

1.11.7 Relative organ weight

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

(Organ weight)/ (Total body weight) X 100

1.11.8 Biochemical Estimation for Oxidative Stress levels

One testis from each rat was homogenized using a Teflon homogenizer (Heidolph Silent Crusher M) and then the homogenates were centrifuged at 10,000g at 4ºC for 15 min. Homogenized tissues were used for catalase (CAT) and glutathione peroxidase (GSH-Px) assay using standard Enzyme-Linked Immunosorbent Assay (ELISA) (See appendix IV and V).

1.11.9 Immunostaining of Testis

The testis was immunostained for (3β-HSD) in accordance with reports from (Makala et al., 2014, Devi et al., 2016). Testes tissues were taken from Bouin's fluid and transferred to 70% ethanol (Latendresse et al., 2002). They were then processed using a graded ethanol series and embedded in paraffin. The paraffin sections were cut into 4 µm-thick slices using a microtome (Microm HM 315 microtome, Walldorf, Germany). Immunohistochemistry was performed using Santa Cruz 3β HSD primary antibody and Dako Envision FLEX kit. The processed and sectioned tissues were dewaxed with 2 changes of xylene and hydrated with decreasing grades of alcohol, and water. The sections were placed in diluted Envision FLEX Target solution for 20 minutes at 95-99ºC. Tissue sections were washed in wash buffer, blocked with peroxidase and incubated with diluted 3β HSD (1:150) from Santa Cruz for 30 minutes and with HRP for 20 minutes, DAB and counterstained in hematoxylin, washed in wash buffer, dehydrated, cleared and mounted on DPX. The sections were viewed and photographed using a 40X objective (Zeiss Axioscope A1 microscope, Carl Zeiss, Germany) with an AxioCam MRc Zeiss digital camera attached.

In order to determine the percentage immunoreactivity, image analysis and capturing was done using AxioVision software (Carl Zeiss, Germany; version 4.8.3). At least six fields of

view per slide were randomly selected and captured using a 20X objective. 3β HSD expression was determined as percentage of immunostaining (brown) within the interstitium of the testis.

1.11.10 Sperm characteristics

Sperm count, motility, and morphology were accessed in accordance with the method reported previously (Rezazadeh-Reyhani et al., 2015, Jalali AS, 2015). Briefly, Epididymal spermatozoa were collected from the cauda epididymis in 1 ml of normal saline and incubated for 10 minutes at 37ºC in an atmosphere of 5% CO_2 incubator to allow sperm to swim out of the epididymal tubules. In order to assess the sperm motility, one drop of sperm suspension was placed on a microscope slide, and a cover slip placed over the droplet. At least 10 microscopic fields were observed at 400x magnification using a phase contrast microscope.

The epididymal sperm counts were obtained by the standard hemocytometer. Briefly, after dilution of epididymal sperm to 1:20 in normal saline, approximately 10 µl of this diluted specimen was transferred to each of the counting chambers of the hemocytometer, which was allowed to stand for 5 minutes in a humid chamber to prevent drying. The cells sedimented during this time and were counted 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). Sperm morphology was accessed by staining dry smeared sperm on a glass slide with eosin-nigrosin staining and observing under a light microscope.

1.11.11 Determination of Reproductive hormones

The serum levels of LH, FSH and testosterone were determined by enzyme immunoassay using standard kits (Oremosu and Akang, 2015) (See appendix VI, VII and VIII).

1.11.12 Sperm mitochondrial DNA fragmentation and deletion test

Epididymal sperm samples were collected for sperm Total DNA fragmentation analysis by means of Comet assay. DNA damage was assessed using the Comet assay with minor modifications (Singh et al., 1988). Two slides per sample were prepared. The first layer consisted of 2% low melting point agarose (LMPA, 37 °C). A second layer of 10 lL of rat spermatozoa from each sample with 1% LMPA (37 °C) and a volume of 1 lL of GelRedTM nucleic acid gel stain (Biotium, California, cat. no. 41003) were added. A third layer of 1% LMPA (37 °C) was then added. Once solidified, the slides were submerged in cold lysing solution [2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris (pH 10), 10% DMSO] and incubated (4 °C, 1 h). Following incubation, the slides were placed in electrophoresis solution [300 mM NaOH, 1 mM Na2EDTA (pH 13)] for 20 min and thereafter electrophoresed (25V, 35 min, RT) using Bio-Rad compact power supply. The slides were then washed 39 (5 min each) with neutralization buffer [0.4 M Tris (pH 7.4)]. The slides were viewed with a fluorescent microscope (Olympus IX51 inverted microscope with 510–560 nm excitation and 590 nm emission filters). Images of 50 cells/comets were captured per sample and the comet tail lengths (lm), Tail Moment (a.u.), and tail DNA (%) were measured (Olive & Banath, 2006; Gyori et al., 2014) using Soft imaging system (Life Science – ©Olympus Soft Imaging Solutions v5) and CASPLAB – COMET ASSAY SOFTWARE (v1.2.3beta2), Poland.

1.11.13 Statistical Analysis

The data were presented as mean ± SEM. The differences were evaluated using one-way analysis of variance (Ciz et al., 2012) followed by Fisher's Least Significant Difference (LSD) procedure for multiple comparisons. $P<0.05$ was considered statistically significant. The Statistical Package for Social Sciences (SPSS) version 23 was used for the analysis.

1.12 Summary of the manuscripts/Publication

Table 4: Summary of the manuscripts/Publication

SN	TITLE	JOURNAL	REMARK
1.	Naringenin attenuates highly active antiretroviral therapy-induced sperm DNA fragmentations and testicular toxicity in Sprague-Dawley rats	Andrology (doi: 10.1111/andr.12439)	Published
2.	Testicular Microanatomical and Hormonal alterations following use of Antiretroviral Therapy in Sprague-Dawley rats: the role of Naringenin	Andrologia (doi: 10.1111/and.13137)	Published
3.	Antiretroviral therapy induced impaired Sertoli cell function and testicular antioxidant activity: the role of Naringenin	Archives of Medical Sciences	Under review
4.	Testicular 3 Beta hydroxysteroid dehydrogenase in Naringenin under Highly Active Antiretroviral Therapy (HAART): preliminary data using Sprague-Dawley rats	European Journal of Anatomy (in press)	Accepted

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BRIDGING

BETWEEN CHAPTERS ONE AND TWO

An intact Hypothalamo-pituitary-testicular axis (HPT) made up of anatomic tissues as well as biochemical agents secreted is necessary for optimal spermatogenesis. The previous chapter reviewed the literature on the male reproductive system and the causes of male factor infertility. An overview of HIV/AIDS as a disease, the drug management of HIV and deleterious effects of antiretroviral therapy in impacting on the fertility potentials of men was subsequently highlighted, however the mechanism of damage and hence protective agents are yet to be deciphered. Phytochemicals in various medicinal plants have been getting a lot of attention in recent times. Their possible role in the management of infertility is being investigated. The next chapter went ahead to confirm the findings of earlier researchers and further highlight the mechanism of injury caused by the short-term effects of antiretroviral therapy on the testicular microanatomy using stereological methods, assay of reproductive hormones as well as its effects on the sperm mitochondrial DNA.

2 CHAPTER TWO

MANUSCRIPT ONE

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

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Published in Andrology (doi: 10.1111/andr.12439)

Accepted: 11-Oct-2017

First Published: 27-Nov-2017

ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

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Keywords:
comet assay, DNA damage, germ cells, testosterone, male infertility, sperm quality, testis

Received: 15-Jun-2017
Revised: 25-Sep-2017
Accepted: 11-Oct-2017

doi: 10.1111/andr.12439

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

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SUMMARY

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

Materials

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

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

Experimental design

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

Weight determination

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

Animal sacrifice and collection of samples

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

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

Sperm motility, sperm count, and sperm morphology

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

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

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

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

Comet assay

DNA damage was assessed using the Comet assay with minor modifications (Singh *et al.*, 1988). Two slides per sample were prepared. The first layer consisted of 2% low melting point agarose (LMPA, 37 °C). A second layer of 10 μL of rat spermatozoa from each sample with 1% LMPA (37 °C) and a volume of 1 μL of GelRed™ nucleic acid gel stain (Biotium, California, cat. no. 41003) were added. A third layer of 1% LMPA (37 °C) was then added. Once solidified, the slides were submerged in cold lysing solution [2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris (pH 10), 10% DMSO] and incubated (4 °C, 1 h). Following incubation, the slides were placed in electrophoresis solution [300 mM NaOH, 1 mM Na_2EDTA (pH 13)] for 20 min and thereafter electrophoresed (25V, 35 min, RT) using Bio-Rad compact power supply. The slides were then washed 3× (5 min each) with neutralization buffer [0.4 M Tris (pH 7.4)]. The slides were viewed with a fluorescent microscope (Olympus IX51 inverted microscope with 510–560 nm excitation and 590 nm emission filters). Images of 50 cells/comets were captured per sample and the comet tail lengths (μm), Tail Moment (a.u.), and tail DNA (%) were measured (Olive & Banáth, 2006; Gyori *et al.*, 2014) using Soft imaging system (Life Science – ©Olympus Soft Imaging Solutions v5) and CASPLAB – COMET ASSAY SOFTWARE (v1.2.3beta2), Poland.

Relative organ weight

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

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

Histomorphometrical studies

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

For histomorphometric analysis, seven vertical sections from the polar and equatorial regions were sampled and as an unbiased numerical estimation of the diameter and cross-sectional area of the seminiferous tubule, the seminiferous epithelial thickness was determined using systematic random sampling fair distribution (Gundersen & Jensen, 1987). Eighteen seminiferous tubules with round profiles were randomly selected on each slide. The vertical and horizontal diameters of each seminiferous tubule were measured as d1 and d2, respectively, and the mean diameter (D) was recorded as an observation. This was performed to minimize longitudinal profiles that exhibit different degrees of damage or irregular shrinkage (Christensen & Peacock, 1980). The height and tubular diameter of the seminiferous tubule epithelium was scanned using Leica SCN 400 (Leica Microsystems GmbH, Wetzlar, Germany), and measurements were taken using the image analyzer and Leica microsystem software.

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

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

Determination of reproductive hormones

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

Statistical analysis

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

RESULTS

Weights

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

Sperm count, motility, and morphology

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

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

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

Group	Initial BW	Final BW	Weight gain	Relative TW
DW	182.00 ± 3.61	287.00 ± 7.57	105.00 ± 4.51	1.01 ± 0.04
H	167.66 ± 4.81	248.33 ± 12.68	80.67 ± 12.42	0.87 ± 0.29
N40	167.33 ± 1.76	267.33 ± 9.70	100.00 ± 11.35	1.09 ± 0.08
N80	208.67 ± 4.84	299.67 ± 6.69	91.00 ± 6.66	0.99 ± 0.02
HN40	167.00 ± 3.61	251.33 ± 6.49	84.33 ± 9.82	1.10 ± 0.04
HN80	177.44 ± 6.47	269.00 ± 6.64	83.33 ± 8.67	1.10 ± 0.01

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

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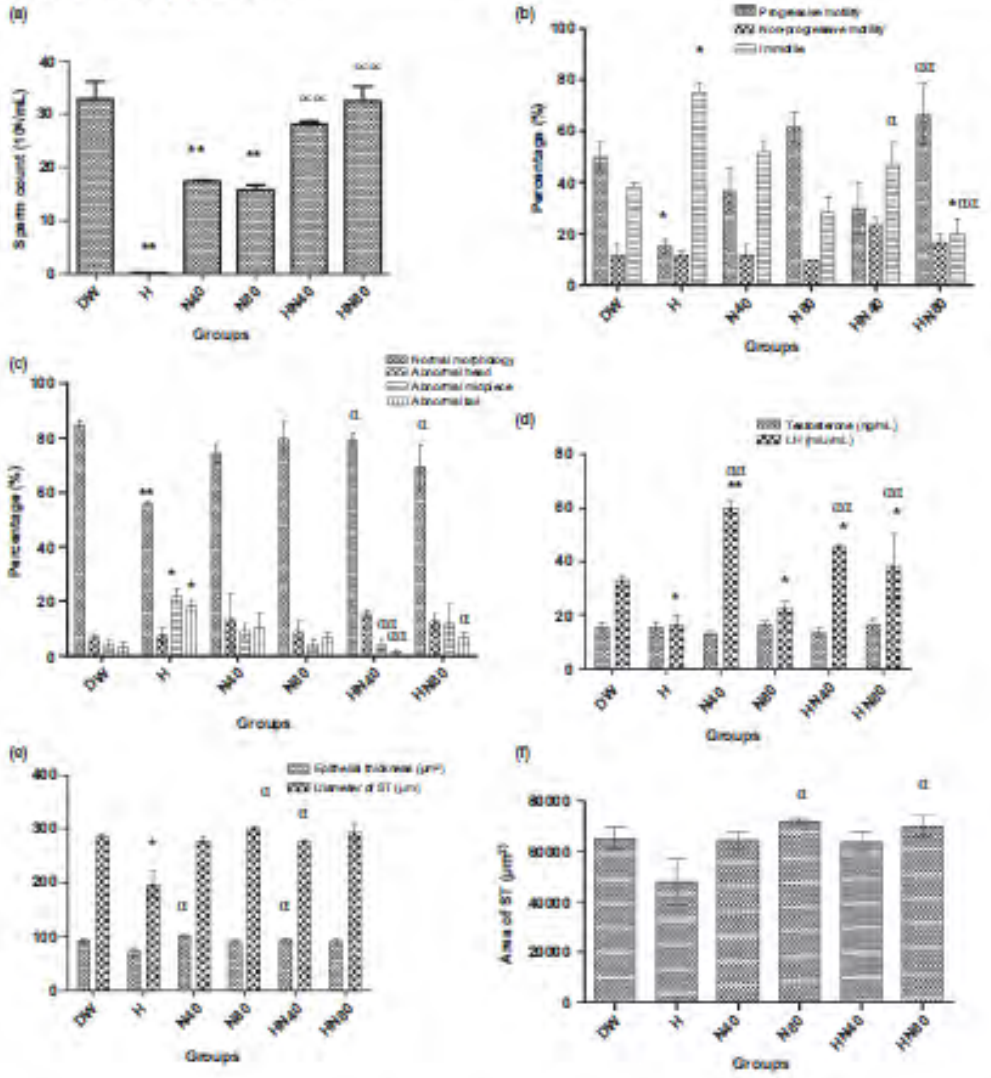
Figure 1 (a) Sperm count, (b) sperm motility, (c) Sperm Morphology, (d) Serum free testosterone and Luteinizing Hormones, (e) Epithelial thickness and diameter of seminiferous tubules (ST), (f) Areas of seminiferous tubules (ST) of SD rats in groups DW (control), H (Atripla, 10 mg/kg), N40 (Naringenin 40 mg/kg), N80 (Naringenin 80 mg/kg), HN40 (Atripla + Naringenin 40 mg/kg) and HN80 (Atripla + Naringenin 80 mg/kg) after 4 weeks of treatment. Bars indicate mean ± SD. *Represents significant difference ($p < 0.05$) between the group and control. **Represents significant difference ($p < 0.001$) between the group and control. a Represents significant difference ($p < 0.05$) between the group and H group. aa Represents significant difference ($p < 0.001$) between the group and H groups.



The normal sperm morphology in H group was also significantly different ($p < 0.001$) compared to the control. In addition, the abnormalities in the tail and midpiece of rats in H group increased significantly compared to the control (Fig. 1c). Sperm cells with normal morphology increased significantly ($p < 0.05$) in HN40 and HN80 compared to the control. In addition, there was a significant decrease ($p < 0.001$) in both abnormal midpiece and abnormal tail in HN40 compared to H group while in

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

Sperm DNA fragmentation

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

Reproductive hormones

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

Histology and morphometric parameters

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

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

DISCUSSION

The stage of once daily all-in-one single pill combination HAART has gained remarkable significance in the fight against the human immunodeficiency virus (HIV). This newer therapy evolved in an attempt to improve compliance, reduce resistance, improve efficacy, and reduce side effects. Although these drugs are considered safer, they are not completely devoid of adverse effects (Chersich *et al.*, 2006; Venter *et al.*, 2017). Highly active antiretroviral therapy is a combination of three of the most efficacious and safest first-line antiretroviral drugs consisting of two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI)-efavirenz and emtricitabine and one non-nucleoside reverse transcriptase inhibitor (NNRTI)-Tenofovir (Esté & Cihlar, 2010).

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

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

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

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

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

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

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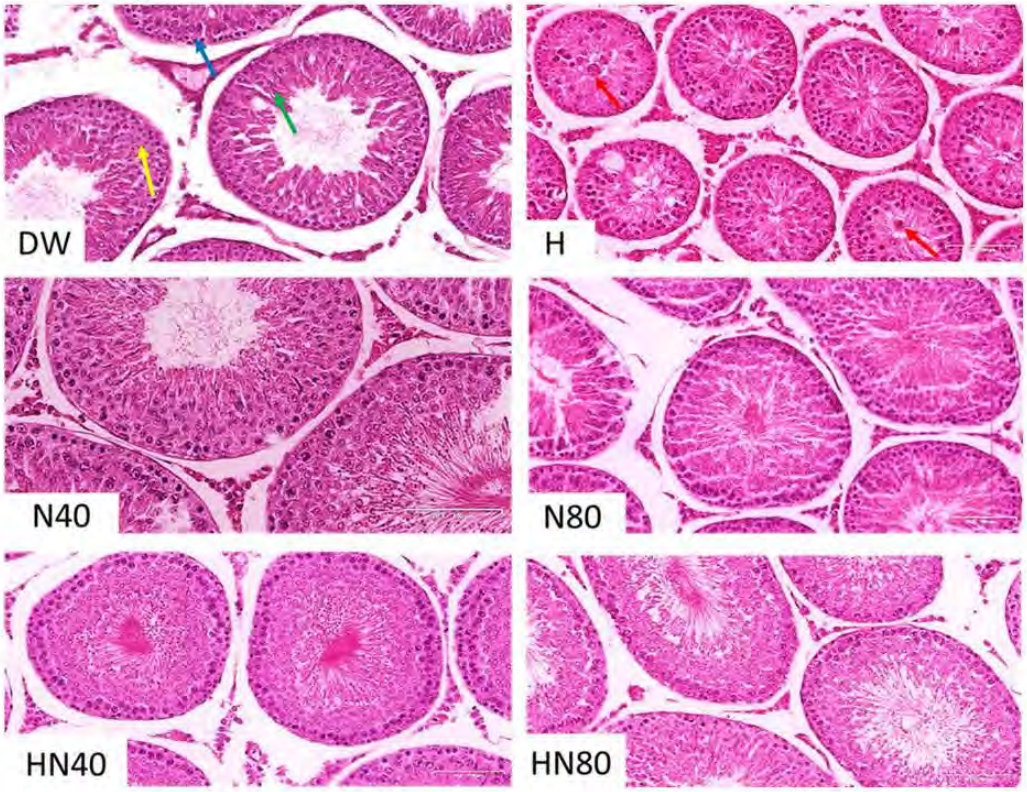
Figure 2 Representative photomicrographs of H&E stained testicular tissues. Distilled water (DW) Testis of control rats showing normal histological structure of active mature functioning seminiferous tubules (ST) associated with complete spermatogenic series. The peripheral layer of cells is composed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



with HAART decreased very significantly so much that values were less than 1% of the untreated/control animals, while the progressive motile sperm cells had comparatively decreased by 70% to the HAART-treated group. Likewise, the sperm morphology showed a marked increase in abnormalities especially tail abnormalities. Our findings may be an offshoot of the numerous aberrations observed in the testicular histology. In addition, the excessive accumulation of the polyunsaturated fatty acid (PUFA) on the plasma membrane of sperm cells makes it a target for oxidants (Oremosu & Akang, 2015; Aitken *et al.*, 2016). Increased lipid peroxidation impairs the flexibility of the sperm's flagellum thereby decreasing the sperm motility (Moazamian *et al.*, 2015). Moreover, oxidative stress is thought to play a role in many health conditions in both animal models and clinical trials by contributing to cellular dysfunction (Uygur *et al.*, 2013; Sies *et al.*, 2017). Reactive oxygen species (ROS) may have distorted the normal process of spermatogenesis resulting in the marked reduction in sperm count and increased morphological disorders. It has been reported that ROS may affect the fluidity of the plasma membrane of the sperm cell resulting in germ cell apoptosis, thereby affecting the sperm count (Aitken & Fisher, 1994; Agarwal *et al.*, 2003). Although our study never assessed the effects of the individual component of HAART, Efavirenz has been reported to negatively affect sperm motility (Frapsauce *et al.*, 2015; Jerónimo *et al.*, 2017).

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

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

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

Antiretrovirals, specifically TFV and FTC, provide protection against the sexual transmission of HIV (Trezza & Kashuba, 2014). To achieve pregnancy and protection against transmission of HIV especially in a serodiscordant couple, the World Health Organisation (WHO) has laid down a consolidated guideline on sexual and reproductive health and rights of women living with HIV (WHO, 2017). This includes several options to support achieving pregnancy with minimal risk of HIV transmission in HIV serodiscordant relationships (Saleem *et al.*, 2017), such as antiretroviral therapy (ART) use by the person living with HIV to suppress viral load; use of oral pre-exposure prophylaxis (PrEP) by the uninfected partner, which are also interventions that are important for health and well-being beyond attempting pregnancy and semen insemination or other assisted reproductive interventions. However, the result of this research and other previous ones (Ogedengbe *et al.*, 2016; Jegede *et al.*, 2017) has implications for the successful protection of male fertility. Thus, there may be need to include an adjuvant therapy with PrEP in the form of a phytochemical such as a flavonoid especially in those desirous of pregnancy to protect against the reproductive toxicity associated with the use of some ARTs. This can be either through the improvement in the oxidative status (Dikshit *et al.*, 2016; Kumar & Tiku, 2016) or through other pathways such as antihypercholesterolemic (Dikshit *et al.*, 2016), antiangiogenic, anti-ischemic, inhibition of platelet aggregation, and anti-inflammatory activities (Chu *et al.*, 2017).

CONCLUSION

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

ACKNOWLEDGMENTS

The College of Health Sciences, University of KwaZulu-Natal, is acknowledged for Operational funds to Postgraduate student Adana MY and Postdoc fellow Akang EN. The technical support from Drs. Sanil D Singh and Linda Bester of Biomedical Resource Unit, University of KwaZulu-Natal, is appreciated. The South African National Research Foundation (NRF) is also acknowledged for support to senior author vide Grant Unique Number U95874. The views expressed in this research are entirely those of the authors and do not represent the opinion of the NRF.

CONFLICT OF INTEREST

None.

AUTHORS' CONTRIBUTIONS

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

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BRIDGING

BETWEEN CHAPTERS TWO AND THREE

The previous chapter described the short-term damaging effects of HAART on the sperm DNA and the testicular microanatomy using Sprague Dawley rats. Currently, there has not been a cure for HIV and the new WHO guideline recommends the commencement of antiretroviral drug soon after diagnosis in all individuals irrespective of age, gender, CD4 lymphocyte cell count nor viral load. The use of antiretroviral therapy is, therefore, a lifelong issue in people living with HIV/AIDS. Many of these people are in their reproductive age group and are likely to desire their own biological children at some point in their lives. This informed the second phase of the study in which effects of long-term use of HAART on the testicular histoarchitecture as well as the Hypothalamo-pituitary testicular (HPT) axis was studied.

3 CHAPTER THREE

MANUSCRIPT TWO

Testicular Microanatomical and Hormonal alterations following use of Antiretroviral Therapy in *Sprague-Dawley* rats: role of Naringenin

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Published in Andrologia: 20-September-2018 (DOI: 10.1111/and.13137)

Accepted: 29-July-2018

Andrologia

Decision Letter (AND-18-207.R1)

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Subject: Andrologia - Decision on Manuscript ID AND-18-207.R1

Body: 29-Jul-2018

Dear Dr. Adana:

It is a pleasure to accept your manuscript entitled "Testicular Microanatomical and Hormonal alterations following use of Antiretroviral Therapy in Sprague-Dawley rats: role of Naringenin" for publication in Andrologia. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

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Received: 19 April 2018 | Revised: 9 July 2018 | Accepted: 29 July 2018

DOI: 10.1111/and.13137

ORIGINAL ARTICLE

WILEY ANDROLOGIA

Testicular microanatomical and hormonal alterations following use of antiretroviral therapy in *Sprague Dawley* rats: Role of Naringenin

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Funding information
College of Health Sciences, University of KwaZulu-Natal; South Africa National Research Fund, Grant/Award Number: U99053 CPRR

Abstract

Human immunodeficiency virus-infected man may require assisted reproductive technology not just for safer conception but also due to subfertility. The study investigated the effect of antiretroviral drugs on the fertility potentials of males and the possible protective role of Naringenin, using Sprague Dawley rats. Thirty adult male Sprague Dawley rats were grouped into—A: Distilled water; B: Highly Active Antiretroviral Therapy (HAART); C: Naringenin 40 mg/kg; D: Naringenin 80 mg/kg, E: HAART + Naringenin 40 mg/kg; F: HAART + Naringenin 80 mg/kg. The rats were euthanised after 10 weeks. Results showed a significant decrease in sperm count in group B when compared to the control and other groups. Spermatozoa with normal morphology also reduced significantly in the B group and progressive sperm motility reduced when compared to the control, D and the F group. The serum testosterone was not significantly different between groups A and B, however the groups C and D displayed significant increase when compared to groups A and B. The serum luteinising hormone was significantly higher in group B when compared to groups A, E and F. Our data suggest that Naringenin improves the male reproductive anatomy and function, therefore, it promises to be a beneficial adjuvant for mitigating HAART testicular and reproductive perturbations.

KEYWORDS
antiretroviral therapy, hormones, male infertility, semen parameters, Sprague Dawley, testis

1 | INTRODUCTION

Even though infection with Human Immunodeficiency Virus (HIV) depresses the health status of men, it does not eliminate the desire for future fertility (Cooper et al., 2009; Heys, Kipp, Jhangri, Alibhai, & Rubaale, 2009; Iliyasu et al., 2009; Tesfaye, Admassu, Getachew, & Sharma, 2012; Wekesa & Coast, 2014) yet testicular toxicity has been a major complication associated with Highly Active Antiretroviral Therapy (HAART). The Nucleoside reverse transcriptase inhibitors (NRTIs) are particularly a major culprit. Reports suggest that most people living with HIV and Acquired Immune Deficiency Syndrome (AIDS) (PLWHA) are in their reproductive age (Azu et al., 2014; Lewis, Day, & Copeland, 2003; van Leeuwen et al., 2008). The vigorous efforts directed to reducing and treating HIV infection has recorded success in improving the life expectancy and aspirations among PLWHA (Iliyasu et al., 2009; Nakagawa et al., 2012). Reports of The Joint United Nations Program on HIV/AIDS (UNAIDS) suggests that approximately 1.8 million people were newly infected in 2016. The number of people accessing antiretroviral has increased steadily over the years (UNAIDS, 2016).

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An estimated 19 million people were accessing antiretroviral drugs globally as at the end of 2016 (UNAIDS, 2016). According to this report, South Africa had the highest figure of 3.4 million people on treatment. HAART has been associated with deleterious organ toxicity especially as it affects the gonads (Azu et al., 2014; Bujan et al., 2007; Semprini et al., 2013).

HAART-induced toxicity has been reported to cause deleterious histopathological changes in the testes. Changes observed include tubular atrophy with altered morphometric indices (Adana et al., 2017; Azu et al., 2014). Autopsy studies on individuals on HAART revealed testicular histomorphologic abnormalities, and testicular atrophy in more than 95% of cases (Da Silva et al., 1990). Earlier study carried out by researchers equally reveal that HAART-induced alterations result in significantly decreased total sperm count, progressive motility, and increased numbers of abnormal forms (Nicopoullos, Almeida, Vourliotis, & Gilling-Smith, 2011). In addition, structural integrity of the testis is essential for maintenance of the hormonal equilibrium including testosterone production. Therefore, with altered androgen levels linked to HAART protocols (Adana et al., 2017; Muthiah, Kallikadavil, Shivaswamy, & Menon, 2016), fertility issues become very rife in men on HAART.

An intact testicular microanatomical structure is essential for normal testosterone production. The exact mechanism for HAART-induced organ toxicity is still unclear however the most popularly proposed pathway is through initiation of a cascade of events involving the oxidant-antioxidant pathway (Azu et al., 2014; Olaniyan, Maduagwu, Akintunde, Oluwayelu, & Brai, 2015). Earlier studies have reported that increased activities of reactive oxygen species (ROS) overwhelm tissue's total antioxidant status leading to oxidative stress (Aitken & Curry, 2011; Aitken & De Iuliis, 2007; Aitken & Roman, 2008). The overall effect is reduced fertility of men on HAART. This calls for an intervention strategy to protect against declining fertility.

Currently, the most specialised form of treatment of infertility is assisted reproductive techniques especially in vitro fertilisation. However, availability and access to these techniques are hindered by the relatively high cost (Chambers, Sullivan, Ishihara, Chapman, & Adamson, 2009) and availability of trained personnel. In addition, failure rates are high even when the male appears to have normal spermiograms (Liu & Baker, 2000; Younes et al., 2016). Therefore there is need for continued search for alternative effective, affordable and easily accessible therapies.

The use of medicinal plants for the treatment of diseases is gaining more popularity in the 21st century especially in Africa (Kayode & Kayode, 2011; Mahlangeni, Moodley, & Jonnalagadda, 2014) This is consequent on their various biologically active phytochemicals (Krishnaiah, Sarbatly, & Nithyanandam, 2011; Rahmatullah et al., 2010). Flavonoids mitigate free radical/oxidative stress induced tissue damage (Chen et al., 2015; Salem et al., 2016).

Bioflavonoids are known for their immense health benefits in many human tissues (Çetin, Çiftçi, & Otlu, 2016; Ma, Zhang, & Jiang, 2017; Shoskes, 1998; Tirkey, Pilkhwal, Kuhad, & Chopra, 2005) and particularly in the reproductive functions in males (Ciftci, Ozdemir, Aydin, & Beytur, 2012; Jamalan, Ghaffari, Hoseinzadeh, Hashemitabar, & Zeinali, 2016). Naringenin, a bitter colourless flavone extract from citrus species (Felgines et al., 2000; Yáñez et al., 2008) has been reported to have a wide variety of bioactivity. Naringenin and closely related bioflavonoids are reported to be beneficial for protecting and ameliorating testicular toxicity (Ganaie, 2015; Jamalan et al., 2016; Roy, Rahaman, Ahmed, Metya, & Sannigrahi, 2013; Sahin, Ozkaya, Cuce, Uckun, & Yologlu, 2017). This has been attributed to their free radical scavenging properties (Ganaie, 2015; Roy et al., 2013). This study was designed to quantify testicular damage associated with use of HAART and investigated the effect of naringenin on these changes. This was carried out by assessing the semen characteristics, serum biochemical parameters, pregnancy occurrences and testicular histoarchitectural changes in *Sprague Dawley* rats following long-term use of HAART.

2 | MATERIALS AND METHODS

2.1 | Materials

Thirty (30) adult male and 18 female Sprague Dawley (Aitken & Roman, 2008) rats, weighing 200–220 g (Jegede, Offor, Onanuga, Naidu, & Azu, 2017), were obtained from the animal house of the Biomedical Resource Unit, University of KwaZulu–Natal, South Africa. They were housed in standard cages under controlled environmental conditions (25°C and a 12-hr light/dark cycle). The animals had free access to pulverised standard rat pellet food and tap water. The approval of the Animal Research Ethical Committee (AREC), UKZN, South Africa was sought and gotten with reference number AREC/046/016D. A fixed-dose combination (FDC) of HAART containing 600 mg of Efavirenz (EFV), 200 mg of Emtricitabine (FTC) and 300 mg of Tenofovir (TDF) [TDF/FTC/EFV] was administered daily. The therapeutic dose of the FDC was adjusted for animal weight using the human therapeutic dose equivalent for a rat model (7.5/5/15 mg/kg) (Meintjes et al., 2014). The drug was procured from Pharmacare Ltd, Port Elizabeth, South Africa. Naringenin was procured from Sigma Aldrich Avengelee.

2.2 | Experimental design

Thirty (30) disease-free adult male SD rats were randomly divided into 6 equal number groups. Group A served as control and was given distilled water, B was given the FDC of HAART containing TDF/FTC/EFV, C had naringenin, 40 mg/kg, D had naringenin, 80 mg/kg (Adana et al., 2017). The last two groups, E and F had TDF/FTC/EFV and naringenin 40 and 80 mg/kg respectively.

All administrations were through the oral route and were performed between 8:00 a.m. and 10:00 a.m. for a period of 10 weeks (March 13th to 23rd of May 2017). Animals were examined and weighed on daily for signs of toxicity and dose adjustments. Weights were taken between 8:00 and 10:00 a.m.

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2.3 | Determination of fertility index

At the end of the 10-week period of drug administration to male rats, eighteen female animals in the proestrous or oestrus phase were randomly divided into 6 groups of 3 animals each and labelled according to the male animal grouping (A^1, A^2, A^3; B^1, B^2, B^3; C^1, C^2, C^3; D^1, D^2, D^3; E^1, E^2, E^3; F^1, F^2, F^3) described above. They were then introduced to the corresponding males for copulation to take place. The morning on which spermatozoon is found in the vagina lavage was designated gestational day zero (gd 0) (Hafez 1970). Female animals were studied at day 20 of pregnancy. The number of pregnancies and subsequently number of litters per group were noted. Fertility index (FI) was calculated as follows:

$$FI = (\text{number of pregnancies})/(\text{number of mated females}) \times 100$$

(Hood, 2016).

2.4 | Animal sacrifice and collection of samples

Five days after mating, each male SD rat was euthanised under excess halothane anaesthesia and blood samples were collected via transcardial puncture. Five millilitre (5 ml) of blood sample was obtained from each rat and divided into two: One half in a plain bottle and the other half in an ethylenediamminetetraacetic acid (EDTA) bottle. Plasma and serum were obtained by centrifugation at 1401 x g for 20 min. The testes were excised, weighed and fixed in Bouin's fluid for histological analysis. The cauda epididymis was gently minced and assayed for sperm count, sperm motility and sperm morphology. The relative testicular weights were calculated using the following formula:

$$\text{Organ weight}/\text{Final body weight} \times 100$$

(El-Aziz, El-Fark, & Hamdy, 2016; Patrick-Iwuanyanwu, Udowelle, & Okereke, 2016)

2.5 | Sperm characteristics

Sperm count, motility and morphology were assessed as described by Amniattalab and Razi (2015) and Jalali, Najafi, Hosseinchi, and Sedighnia (2015). In brief, Epididymal spermatozoa were collected from the cauda epididymis in 1 ml of human tubular fluid (HTF) solution and incubated for 10 min at 37°C in an atmosphere of 5% CO_2 incubator to allow sperm to swim out of the epididymal tubules.

2.5.1 | Motility

To assess the sperm motility, one drop of sperm suspension was placed on a microscope slide, and a coverslip was placed over the droplet. Ten (10) microscopic fields were observed at 400x magnification using a phase contrast microscope.

2.5.2 | Count

The epididymal sperm counts were obtained by the standard hemocytometric method using the Biorad automated counting chamber and equipment. In brief, after dilution of epididymal spermatozoa to 1:20 in HTF medium, approximately 10 microlitre (µl) of this diluted specimen were transferred to each of the counting chambers for automatic counting.

2.5.3 | Morphology

Sperm morphology was accessed by staining dry smeared spermatozoa on glass slide with eosin-nigrosin staining. Morphology was described as normal, abnormal head, abnormal midpiece, and abnormal tail (Adana et al., 2017; Azu et al., 2014).

2.6 | Determination of reproductive hormones

The serum levels of luteinising hormone (Hegazy, Ali, & Sabry, 2016), and free testosterone were determined by Elabscience rat specific kits with catalogue numbers: E-EL-R0026 and E-EL-R0389 respectively. Procedure was carried out in accordance with manufacturers' instructions.

2.7 | Histomorphometric studies

For histopathological examination, fixed testicular tissues were dissected. Samples were then processed using a graded ethanol series and embedded in paraffin. The paraffin sections were cut into 5 µm-thick slices using a microtome (microm HM 315 microtome, Walldorf, Germany) and stained with haematoxylin and eosin for histological examination. The sections were viewed and photographed using an Olympus light microscope (Olympus BX51, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Optical Co. Ltd., Japan).

A ten millimetre (10 mm) grid was placed on the 20 µm haematoxylin and eosin stained testicular tissue sections. Eleven sections per animal were selected using the Leica slidePath gateway LAN software. Selections were carried out using the systematic random sampling method with a random start. Four blocks per testes were used. The same number of grid points was allowed on all sections. The number of grid points falling on each component of the seminiferous tubules (ST)- germinal epithelium (GE), lumen (L) and interstitium (I) were noted. The volume fraction (Vv) of each component was calculated as follows:

$$Vv(\text{Component}/\text{Ref Volume}) = \sum \text{Points component} / \sum \text{Points reference} \times 100$$

(Bordbar, Esmaeilpour, Dehghani, & Panjehshahin, 2013; Howard & Reed, 2004).

2.8 | Statistical analysis

The data were presented as mean ± *SEM*. Variables were subjected to Shapiro–Wilk test to check for normality. The differences were

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TABLE 1 Body weights (BW) and relative testicular weights (TW) in animals in groups A (control), H (HAART), C (Naringenin 40 mg/kg), D (Naringenin 80 mg/kg), E (HAART + Naringenin 40 mg/kg) and F (HAART + Naringenin 80 mg/kg) after 10 weeks of treatement. Bars indicate mean ± *SD*

Group	Initial BW	Final BW	Weight gain	Relative TW
A (control)	147.40 ± 7.24	341.60 ± 15.16	194.20 ± 15.89	1.02 ± 0.04
B (HAART)	164.40 ± 1.33	339.80 ± 15.50	175.4 ± 15.56	1.01 ± 0.04
C (naringenin 40 mg/kg)	161.20 ± 8.31	359.60 ± 14.50	198.40 ± 13.73	0.97 ± 0.02
D (naringenin 80 mg/kg)	165.00 ± 7.19	370.80 ± 9.32	205.80 ± 9.69	1.04 ± 0.02
E (HAART + Naringenin 40 mg/kg)	152.20 ± 3.99	361.00 ± 11.15	208.80 ± 13.95	1.03 ± 0.03
F (HAART + Naringenin 80 mg/kg)	149.80 ± 5.01	355.80 ± 5.68	206.00 ± 2.26	1.03 ± 0.03

TABLE 2 Semen parameters

Group	Sperm count ($x10^6$)	Progressive motility (%)	Abnormal spermatozoa (%)
A (Control)	29.28 ± 0.66	84 ± 2.92	5.5 ± 1.50
B (HAART)	20.05 ± 1.58**	58 ± 3.74**	12.26 ± 5.03
C (naringenin 40 mg/kg)	25.56 ± 1.78†	62 ± 3.74**††	4.64 ± 1.18
D (naringenin 80 mg/kg)	26.12 ± 1.64	76 ± 2.92††	8 ± 3.78
E (HAART + Naringenin 40 mg/kg)	24.92 ± 2.06	65 ± 2.24**††	5 ± 0.77
F (HAART + Naringenin 80 mg/kg)	25.04 ± 1.17	74 ± 2.92††	5.6 ± 1.17

Note. Semen parameters in animals in groups A (control), H (HAART), C (Naringenin 40 mg/kg), D (Naringenin 80 mg/kg), E (HAART + Naringenin 40 mg/kg) and F (HAART + Naringenin 80 mg/kg) after 10 weeks of treatment. Bars indicate mean ± *SD*.
*$p \leq 0.05$ between the group and control. **$p \leq 0.001$ between the group and control. †$p \leq 0.05$ between the group and HAART group while. ††$p \leq 0.001$ between the group and HAART group.

then evaluated using one-way analysis of variance (Ciz et al., 2012) followed by Fisher's Least Significant Difference (LSD) procedure for multiple comparisons. $p < 0.05$ was considered statistically significant and <0.01, very significant using Statistical Package for Social Sciences (SPSS) version 23.

3 | RESULTS

3.1 | Body weight and relative testicular weight

There were no significant differences in the weight gain [ANOVA $p = 0.472$] and relative testicular weights [ANOVA $p = 0.506$] across the groups (Table 1).

3.2 | Semen parameters

Sperm count was significantly different between the groups [ANOVA $p = 0.019$]. There was a significantly lower count in group B (HAART) compared to A (control) [$p = 0.01$] and C [$p = 0.032$]. There were significantly lower progressive spermatozoa in group B when compared to A [$p < 0.0001$], C [$p = 0.001$], D [$p = 0.00$], E [$p = 0.00$] and F (HAART + Naringenin 80 mg/kg) [$p = 0.001$]. Group C [$p = 0.001$] and E [$p = 0.002$] also displayed significantly lower progressive motility when compared to A (Table 2).

3.3 | Fertility index

Statistical comparison was not made for fertility index. However, the fertility index was higher in the A group than in the B and C groups. The number of pubs per group was also higher. The average number of pubs per pregnant female were lower in the B and C groups (Table 3).

3.4 | Reproductive hormones

Serum testosterone levels were not significantly different [$p = 0.344$] between groups A and B. Groups that were administered only naringenin- C and D displayed significant increase [$p = 0.025$] and [$p = 0.006$], respectively, when compared to groups A. Testosterone level were higher in the D was compared to B group [$p = 0.051$]. The serum luteinising hormone was significantly higher in group B when compared to groups A [$p = 0.027$], E [$p = 0.017$] and F [$p = 0.018$] Figure 1a,b.

3.5 | Histomorphometry

There were significant differences in the volume fractions of the seminiferous epithelium (SE) [ANOVA $p = 0.027$] and lumen [ANOVA $p = 0.024$]. The volume fraction of SE was higher in the B

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TABLE 3 Fertility index in animals in groups A (control), H (HAART), C (Naringenin 40 mg/kg), D (Naringenin 80 mg/kg), E (HAART +Naringenin 40 mg/kg) and F (HAART + Naringenin 80 mg/kg) after 10 weeks of treatement. Bars indicate mean ± *SD*

Group	No of pregnancies	Fertility index (FI)	No of resorptions	No of pubs
A (control)	5	100.00	0.67 ± 0.58	8.67 ± 2.08
B (HAART)	3	60.00	0.67 ± 1.15	6.67 ± 5.77
C (naringenin 40 mg/kg)	3	60.00	0.33 ± 0.58	6.00 ± 5.57
D (naringenin 80 mg/kg)	5	100.00	0.00	9.67 ± 1.53
E (HAART + Naringenin 40 mg/kg)	5	100.00	0.67 ± 0.58	9.33 ± 1.15
F (HAART + Naringenin 80 mg/kg)	5	100.00	0.33 ± 0.56	9.33 ± 1.15

group than all groups except the C group which was significantly higher [$p = 0.017$]. The lumen in group B was significantly smaller than those of D [$p = 0.007$], E [$p = 0.023$], F [$p = 0.002$] (Table 4).

3.6 | Histomorphology

The seminiferous tubules (ST) of the control animals displayed the normal progression of cells of the spermatogenic series with sperm cells in the lumen and Leydig cells in the interstitium (Figure 2). Group b had apparently smaller ST with scanty cells in the lumen of some of the ST, whereas the c, d, e, and f displayed larger ST with the normal progression of cells of the spermatogenic series. Leydig cells in the interstitium and mature sperm cells in the lumen were similar to control (Figure 2).

4 | DISCUSSION

Long-term use of HAART has become the cornerstone of the success achieved in the prevention and management of HIV infections. It is associated with decline in AIDS incidence and death rates across different parts of the world (Antiretroviral Therapy Cohort Collaboration, 2008; Kitahata et al., 2009; Liu, 2016; Liu & Baker, 2000). However, it is also associated with myriads of biochemical, physiological and structural changes and their attendant complications (Azu et al., 2014; del Valle, Hernández, & Ávila, 2013; Mudie et al., 2014). This study investigated the effects of HAART on the male reproductive potential and the protective role of naringenin in animals.

4.1 | Semen parameters and fertility rate

This study reports that HAART-induced alterations in semen parameters in rats. This was evident from the decrease in the sperm count, sperm motility and an increase in abnormal forms with the use of TDF/FTC/EFV combination. This is in tandem with the findings of earlier studies (Azu et al., 2014; Kehl et al., 2011; Ogedengbe et al., 2016) who also reported semen abnormalities. The effect on sperm parameters could explain the lesser number of pregnancies and subsequently pubs in the rats on TDF/FTC/EFV combination. The co-administration of naringenin (80 mg/kg) and HAART restored

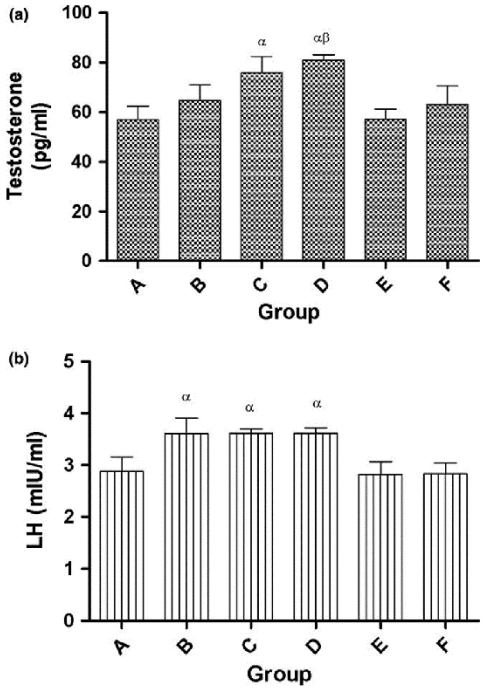

FIGURE 1 (a) Serum Testosterone and (b) Luteinising hormone in groups A (control), B (HAART), C (Naringenin 40 mg/kg), D (Naringenin 80 mg/kg), E (HAART + Naringenin 40 mg/kg) and F (HAART + Naringenin 80 mg/kg) after 10 weeks of treatment. Bars indicate mean ± *SD*. $^{\alpha}p \leq 0.05$ between the group and control. $^{\beta}p \leq 0.05$ between the group and HAART group

the fertility rate and did prevent alterations in the semen parameters as they were comparable to those of the control. This suggests that naringenin may protect against HAART-induced testicular toxicity. Roy et al., 2013 earlier reported the ameliorative properties of naringenin in diabetes-induced testicular toxicity (Roy et al., 2013). The protective property of naringenin has been attributed to its ability to prevent antioxidant enzyme inactivation by potential

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TABLE 4 Volume fraction of the germinal epithelium lumen and interstitium

Group	Vol. fraction of seminiferous epithelium (%)	Vol. fraction of lumen (%)	Vol. fraction of interstitium (%)
A (control)	56.67 ± 1.52	16.67 ± 2.91	25.00 ± 1.96
B (HAART)	57.23 ± 2.86	11.11 ± 1.76*	31.67 ± 2.58
C (naringenin 40 mg/kg)	61.66 ± 2.04	15.00 ± 2.07	23.33 ± 1.11
D (naringenin 80 mg/kg)	57.22 ± 1.67**	21.67 ± 1.36**	21.11 ± 1.11
E (HAART + naringenin 40 mg/kg)	56.67 ± 2.99**	18.89 ± 1.84**	24.44 ± 2.22
F (HAART + naringenin 80 mg/kg)	48.33 ± 1.88**	20.55 ± 2.99**	31.11 ± 4.60

Note. Volume fraction of in groups A (control), B (HAART), C (Naringenin 40 mg/kg), D (Naringenin 80 mg/kg), E (HAART + Naringenin 40 mg/kg) and F (HAART + Naringenin 80 mg/kg) after 10 weeks of treatment. Bars indicate mean ± *SD*.
*Represents significant difference ($p < 0.05$) between the group and control. **Represents significant difference between the group and B group.

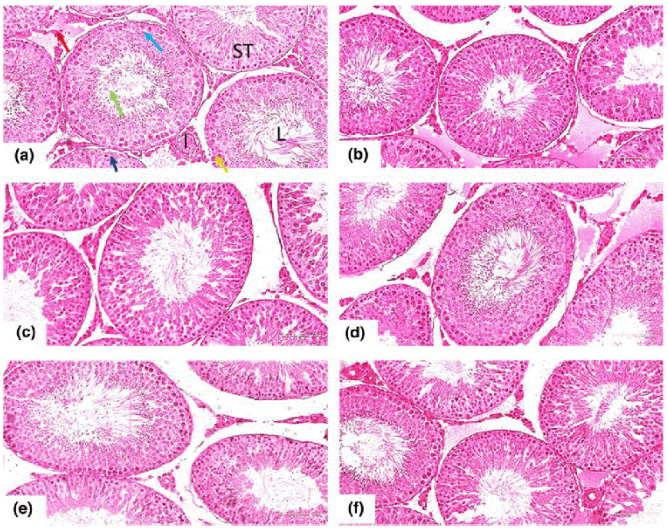

FIGURE 2 Haematoxylin and eosin stained histological sections in groups (a; control), (b; HAART), (c; Naringenin 40 mg/kg), (d; Naringenin 80 mg/kg), (e; HAART + Naringenin 40 mg/kg) and (f; HAART + Naringenin 80 mg/kg). The seminiferous tubules (ST) of the control animals displayed the normal progression of cells of the spermatogenic series with sperm cells in the lumen and Leydig cells in the interstitium. Group (b) had apparently smaller ST with scanty cells in the lumen, whereas the (c), (d), (e), and (f) displayed larger ST with the normal progression of cells of the spermatogenic series. Leydig cells in the interstitium and mature sperm cells in the lumen were similar to control

prooxidants, prevention of excessive lipid peroxidation, and prevention of structural and ultrastructural tissue changes induced by toxic agents (Hermenean et al., 2013). The ability to scavenge free radical was explained by its 5-hydroxy and 4-carbonyl groups in the C ring, which may interact with Cu and Fe ions (Podder, Song, & Kim, 2014). Naringenin has also been shown to be effective in inducing the expression of many antioxidant-related genes (Renugadevi & Prabu, 2009) and inhibit the activity of ROS-forming enzymes such as NADPH oxidase (Ciz et al., 2012).

4.2 | Reproductive hormones

In the present study, serum testosterone levels were reduced with exposure to HAART. Sperm function and hence the fertility potential

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of a male is highly dependent on the state of his hypothalamopituitary testicular axis (HPT) (Gandhi et al., 2017). This can be reflected by the circulating level of the reproductive hormones including testosterone and the gonadotropins. A well balanced HPT axis is essential for a successful reproduction; however, the balance can be offset by several factors such as the existing medical or surgical conditions and drugs used (Whirledge & Cidlowski, 2010).

This is contrary to the findings of Collazos, Mayo, Martínez, & Ibarra, 2002 who recorded an increase in testosterone levels. This conflict could be attributed to the difference in class of drug and mechanism of organ toxicity (Collazos et al., 2002). The earlier study associated a use of protease inhibitor with an increase in circulating testosterone (Collazos et al., 2002). This was however accompanied by sexual dysfunction. Another study reported a similar reduction in testosterone levels (Rochira et al., 2011). Naringenin conversely, induced a significant increase in serum levels of testosterone when compared to the control and HAART administered animals. The combination of naringenin and HAART used in this study has minimal effects on the serum levels of testosterone. This may explain the maintenance of the testicular weight and suggests other mechanisms of altered sperm function.

Naringenin significantly increased the level of testosterone in a dose-related manner. This may explain the high sperm count and motility (although not better than control) and the less abnormal forms observed in the naringenin administered group. This is similar to the findings from other studies (Roy et al., 2013; Sahin et al., 2017) and contrary to others (Papiez, 2003; Ranawat & Bakshi, 2017). In study examining the effects of naringenin on diabetes, naringenin-treated diabetic rats showed an improved histological appearance, sperm parameters and serum testosterone levels (Roy et al., 2013). An earlier study revealed that naringenin also caused minor changes in metabolic processes in the testes of the rats but did not significantly affect the synthesis of androgens (Papiez, 2003).

The serum luteinising hormone levels increased significantly in HAART administered animals. A normal serum level and intratesticular concentration are required for optimal testicular weight and function (Harada et al., 2016).

4.3 | Histomorphometry

Structural changes were observed across the groups. We observed increase in the volume fractions of the interstitium with the use of HAART. The seminiferous tubules (ST) of the control animals displayed the normal progression of cells of the spermatogenic series with sperm cells in the lumen and Leydig cells in the interstitium. This was also observed in the HAART administered group but there was an apparently smaller ST with scanty cells in the lumen of some of the ST. On the other hand, the other experimental groups displayed larger ST with the normal progression of cells of the spermatogenic series. Leydig cells in the interstitium and mature sperm cells in the lumen were similar to control. Minimal changes were detectable in the tubular structure of the testis associated with HAART use in this study, although a reduced intertubular space may suggest oedema of the testes induced by tissue inflammation. This, according to prior studies, preceed changes in the seminal epithelium (Chiquoine, 1964; Heuser, Mecklenburg, Ockert, Kohler, & Kemkowski, 2012; Hijazi, Ahmed, Ul Haq, Naqvi, & Parveen, 2016). Likely mechanism of toxicity is a disturbance of fluid homeostasis (Heuser et al., 2012). These changes may be better delineated using transmission electron microscope studies. The study made use of viral free animal models. The co-administration of naringenin and HAART is intended for HIV infected men. The immunological reaction to the virus may alter the observed interactions.

5 | CONCLUSION

The study has shown that use of the FDC-TDF/FTC/EFV induced changes in serum reproductive hormones, local tissue inflammation and disturbed fluid homeostasis in *Sprague Dawley* rats. These changes resulted in reduced sperm count and motility as well as an increase in abnormal forms, and subsequently reduced fertility index. Naringenin demonstrated some protection against these changes. Further studies on the testicular ultrastructure may be able to authenticate the beneficial role of this bioflavonoid as an adjuvant in the management of men on HAART.

ACKNOWLEDGEMENTS

The authors acknowledge Dr Oluwole Alese and Mr Denis Makhubela of Physiology Department University of KwaZulu-Natal for their support and for making available laboratory equipment during the course of the laboratory work. Profound gratitude goes to the Professor Sanil Singh and Dr Linda Bester of the Biomedical Resource Unit of the University of KwaZulu-Natal for their assistance with animal handling and sacrifice.

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How to cite this article: Adana MY, Akang EN, Naidu ECS, et al. Testicular microanatomical and hormonal alterations following use of antiretroviral therapy in *Sprague Dawley* rats: Role of Naringenin. *Andrologia*. 2018;e13137. https://doi.org/10.1111/and.13137

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BRIDGING

BETWEEN CHAPTERS THREE AND FOUR

Chapter three addresses the effects of antiretroviral therapy used over a longer period that lasts 10 weeks on the testicular microanatomy using histologic and histomorphometric methods. The changes associated were compared to changes in semen parameters (sperm count, sperm motility and percentage with normal morphology) alterations in the serum level of reproductive hormones. The flavonoid, Naringenin was administered to reverse/ protect against reproductive toxicity. This was done by checking the pregnancy rates following administration of drugs and phytochemical extract. The chapter reports alteration in semen parameters as well as testicular histoarchitecture. This suggests an underlying Sertoli cell dysfunction. Next chapter describes the testicular ultrastructure following the use of HAART as wells as the testicular antioxidant exam activity, an important protective mechanism in the male gonads.

4 CHAPTER FOUR

MANUSCRIPT THREE

Antiretroviral therapy induced impaired Sertoli cell function and testicular antioxidant activity: the role of Naringenin

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Submitted to Archives of Medical Sciences (AMS-08220-2018-01): 08-May-2018

Antiretroviral therapy induced impaired Sertoli cell function and testicular antioxidant activity: the role of Naringenin

Abstract

Background: Highly Active Antiretroviral Therapy (HAART) induced histopathological changes in the testes has been linked to increased activity of reactive oxygen species that ultimately overwhelms the tissue`s total antioxidant status.

Objective: This study investigated testicular ultrastructural changes and antioxidant enzyme activity following use of antiretroviral therapy and Naringenin, a bioactive in Sprague Dawley rats.

Materials and Methods: Thirty male Sprague Dawley rats weighing 200–220g, were assigned into 6 groups; **DW**: Distilled water, **H**: HAART, **N40**: Naringenin, 40 mg/kg, **N80**: Naringenin, 80 mg/kg, **HN40**: HAART+Naringenin, 40 mg/kg and **HN80**: HAART+Naringenin, 80 mg/kg. Oxidative enzyme activities were assayed using enzyme linked immunoassay and testicular ultrastructural changes were noted via electron microscopy.

Results: Results revealed that the testicular glutathione peroxidase levels were lower in H (P=0.000), HN40 (P=0.001) and HN80 (P=0.016) than DW. Catalase activity in H group was significantly lower than groups DW (p=0.024) and N80 (p=0.003). Group H displayed abnormal ultrastructural appearance including distortion of cellular progression in the germinal epithelium, degeneration of nuclear membrane and widening of internuclear space of Sertoli cells. Co-administration of Naringenin restored the normal cellular progression.

Discussion: Sertoli cell dysfunction has been identified as a manifestation of testicular toxicity from free radicals. Electron donation and possibly mop up free radicals are the proposed mechanism underlying the restoration of the oxidant-antioxidant balance in the testes.

Conclusion: This study suggests that HAART has deleterious effects on testicular function and male fertility. Naringenin, a bioflavonoid may be a useful adjuvant therapy in protecting against testicular toxicity.

Keywords: testis; antiretroviral therapy; glutathione; catalase; spermatogenic series; Sertoli cell

Introduction

The testes, an organ straddled with dual functions of steroidogenesis and sperm production is greatly dependent on oxygen to drive spermatogenesis and yet extremely vulnerable to the toxic effects of reactive oxygen metabolites (1-3). As a result the testis devises an efficient antioxidant systems involving both enzymatic and non-enzymatic components. The enzymatic component is activated during the process of spermatogenesis. At this time superoxide anion ($O2^-$) is generated from high rates of mitochondrial oxygen consumption by the germinal epithelium. This is then converted to hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase (SOD) in order to prevent the superoxide anion from forming the highly toxic hydroxyl radicals. The H_2O_2 generated is also a powerful membrane permeant oxidant which must be promptly removed from the cell so as to limit oxidative damage to lipids, proteins and DNA. The elimination of H_2O_2 is either achieved by catalase or glutathione peroxidase activity (4, 5).

The seamless oxidant-antioxidant balance in the testes may be perturbed by a number of factors. These factors induce a state of oxidative stress in the testes. Free radical injury is currently being regarded as the most significant cause of impaired testicular function associated with the pathogenesis of many male factor infertility ranging from varicocele (6), to diabetes (7, 8), testicular torsion (9, 10), xenobiotic exposure (11-13) as well as idiopathic cases (14-17). In a study inducing testicular torsion in animal models, severe histopathological damage, indicated by oxidative stress index and oxidants (malondialdehyde and total oxidant status) were increased with an attendant decrease in the antioxidants (glutathione peroxidase and total antioxidant status) in the torsion/detorsion group (10).

Antiretroviral therapy has been associated with deleterious histopathological changes in the testes causing tubular atrophy and altered morphometric manifestations (18-20). An ultrastructural study of rats' testes administered with a combination of antiretroviral drugs revealed significant increase in the basement membrane (BM) thickness when compared with control group. The HAART-treated group showed mitochondrial cristae collapse, and Sertoli cells cytoplasmic vacuolations (21). This study investigated testicular ultrastructural changes and antioxidant activity following use of antiretroviral therapy and Naringenin, a bioactive flavonoid in Sprague Dawley rats.

Materials and Methods

Materials

Thirty (30) adult male weighing 200–220g, were obtained from the animal house of the Biomedical Resource Unit, University of KwaZulu- Natal, South Africa. They were housed in standard plastic cages. Animals were kept under controlled environmental conditions (25ºC and a 12-h light/dark cycle) and they had free access to pulverized standard rat pellet food and tap water. Animal care and handling was in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

A fixed dose combination (FDC) of HAART containing Efavirenz (EFV) 600 mg, Emtricitabine (FTC) 200 mg and Tenofovir (TDF) 300 mg was procured from Pharmacare Ltd, Port Elizabeth, South Africa and administered to rats daily (Adult antiretroviral therapy guidelines 2014). The dose of the FDC was adjusted for animal weight using the human therapeutic dose equivalent for a rat model (7.5/5/15 mg/kg). Naringenin was procured from Sigma Aldrich Avengelee.

Experimental Design

Thirty SD rats were randomly divided into 6 equal number groups viz- Groups DW served as control and was given distilled water, H was given the FDC of HAART containing TDF/FTC/EFV, N40 had Naringenin, 40 mg/kg, N80 had Naringenin, 80 mg/kg. The last two groups, HN40 and HN80 had TDF/FTC/EFV and Naringenin 40mg/kg and 80mg/kg respectively.

All administrations were through the oral route and were done between 8:00 a.m. and 10:00 a.m. for a period of 10 weeks. Animals were weighed on alternate days for dose adjustments.

Animal Sacrifice and Collection of Samples

Following 10 weeks of treatment, the rats were euthanized. The testes were excised, weighed and fixed in glutaraldehyde for ultrastructural analysis.

Ultrastructural Studies

The tissues were fixed overnight at 4ºC in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and transferred to 0.1 M phosphate buffer (pH 7.2). The cells were postfixed in 1% osmium tetroxide in S-collidine, dehydrated in graded ethanols, transferred to propylene oxide, and embedded in Epon 812. Semi-thin sections were stained with 1% methylene blue. The samples were further sectioned into ultrathin slices (75 nm), contrasted with uranyl acetate and lead citrate and observed on a transmission electron microscope (Bressenot et al., 2009)

Biochemical Estimation for Oxidative Stress levels

One testis from each rat was homogenized using a Teflon homogenizer (Heidolph Silent Crusher M) and then the homogenates were centrifuged at 10,000g at 4ºC for 15 min. Homogenized

tissues were used for catalase (CAT) and glutathione peroxidase (GSH-Px) assay using standardised Elabscience enzyme-linked immunoassay (ELISA) rat specific kits with catalog numbers: E-EL-R2456 and E-EL-R2491 respectively.

Statistical Analysis

The data was presented as mean ± standard error of mean (SEM). The statistically significance of differences were evaluated by using one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) procedure for multiple comparisons. $P \leq 0.05$ was considered statistically significant and ≤ 0.01, very significant using SPSS version 23 for windows.

Results

Antioxidant enzymes

The testicular glutathione peroxidase levels was higher in the N40 and N80 groups when compared to H group. Lower levels were also observed in groups DW, HN40 and HN80 when compared to Naringenin-only groups. The N80 group showed significantly higher level of testicular catalase activity than groups DW ($p=0.024$) and H ($p=0.003$) see fig 1 and fig 2. The level in the HN40 was significantly higher than that of H ($p=0.019$).

Ultrastructural study

Group DW (Distilled water)

Basal and ad luminal compartments

Ultrastructure of control group (DW) displays cells in the different stages of maturation. Early and late cellular stages of spermatogenesis are presented with well circumscribed nuclear membrane. The Sertoli cells are seen with prominent nucleolus next to the myoid cells of the BM with normal thickness. The primary spermatocytes appear rounded with large nuclei [Fig. 2 (a) - (d)]. The germinal epithelium depicting normal testicular architecture with orderly progression of cells of the spermatogenic series is indicative of active spermatogenesis. Shown in Fig. 2 (e) - (g) are the ad luminal compartments displaying round and elongating late spermatids surrounded by residual cytoplasm with several microtubules of the midpieces, suggesting multiple spermatozoa. Each piece depicts a typical flagella axonemes composed of an array of microtubules, containing nine doublets arranged radially around two single central ones. Mitochondria sheaths are seen in

the mid pieces. Also observed are the lysosomes, ribosomes, smooth endoplasmic reticulum.

Interstitium

The interstitium houses the interstitial cells of Leydig (Ly) with nucleus having slight indentation and rich in euchromatin. The nuclear membrane is lined by a rim of heterochromatin. A large lymphatic vessel is seen traversing the interstitium. There was abundance of mitochondria and lipid droplets suspended within the cytoplasm of the Leydig cell. Other organelles such as lysosomes and smooth endoplasmic reticulum are dispersed in the Leydig cell cytoplasm. [Fig. 2 (h) - (i)].

Group H (HAART)

Basal and ad luminal compartments

Shown in Fig. 3 are the electron micrographs of ultrastructural sections of seminiferous tubule portion of group H. This group has several ultrastructural abnormalities. There are alterations in the normal progression of cells in the germinal epithelium. The more matured cells belonging to the ad luminal compartment - early and late spermatids appear in the basal compartment. The outline of the BM displays irregularities and increased thickness with hypertrophied myoid cells. There is widening of the internuclear space between Sertoli cells and there are scanty spermatogonic cells along the BM [Fig. 3 (a) - (d)]. Seen are spermatocytes showing discontinuous nuclear membrane indicating degenerative processes in the cells. Also observed are abnormally formed spermatid heads. The axonemes of the midpieces appears disorganised in cross section. The germinal epithelium appeared scanty with spermatocytes surrounded by abundance of residual cytoplasm suggestive of germ cell

atrophy. Cross section through the tail of the spermatozoa revealed axonemal defects in the form of lost organizations [Fig. 3 (e) - (h)].

Interstitium

The interstitium presents Leydig cells appearing smaller with reduced nuclear diameter and slight nuclear indentation [Fig. 3(i)]. There is less euchromatin within the nucleus and the cytoplasm appeared scantier with fewer suspended organelles.

Group N40 (Naringenin 40mg/kg)

Basal and ad luminal compartments

Ultrastructural sections of the testes of rats in N40 (Naringenin 40mg/kg) were similar to DW, showing normal progression of cells of the spermatogenic series. The spermatogonia and Sertoli cells were resting on BM. The cells have well circumscribed nuclei. The BM demonstrates normal thickness and the myoid cells. [Fig. 4 (a) - (c)]. The different stages of development of the spermatids were seen. The round spermatids and the late spermatids (LSt) were observed displaying normal appearance. The residual cytoplasm of the late spermatids were present close to the lumen with the midpieces suspended within. The nuclei appeared to be rich in euchromatin [Fig. 4 (d) - (f)].

Interstitium

The Leydig cells are seen with slight nuclear indentations. The nucleus appeared large and rich in euchromatin. There is an abundance of cellular organelles suspended within the cytoplasm. Blood vessel were observed traversing the interstitium [Fig. 4 (g) - (i)]. No vacuolation observed.

Group N80

Basal and ad luminal compartments

Electron sections of the testes of rats in N80 (Naringenin 80mg/kg) showing normal progression of cells of the spermatogenic series. The spermatogonia and Sertoli cells are aligned along the BM. Cells have well circumscribed nucleus and normal internuclear space. The BM demonstrates normal thickness. Also seen are the primary spermatocytes [Fig. 5 (a) - (d)]. The cross section of the ad luminal compartment revealed a large population of spermatozoa tails close to the lumen showing midpieces and flagellum made up of the principal pieces and the end pieces. The axonemes of the pieces are well organised. Also seen are the residual cytoplasm and vacuoles accommodating the midpieces [Fig. 5 (e) - (g)].

Interstitium

The Leydig cells (Ly) are seen in the interstitium. The nuclei are large and rich in euchromatin. The cells possess abundant mitochondria and lipid droplets within their cytoplasm. A large blood vessel is seen containing red and white blood cells in the interstitium [Fig. 5 (h) - (i)]. Other cell types are observed within the interstitial tissue.

Group HN40

Basal and ad luminal compartments

Observation from testes of HN40 group presented some improvement in the ultrastructural appearance when compared to the H group. Electron sections show Sertoli and spermatogonic cells lined along the BM. The myoid cells of the BM are in close relation with the spermatogonic cells. There is slight thickening of the BM. The primary

spermatocytes presents with some degenerative changes in the nuclear membrane [Fig. 6 (a) - (f)]. The cross section of the tails of late spermatids shows the midpiece, principal piece and end piece. There are a few midpieces with disorganised axonemes and abnormal spermatid heads [Fig. 6 (g) - (h)].

Interstitium

The Leydig cells appear with deep nuclear indentations. Nuclei present with normal chromatin condensations. Cytoplasmic organelles are seen suspended. Blood vessels are seen traversing the interstitium. Improvement on observations from the H group was noted [Fig. 6 (i)].

Group HN80

Basal and ad luminal compartments

Ultrastructure of testes of group HN40 reveals normal progression of cells of the spermatogenic series. The Sertoli and Spermatogonic cells lie on the BM with myoid cells in the adjacent BM. The BM has a relatively straight outline with slight thickening [Fig. 7 (a) - (d)]. Round spermatids displays acrosomal cap and occasional degeneration of nuclear membrane. The Late spermatids in the process of spermiation are observed. The lumen of seminiferous tubule contains cross section of tails showing abundant midpieces and principal pieces. There is some residual cytoplasm around the midpieces. Occasional abnormal forms in the late spermatids were observed [Fig. 7 (e) - (h)].

Interstitium

Leydig cells lie amidst scanty cytoplasmic organelles. There is reduced chromatin condensation. No vacuoles were observed. There is slight nuclear indentation in the Leydig cells [Fig. 7 (i)].

Discussion

ART is recommended for all individuals with HIV, regardless of CD4 cell count, to reduce the morbidity and mortality associated with HIV infection and to prevent transmission. Commencement of Highly Active Antiretroviral Therapy (HAART) however mean lifelong use despite the organotoxic properties of the regimen. This study describes antiretroviral therapy- induced alterations in testicular ultrastructure and antioxidant activity of adult male Sprague Dawley rat and evaluated the possible protective role of Naringenin.

In this study, administration of HAART was associated with a reduction in the activity of testicular antioxidant enzymes. The testicular activity of glutathione peroxidase and catalase improved with the co-administration of Naringenin and HAART in a dose dependent manner. The improved activity of antioxidant enzymes in tissues may explain the earlier identified free radical scavenging properties of Naringenin as previously described. (22-24) In a manner similar to Naringenin, Rutin, a citrus flavonoid has been shown to increase testicular Glutathione peroxidase and Glutathione activities in a dose-dependent manner. (25, 26)

Germ cell injury has been identified as the most important morphologic event underlying testicular dysfunction following administration of cytotoxic compounds. These morphologic changes usually offer an explanation of the mechanism of action of the gonadotoxic agent. (27, 28) This study revealed alteration of the normal ultrastructure of the testes with use of HAART. This was evidenced by irregular contour and thickening of the BM. A result of cellular infiltration of the BM. Tubular degeneration has been linked with thickening of BM by hindering metabolic exchange between the germinal epithelium and the interstitium(29) A thickened BM is a common finding associated with the degeneration

in the seminiferous tubule of crypto-orchid testes. This has been linked to impaired metabolic exchange and subsequently gonadotrophin deficiencies.(30, 31) Similar pathology has been noted in other atrophic testes with a different aetiology (32). The altered exchange of materials within the testicular tissue will further induce BM infiltration and hence thickening. Aging is another situation related to a convoluted and thickened BM(33). An increase in the expression of BM genes has been implicated in this process. This was found to precede atrophy of the seminiferous tubules .(33) Both the seminiferous tubule and the myoid cells are required for appropriate deposition of BM in the testes, an abnormality in the BM may suggest a malfunction of either the myoid cell or Sertoli cell.(33)

The normal progression of the cells in the seminiferous germinal epithelium was altered following use of antiretrovirals. This was evidenced by the presence of elongating spermatids being retained and resorbed into basilar Sertoli cell cytoplasm within the basal compartment. Other studies that described the morphologic changes in the rat testes such as spermatid retention and degeneration of cells linked it with decreased intratesticular testosterone levels. (34, 35)

The spermatogonic cells along the BM was observed to be scanty following the use of HAART. However the Sertoli cells seem unaffected, there was an associated reduction in the population of primary spermatocytes. The spermatocytes and round spermatids displayed incomplete nuclear membrane. This suggests some degenerative process ongoing within the cell. It has also been observed that Sertoli cell injury is expressed by loss of function rather than loss of Sertoli cells themselves. The many roles that Sertoli cells play in spermatogenesis are reflected in the variety of morphologic manifestations associated with Sertoli cell toxicity. An impaired Sertoli cell function expresses as germ cell

degeneration. Similarly degeneration of seminiferous epithelium may either present as endocrine toxicity or direct germ cell injury following penetration of the toxic compound through the blood testis barrier. (36)

The co-administration of Naringenin proved beneficial. The normal cellular progression was observed and cells of the spermatogenic series were well represented however there were occasional defects in the continuity of the nuclear membrane. This restorative potential associated with use of high dose Naringenin is in tandem with earlier observations. (20) Kwatra et al (2016) revealed that naringin usage, a closely related flavonoid ameliorated the behaviour alternation in Doxorubicin treated rats, oxidative stress, proinflammatory cytokines, mitochondrial dysfunction and improved monoamines contents in brain hippocampus of rats (37). Addition of Naringenin to oseltamivir treatment improved the neurofunctions of the brain through improving the Y maze task and reductions in the pathophysiological effect of oseltamivir. This was attributed to increasing total antioxidant capacity, brain fatty acid binding proteins FABP7 and Ca-ATPase and reducing oxidative (38). In the same study Naringenin was proposed to have reduced total nitric oxide, total oxidant capacity and total cytochrome P450 (CYP450) contents (38).

Some studies have proposed an improvement in the antioxidant activity. Supplementation of Naringenin and its synthetic derivative enhanced antioxidant enzyme activities of erythrocyte and liver in high cholesterol-fed rats by enhancing the antioxidant defence system (24). In cisplatin-Naringenin combined treated rats , the activities of superoxide dismutase, glutathione peroxidase, and catalase were significantly increased (23).

Naringenin has also been regarded as a chelating agent capable of binding to toxic metal ions to form complex structures which are easily eliminate from intracellular or

extracellular spaces. Cavia-Saiz et al (2010) reveals that Naringenin acts as an active chelator of metallic ions and inhibitor of the enzyme xanthine oxidase. These ameliorative effects of Naringenin may be attributed to its chemical structure. The hydroxyl groups present are able to donate electrons and mop up generated free radicals from the altered oxidative status thereby restoring the oxidant-antioxidant balance in the testes. This provides an insight into the mechanism of gonadotoxic effect of antiretroviral therapy. Ranawat et al (2017) however revealed opposing views when Naringenin was administered intraperitoneally to mice. They suggested that Naringenin despite being a potent antioxidant may also act as pro-oxidant in a dose dependent fashion, hence causing damaging effects in the testes observed as decreasing sperm motility as well as concentration and inducing alterations in testicular histomorphology (39). This difference can be attributed to the increased bioavailaibility of Naringenin using the intraperitoneal route. Additionally, the Naringenin vehicle used in the study Dimethyl sulfoxide exhibit a range of pharmacological activity including ability to penetrate biological membranes increasing the concentration of pharmacological agents within the tissue.

Conclusion

The present study reports that HAART induced ultrastructural changes in the testicular tissue due to Sertoli cell dysfunction and altered antioxidant activity in the testes of adult rats. The mechanism of injury is similar to those observed in cryptorchidism and aging. Some restoration of structure and function was achieved by Naringenin, a bioactive flavonoid. Researchers suggests that the protective potential of Naringenin in ART induced gonadotoxicity might be due to its antioxidant and metal chelating properties, which could

present it as a useful adjuvant in the treatment of HIV patients especially those desirous of future fertility.

Acknowledgements

Authors acknowledge Subashen Naidu of Microscopy and Microanalysis unit of University of KwaZulu-Natal for the technical support and for making available laboratory equipment during the course of the work. Profound gratitude goes to the Professor Sanil D. Singh and Dr Linda Bester of the Biomedical Resource Unit of the University of KwaZulu-Natal for their assistance with animal handling and sacrifice.

Conflict of interest

The authors declare no conflict of interest.

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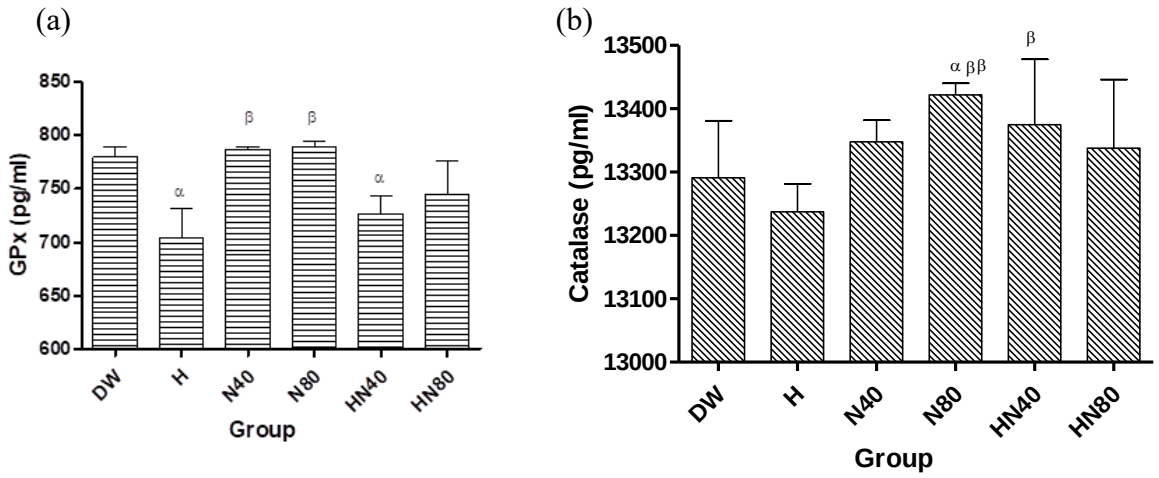

Fig. 1 (a) Testicular glutathione peroxidase and (b) Catalase in groups DW (control), H (HAART), N40 (Naringenin 40mg/kg), N80 (Naringenin 80mg/kg), HN40 (HAART + Naringenin 40mg/kg) and HN80 (HAART + Naringenin 80mg/kg) after 10 weeks of treatment. Bars indicate mean±SD. $^{\alpha}$ represents significant difference ($p<0.05$) between the group and control, ^{ß} represents significant difference between the group and H group. ^{ßß} represents very significant difference ($p<0.01$) between the group and H group.

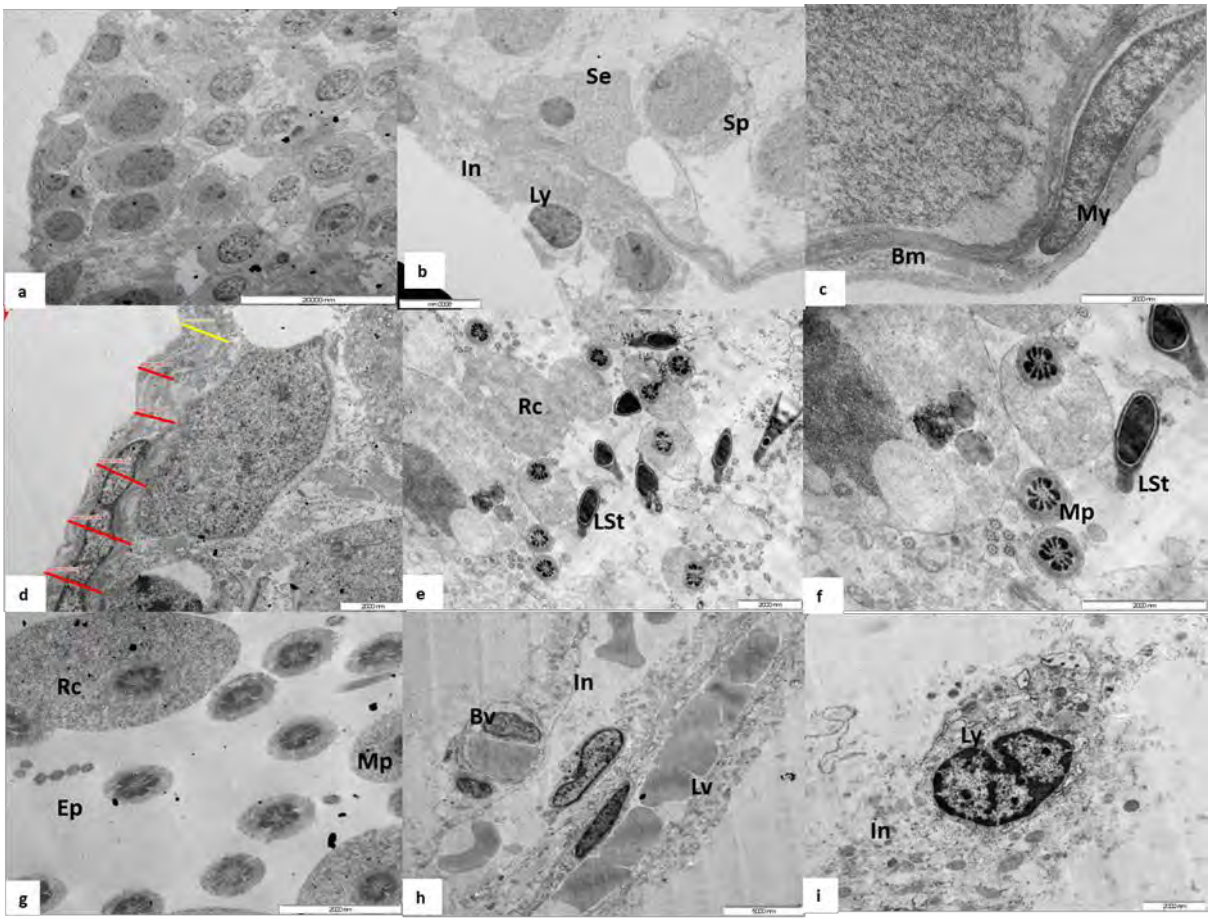

Figure 2: Ultrastructure of group DW (control) depicts normal testicular architecture with orderly progression of cells of the spermatogenic series (a) shows early and late cellular stages of spermatogenesis with well circumscribed nuclear membrane (b) shows the Sertoli cell (Se) with prominent nucleolus (c) seminiferous tubule with BM (Bm), myoid cells (My) (d) shows the BM with normal thickness (e), (f) and (g) show elongating late spermatid (St) surrounded by residual cytoplasm and midpieces, several microtubules of the midpiece (Mp) (h) and (i) show the interstitium (In) containing the interstitial cells of Leydig (Ly) with nucleus having slight indentation, blood vessel (Bv) containing red blood cells and lymphocytes. Also seen is the lymphatic vessel (Lv). Scale bar for a, c, d, e, f, h, i is 2000nm and 5000nm for b,g

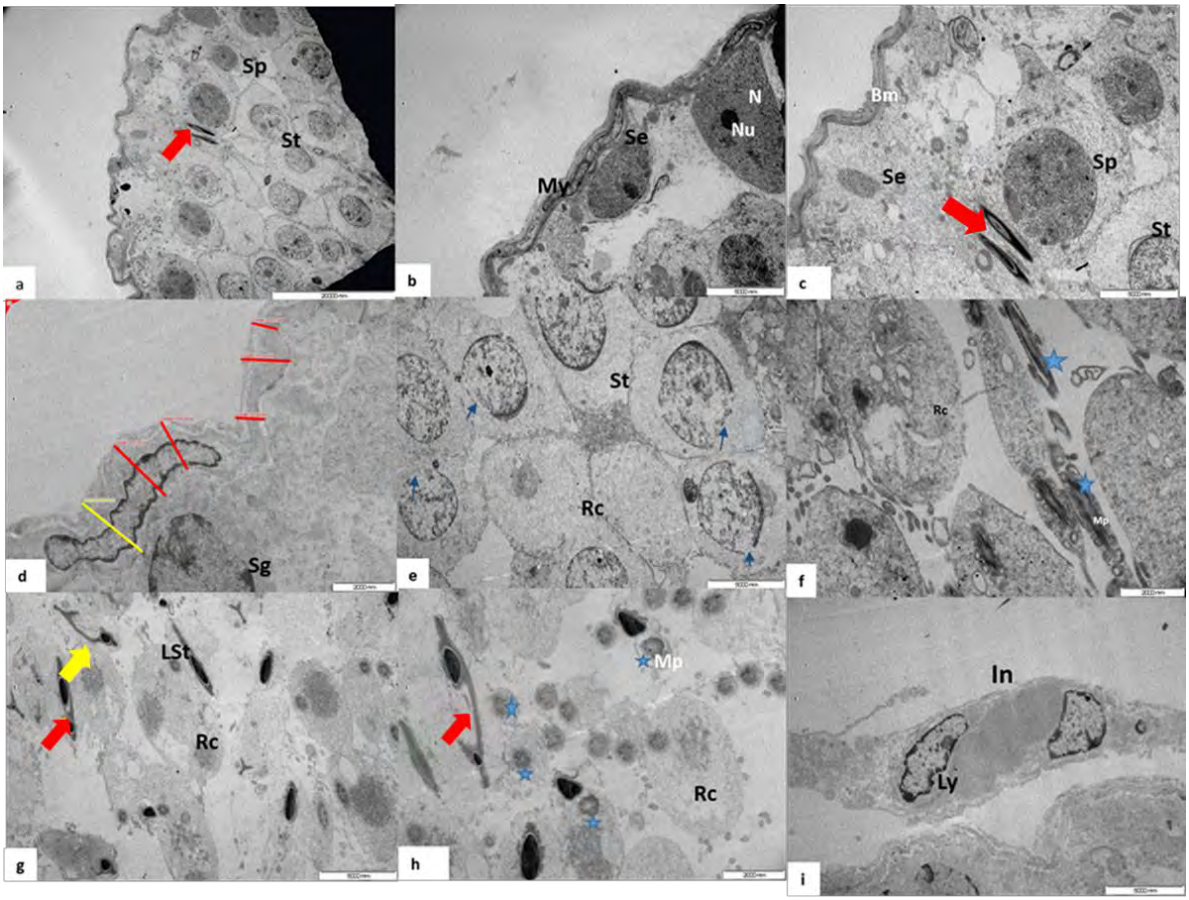

Fig. 3: Ultrastructural micrograph of seminiferous tubule portion of group H highly active antiretroviral therapy ([HAART] only). This group has several ultrastructural abnormalities and disorganised germinal cells (a) alteration in the normal progression of cells in the germinal epithelium. Round Spermatid (St) and late spermatids (red arrow) appearing in the basal compartment. There is also depletion of the spermatogonic cells (b) and (c) showing irregularity in the outline of the BM (d) showed increased thickness in BM (Bm) and myoid cell (My), there is widening of the internuclear space between Sertoli cells (Se) (e) shows degenerative changes in the nuclear membrane (blue arrow) (f), (g) and (h) shows abnormally formed spermatid heads (yellow arrow) and disorganised axonemes in cross section of midpiece (blue star) surrounded by abundance of residual cytoplasm (Rc) (i) shows the interstitium with normal Leydig cells having slight nuclear indentation. Scale bar for a, d, f, h is 2000nm and for b, c, e, g, i is 5000nm.

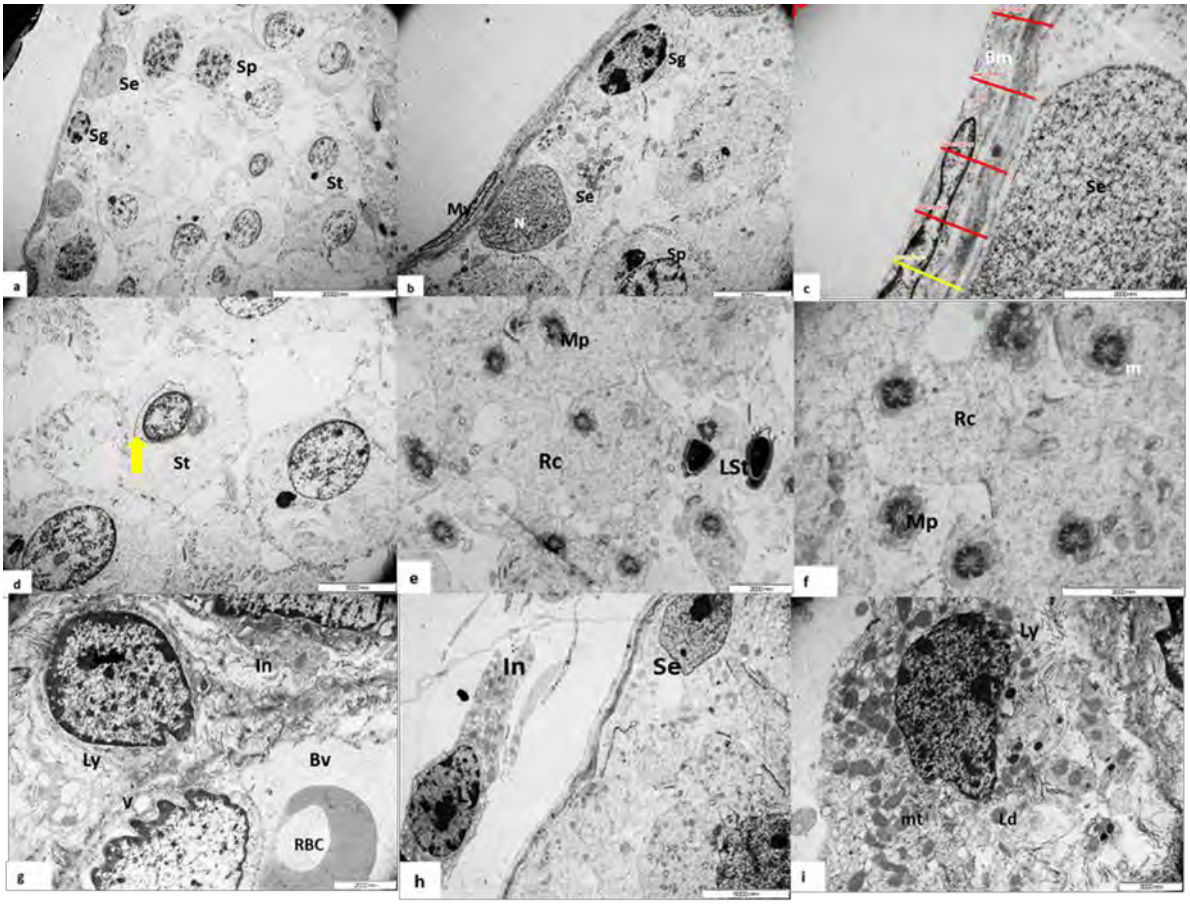

Fig 4: Ultrastructure of the testes of rats in N40 (Naringenin 40mg/kg) (a) showing normal Progression of cells of the spermatogenic series (b)The spermatogonia (Sg) and Sertoli cells (Se) are resting on BM, the spermatogonia (Sg), spermatocytes (Sp) have well circumscribed nucleus (N) and well defined nuclear membrane (c) The BM demonstrates normal thickness and myoid cell with the nucleolus of the Sertoli cell resting on it (d) (e). The different stages of development of the spermatids are seen, the round spermatids (St) and the late spermatids (LSt) The residual cytoplasm of the late spermatids are present close to the lumen with the midpiece (Mp) suspended within (g) The Leydig cells (Ly) are seen in the interstitium (h) and (i) shows the Leydig cell (Ly) in the interstitium (In) with abundant mitochondria and lipid droplets. Scale bar for a, c, e, f, g, i is 2000nm and b, d, h are 5000nm.

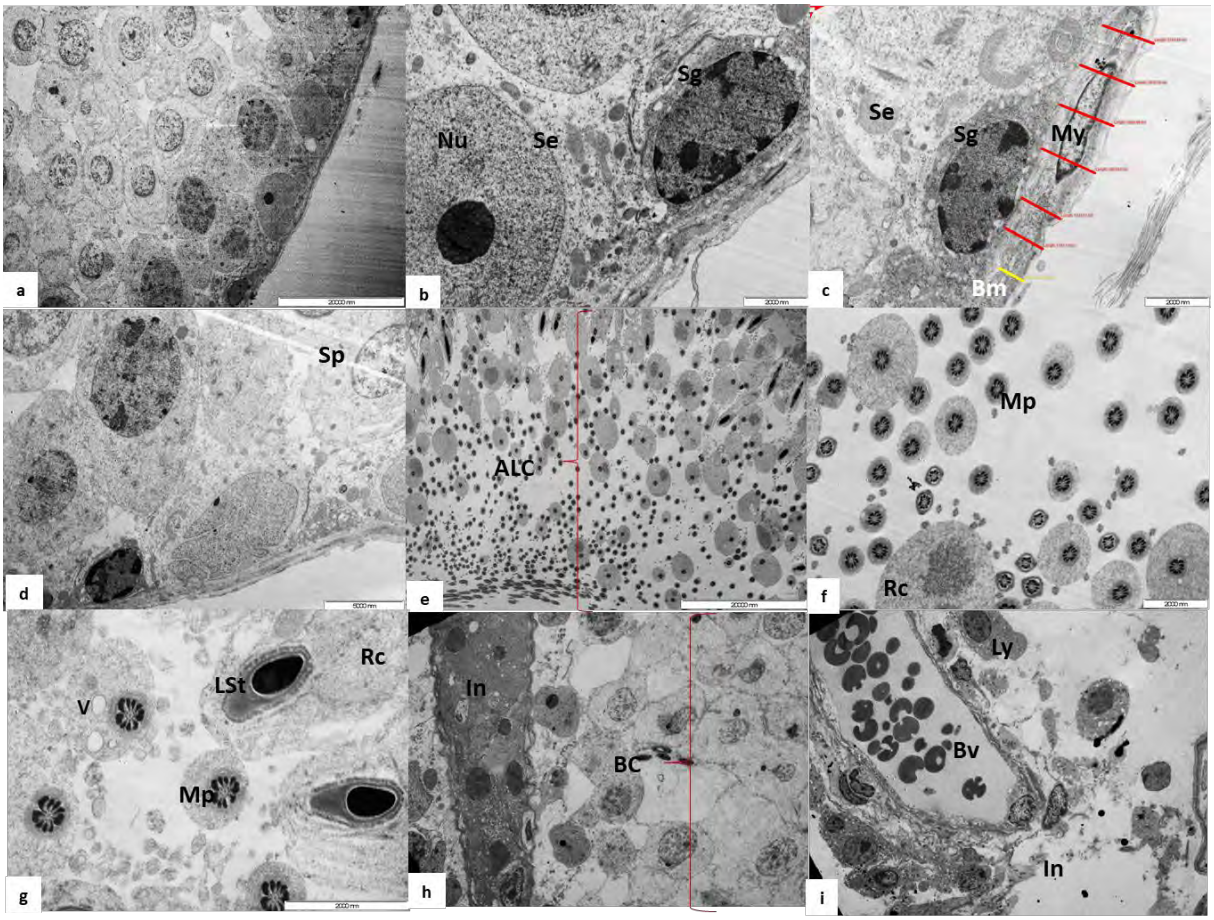

Fig 5: Ultrastructure of the testes of rats in N80 (Naringenin 80mg/kg) (a) showing normal Progression of cells of the spermatogenic series (b)The spermatogonia (Sg) and Sertoli cells (Se) are resting on BM, the spermatogonia (Sg), and Sertoli cells (Se) have well circumscribed nucleus (Nu) and normal internuclear space (c) The BM demonstrates normal thickness and myoid cell (d) is showing the basal compartment consisting of the primary spermatocyte (Sp), the Sertoli cell and the spermatogonia (e), (f) and (g) presents the ad luminal compartment (ALC) with the cross section of the midpiece and flagellum made up of the principal pieces and the end pieces. Also seen is the residual cytoplasm (Rc) and vacuoles (v) surrounding the midpiece (g) The Leydig cells (Ly) are seen in the interstitium (h) and (i) shows the Leydig cell (Ly) in the interstitium (In) with abundant mitochondria, lipid droplets and a blood vessel (Bv) containing red and white blood cells. Scale bar for a, b, c, e, f, g, h, i is 2000nm and d is 5000nm.

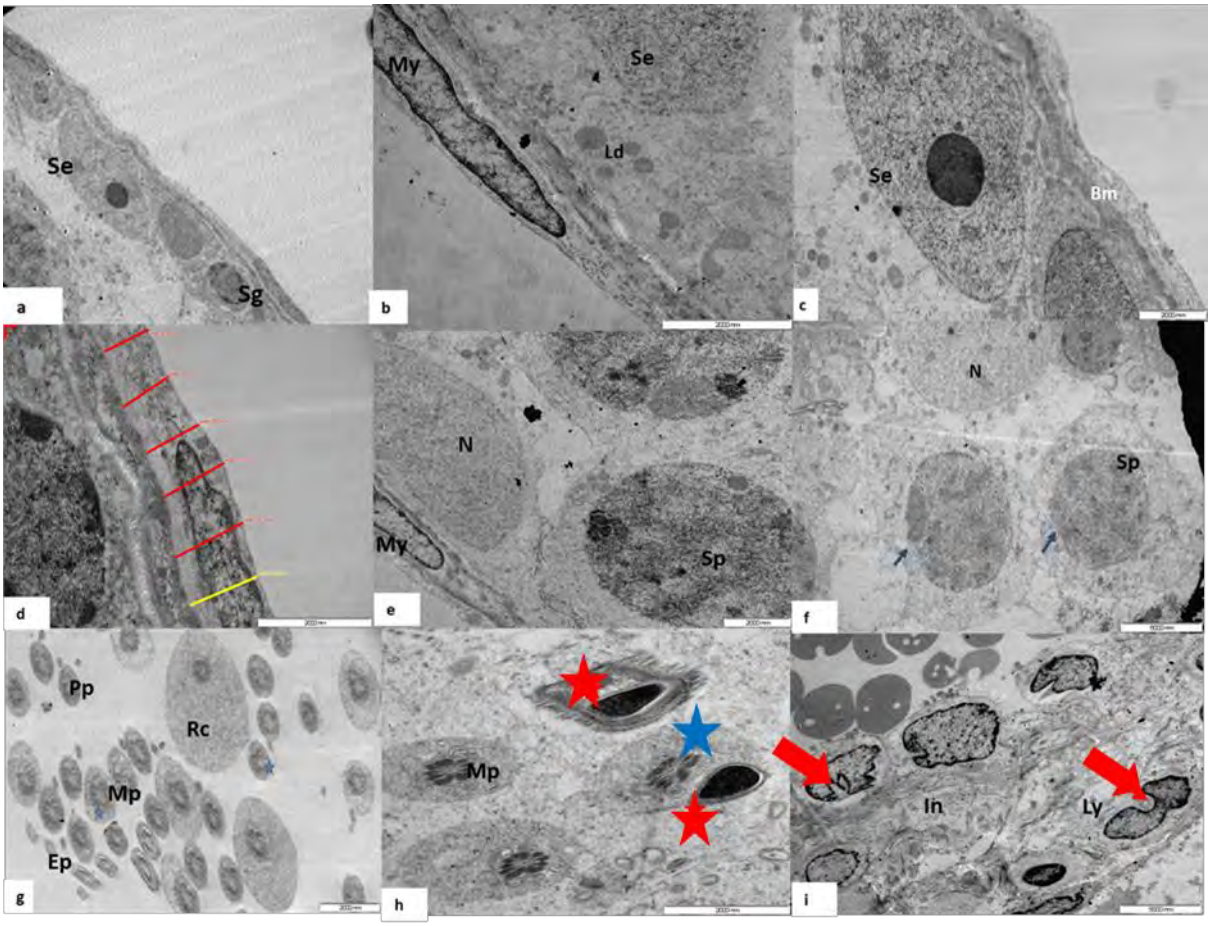

Fig. 6: Ultrastructure of testes of group HN40 (a) Sertoli (Se) and spermatogonic cells (Sg) lined along the BM (Bm) (b) and (c) myoid cells of the BM in close relation with the spermatogonic cells (Sg) (d) shows slight thickening of the BM (e) and (f) presents primary spermatocytes showing some degenerative changes in the nuclear membrane (blue arrow) (g) cross section of the tails of late spermatids showing the midpiece (Mp), principal piece and endpiece (Ep), there are a few midpieces with disorganised axonemes (blue star) (h) shows abnormal spermatid heads (red star) (i) presents the Leydig cells with deep indentations and blood vessels in the iinterstitium. Scale bar for a, b, c, d, e, g, h are 2000nm and 5000nm for f, and i.

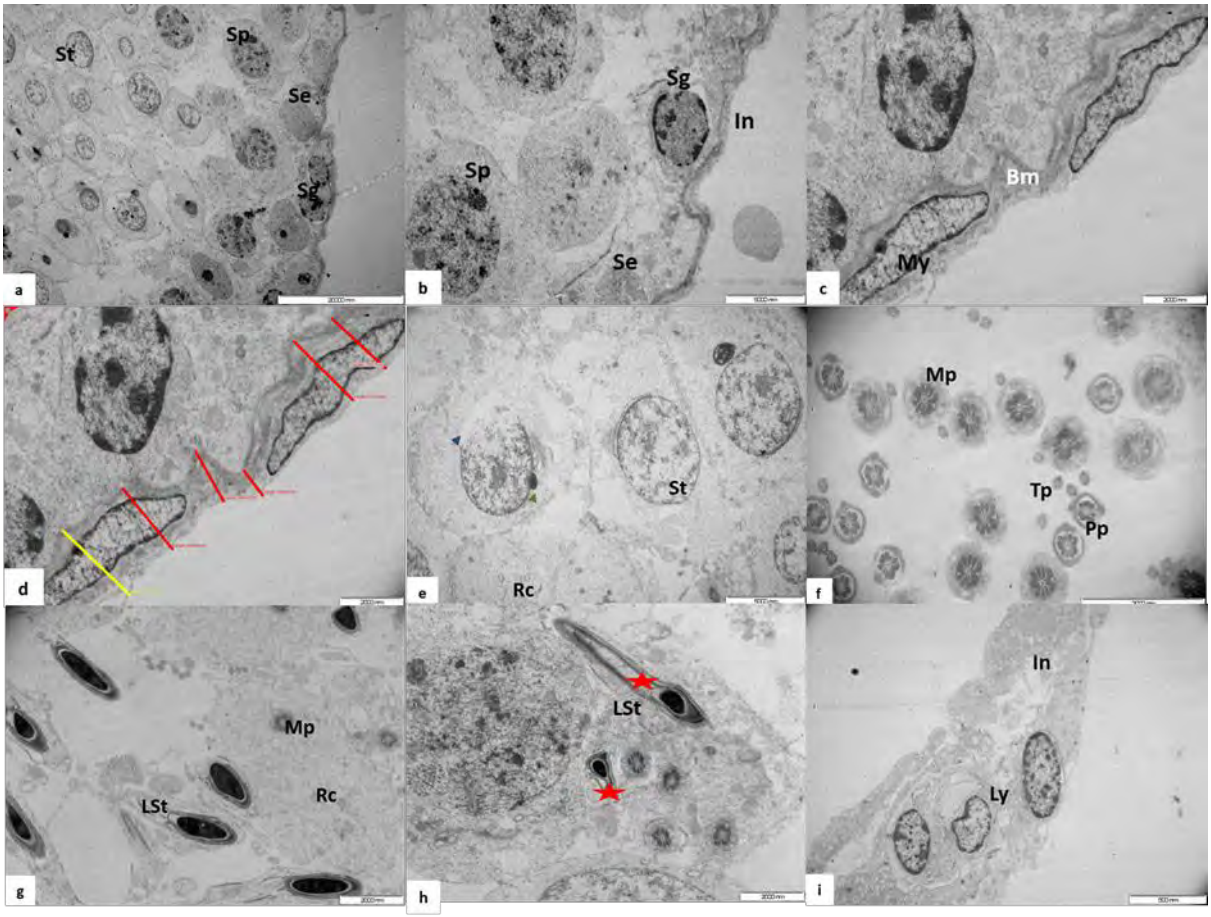

Fig 7: Ultrastructure of testes of group HN40 (a) Normal progression of cells of the spermatogenic series (b) & (c) The Sertoli (Se) and Spermatogonic (Sg) cells lie on the BM (Bm) with myoid cells (d) The BM has a relatively straight outline with slight thickening (e) Round spermatids (St) displays acrosomal cap and occasional degeneration of nuclear membrane (f) Lumen of seminiferous tubule containing cross section of tails showing the midpieces (Mp) and the principal pieces (Pp) (g) Late spermatids (LSt) in the process of spermiation. There is some residual cytoplasm (Rc) around the midpiece (Mp) (h) presents occasional abnormal forms in the late spermatids (red star) (i) Leydig cells (Ly) lie with the interstitium (In). Scale bar for (a), (c), (d), (f), (g), (h) is 2000nm and 5000nm for (b), (e), (i).

BRIDGING

BETWEEN CHAPTERS FOUR AND FIVE

The previous chapter was able to demonstrate the toxic effects of antiretroviral therapy on the oxidative status of the testicular tissue and the attendant effects on the ultrastructural of the organ particularly as it relates to Sertoli and Leydig cell functions. The palliative role of Naringenin was further revealed in the manuscript to further delineate the mechanism of injury to the testes. Testosterone biosynthesis begins from the transfer of cholesterol from the cytoplasm to the inner mitochondrial membrane by StAR protein (Steroidogenic acute regulatory protein), this is followed events under the control of the steroid-synthesizing enzymes in testicular Leydig cells including 3 beta-hydroxysteroid dehydrogenases (3β HSD). The activity of this steroidogenic enzyme and regulatory protein is critical for normal steroidogenesis and the subsequent spermatogenesis. Having observed changes in the Leydig cells, the next chapter clarifies the effects of ARD on the expression of testicular 3β HSD and spermatogenesis.

5 CHAPTER FIVE

MANUSCRIPT FOUR

Testicular 3 Beta hydroxysteroid dehydrogenase in Naringenin under Highly Active Antiretroviral Therapy (HAART): preliminary data using Sprague-Dawley rats

Running title: Naringenin Improves Testicular 3β HSD

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Accepted by European Journal of Anatomy: 24-September-2018

TITLE PAGE

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SUMARRY

HAART has brought relief to many living with HIV/AIDS, decreasing morbidity and mortality rates. In spite of these benefits, the treatment has been associated with reproductive disorders. This study is aimed at investigating the effects of Naringenin (Nar) on the expression of testicular 3β-Hydroxysteroid dehydrogenase (3β HSD) in HAART treated Sprague-Dawley rats. 30 adult male Sprague-Dawley rats were randomly divided into six groups. The rats were fed with 30 mg/kg of HAART (Efavirenz+Embtricitabine+Tenofovir), 40mg/kg and 80 mg/kg of Nar and a combination of both HAART and Nar for a period of 70 days. Thereafter, the animals were euthanized and the testis processed. The results showed a significant decrease (p<0.05) in the expression of 3β HSD in the HAART group compared to controls. However, the co-treatment of HAART with 40 mg/kg Nar, increased significantly (p<0.05) the expression of 3β HSD compared to HAART and control. The relative volume fraction also showed significant increase (p<0.05) in germinal epithelium, lumen and Leydig cells of animals treated with 80 mg/kg Nar, and HAART+40 mg/kg Nar compared to control and HAART respectively. In conclusion, HAART is causes a deficiency in testicular 3β HSD thereby limiting spermatogenesis, however, co-treatment with 40 mg/kg Naringenin increases testicular 3β HSD expression and enhances spermatogenesis.

Keywords: 3β HSD, Testis, Naringenin, Antiretroviral therapy, Leydig cells

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INTRODUCTION

The advent of highly active antiretroviral therapy (HAART) in 1996 [1-2] has brought much succour to persons living with HIV/AIDS (PLWHAS), increasing life expectancy and decreasing the incidence of opportunistic infections [3]. It is expected that by 2020, 90% of diagnosed HIV positive persons will be on HAART [4]. Global statistics shows that over 37 million persons are already infected with the virus [5], and about 40 % are men in their 20s. It is quite unfortunate that despite the relative life expectancy and other health benefits of HAART, it is associated with several comorbidities [6-7] including testicular-toxicity, thereby impairing spermatogenesis [8].

A study by Pilatz et al.[9] comparing the semen quality of HIV patients under stable antiretroviral therapy (ARV) with WHO 2010 reference values, observed impaired semen parameters and altered protein composition among those on ARV. Their findings highlights the need for further studies in the treatment/management of HIV *vis-a-vis* male fertility preservation, especially as most of the men on HAART are in their reproductive age with a strong desire to procreate [10].

Furthermore, studies by Ogedengbe et al.[11] and Jegede et al.[12] demonstrated very toxic effects of HAART on the testis of experimental animals. It is believed that the therapy causes an imbalance in redox activity, promoting the production of excess free radicals which in turn impairs spermatogenesis [13]. However, these authors did not peruse the biosynthesis of testosterone which is critical for the maintenance of spermatogonial numbers, blood-testis barrier integrity, completion of meiosis, adhesion of spermatids and spermiation [14].

Testosterone biosynthesis, which begins from the transfer of cholesterol from the cytoplasm to inner mitochondrial membrane by StAR protein (Steroidogenic acute regulatory protein), is followed by a plethora of events controlled by steroid-synthesizing enzymes in Leydig cells

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[15], including 3 beta hydroxysteroid dehydrogenase (3β HSD) activity. The activity of this steroidogenic enzyme and regulatory protein is critical for normal steroidogenesis and subsequently the process of spermatogenesis [16].

Excessive free radicals have been reported to hinder steroidogenesis [17]. In view of this, antioxidant supplementation is encouraged to stabilize the excesses of mitochondrial free radical synthesis [18]. Flavonoids have a direct scavenging effect on free radicals because they exhibit a wide variety of antioxidant properties [19]. Free radicals are oxidized by flavonoids, resulting in a more stable, less-reactive molecule [19]. Naringenin bioflavonoid (4,5,7-trihydroxyflavon) which is found in abundance in fruits, especially grapefruit and tangerine [20], is considered a safe natural product with reports of mediating decreased testicular oxidative stress by reducing hydrogen peroxidase activity [21]. It also ameliorated oxidative stress-induced hepatic and renal dysfunction [22], and has neuroprotective effects [23-24] However, a report on Naringenin-induced oxidative stress and spermatogenic toxicities [25] necessitates further research on the role of Naringenin on steroidogenesis and spermatogenesis.

This study is therefore aimed at investigating the effects of Naringenin (Nar) on the expression of testicular 3β HSD in HAART treated Sprague-Dawley rats.

MATERIALS AND METHODS

Materials

Thirty (30) adult male Sprague-Dawley (SD) rats weighing 200–220g were randomly distributed into six groups of five each in the study. They were housed at the Biomedical Resource Unit, University of KwaZulu-Natal (UKZN), South Africa (SA). This research was approved by the Animal Research Ethical Committee, UKZN (reference number AREC/046/016D). All procedures were performed in accordance with the 'Principles of Laboratory Animal Care of the National Medical Research Council' and the 'Guide for the

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Care and Use of Laboratory Animals.' The animals were housed in standard cages with dimensions of 20cm long, 20cm wide and 13cm high. The rats were kept under controlled environmental conditions (25^{o}C and a 12-h light/dark cycle) and had free access to standard rat pellets, and tap water. They were allowed to acclimatize for two weeks prior to the commencement of the study.

Natural Naringenin was purchased from Sigma-Aldrich SA, while, Atripla containing efavirenz (EFV, 600 mg), emtricitabine (FTC, 200 mg) and Tenofovir (TDF, 300 mg)[26], was procured from Pharmacare Ltd, Port Elizabeth, SA. The therapeutic dose of Atripla was adjusted for animal weight using the human therapeutic dose equivalent for a rat model.

Experimental Design

Adult male SD rats were randomly divided into 6 groups: Group DW: Control (Distilled water), Group HAART: 30 mg/kg Atripla, Group Nar40: Naringenin, 40 mg/kg [27], Group Nar80: Naringenin, 80 mg/kg [28], Group HNar40: 30 mg/kg Atripla + Naringenin, 40 mg/kg and Group HNar80: 30 mg/kg Atripla + Naringenin, 80 mg/kg. The experiment was conducted between 8:00 am and 10:00 am for a period of 10 weeks and all administrations were done via the oral route using orogastric cannulae.

Animal Sacrifice and Collection of Samples

The animals were euthanized on day 70 by excess Halothane. The testes were excised and separated from the cauda epididymis and fixed in Bouin`s fluid for immunohistochemical analysis.

Immunohistochemical Studies

Testes tissues were taken from Bouin's fluid and transferred to 70% ethanol [29]. They were then processed using a graded ethanol series and embedded in paraffin. The paraffin sections were

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cut into 4 µm-thick slices using a microtome (Microm HM 315 microtome, Walldorf, Germany). Immunohistochemistry was performed using Santa Cruz 3β HSD primary antibody and Dako Envision FLEX kit. The processed and sectioned tissues were dewaxed with 2 changes of xylene and hydrated with decreasing grades of alcohol, and water. The sections were placed in diluted Envision FLEX Target solution for 20 minutes at 95-99^{O}C. Tissue sections were washed in wash buffer, blocked with peroxidase and incubated with diluted 3β HSD (1:150) from Santa Cruz for 30 minutes and with HRP for 20 minutes, DAB and counterstained in hematoxylin, washed in wash buffer, dehydrated, cleared and mounted on DPX. The sections were viewed and photographed using a 40X objective (Zeiss Axioscope A1 microscope, Carl Zeiss, Germany) with an AxioCam MRc Zeiss digital camera attached.

Percentage Immunoreactivity

Image analysis and capturing was done using AxioVision software (Carl Zeiss, Germany; version 4.8.3). At least six fields of view per slide were randomly selected and captured using a 20X objective. 3β HSD expression was determined as percentage of positive reactivity (brown) per interstitial area of testis.

Testicular histology preparation

The testis was harvested and fixed in Bouin's fluid for 24 h after which it was transferred to 70% alcohol for dehydration. The histology of the testis was done by modification of method reported by Akang et al.[30]. The slides were then stained with haematoxylin and eosin. The slides were mounted in DPX. Photomicrographs were taken at a magnification of x200

Morphometric (unbiased stereological) analysis

Morphometric analysis was done with the primary aim of estimating the volumes of seminiferous tubule epithelium (seminiferous epithelium) and interstitial cells of Leydig in the testis. This was done in accordance with methods described by Howard and Reed[31] and Akang

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et al.[30]. Four sections per testis, and six microscopical fields per section, were randomly chosen for analysis using a 20X objective. Fields were sampled as images captured on Zeiss Axioscope A1 microscope (Carl Zeiss, Germany). Volume densities of testicular ingredients were determined by randomly superimposing a transparent grid comprising 35 test points arranged in a quadratic array. Test points falling on a given testis and its ingredients were summed over all fields from all sections. The total number of points hitting on a given ingredient (lumen, germinal epithelium, interstitial cells), divided by the total number of points hitting on the testis sections multiplied by 100, provided an unbiased estimate of its %volume density/volume fraction.

RESULTS

Histomorphological assessment

Cross section of the testis of control animals had a compact interstitium with marked presence of Leydig cells, and normal cellular composition in their germinal epithelium (GE) showing the presence of Sertoli cells and complete cells of spermatogenic series: spermatogonia - primary spermatocytes - secondary spermatocytes – spermatids - and spermatozoa in lumen (L). The photomicrographs of animals that received Naringenin at 40 mg/kg, and 80 mg/kg including those that received both HAART and 40 mg/kg Naringenin had similar cyto-architecture with controls (Figure 1). However, animals that received HAART only and those that received both HAART and 80 mg/kg Naringenin had dilatations in the interstitium and sparse distribution of Leydig cells. Both groups also had compressed seminiferous tubules (Figure 1), with interrupted spermatogenesis in the HAART group (Figure 2).

Morphometric analysis

The relative volume fraction of the germinal epithelium, lumen and interstitial cells of animals that were treated with HAART decreased compared to control though not significantly.

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Whereas, the germinal epithelium, lumen and Leydig cell increased significantly ($p<0.05$) in groups treated with 80 mg/kg Naringenin, and a combined dose of HAART and 40 mg/kg Naringenin compared to control, and HAART treated animals respectively (Table I).

Immunohistochemical Analysis

All the photomicrographs showed expression of 3β HSD in the interstitial cells of Leydig (Figure 2), however, image analysis showed significantly less ($p<0.05$) expression of 3β HSD in the Leydig cells of the animals treated with HAART compared to control. It also showed a significantly higher ($p<0.05$) expression of 3β HSD in animals that received a combination of HAART and 40 mg/kg Naringenin compared to those that received HAART only. While the animals that received a combined dose of HAART and 80 mg/kg Naringenin significantly decreased ($p<0.05$) compared to control (Figure 3).

DISCUSSION

The findings from this study clearly demonstrates a deleterious effect of the combined dose of efavirenz, emtricitabine and tenofovir on the expression of testicular 3β HSD. Efavirenz, which is an active constituent in the first line treatment of HIV positive adults, pregnant and breast feeding mothers, adolescents and children [32], has been reported to increase the production of superoxide anions and morphological alterations of the mitochondria [33]. A study by Ganta et al.[34], showed that EFV-mediated toxicity is initiated via the permeabilization of the outer membrane of the mitochondria and subsequent change in the membrane potential ($\Delta\psi m$) which triggers a series of events like Cytochrome C release which results in a cascade of events, altering cellular homeostasis, including induction of oxidative stress and subsequent autophagy. Moreover, normal steroidogenesis is promoted by the maintenance of normal mitochondrial pH, membrane potential and ATP synthesis [35]. It is therefore plausible to infer that the downregulation testicular 3β HSD by HAART explored the oxidant-antioxidant

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pathway. These findings are in tandem with reports by Wang et al. [36] who reported that the alteration in mitochondrial antioxidants adversely affected 3β HSD expression and Leydig cell steroidogenesis.

Excessive free radical production obstructs the biosynthesis of testosterone, which is essential for the proper functioning of the Sertoli-Sertoli tight junctions also known as the blood-testis-barrier (BTB), and normal spermatogenesis [37]. Thus, a deficiency of this steroid hormone will be counterproductive on spermatogenesis, rarely progressing beyond diplotene spermatocytes stage [38-39]. Our study revealed obstructed spermatogenesis and widening of the interstitium, with fewer Leydig cells in HAART treated animals. The implication thereof, corroborates with our previous study which showed that HAART decreased sperm count, sperm motility and sperm morphology and increased sperm DNA fragmentations [40].

In spite of this deleterious effects of HAART, the Naringenin co-treated animals especially at a lower dose (40 mg/kg) had greater expression of testicular 3β HSD compared to those treated with HAART only. Likewise, the histomorphology and morphometry showed complete spermatogenesis with a very conspicuous presence of spermatozoa in lumen of the seminiferous tubules. During oxidative stress, Naringenin chelates irons and scavenges ROS [41]. 5-hydroxy and 4-carbonyl groups in the C-ring of Naringenin plays a role in ROS scavenging, and Cu and Fe ions interaction. Naringenin also restores mitochondrial membrane potential, reducing mitochondrial dysfunction and subsequent apoptotic cascade [41].This finding confirms the fact that antioxidant supplementation may be useful adjuvants in the treatment of HAART-induced toxicities [12, 18].

Contrary to the report by Ranawat and Bakshi[25], Naringenin improved the histomorphology and morphometry of the testis which may be attributed to the differences in the route of administration. Whereas, Naringenin was administered intraperitoneally by Ranawat and

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Bakshi[25], in this study Naringenin was administered orally which probably reduced tissue absorption rate and the concentrations of total naringenin in the testis [42]. However, at a higher dose (80 mg/kg), the co-treatment with HAART had obvious perturbations on the seminiferous tubule thickness and the expression of testicular 3β HSD resulting in narrowed seminiferous tubule and hypocellularity in interstitial cells. The biphasic nature of Naringenin depends on a host of biological and chemical reactions occurring *in vivo* which may necessitate strong antioxidant or pro-oxidant properties depending on the physiological milieu [43].

In conclusion, HAART is deleterious to the biosynthesis of testicular 3β HSD thereby limiting spermatogenesis, however a co-treatment with a lower dose (40 mg/kg) of Naringenin increases testicular 3β HSD expression and enhances spermatogenesis. Thus, life is more precious than any "devastating" drug effect, it is a moot point contemplating the withdrawal of HAART due to its related toxicities. Rather, our findings suggest co-treatment with Naringenin may be a potential adjuvant to improve on the treatment/management of HIV.

ACKNOWLEDGEMENTS

The College of Health Sciences, University of KwaZulu-Natal is acknowledged for Operational funds to Postgraduate student Adana MY and Postdoc fellow Akang EN. The technical support from Prof T. Naicker and D. Margolis of Optics and Imaging Centre, and Drs. Sanil D Singh and Linda Bester of Biomedical Resource Unit, University of KwaZulu-Natal is appreciated. The South African National Research Foundation Grant U99053 to Senior author OOA is acknowledged.

CONFLICTS OF INTEREST: None

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Figure legends

Figure 1: Photomicrographs of testis (H and E stains). Showing germinal epithelium (GE), interstitial cells of Leydig (arrow heads), Sertoli cells (arrows) and spermatozoa in lumen (L). Note the ballooning of the interstitium of HAART and HNar80 groups with sparse Leydig cells and narrowed/constricted seminiferous tubules Magnification of x200.

Figure 2: Photomicrographs of testis showing immunohistochemistry of 3 beta hydroxysteroid dehydrogenase (3β HSD) in the interstitium of testis (arrow heads) and perturbations (arrows) in the seminiferous tubule of HAART group. Magnification of x400

Figure 3: Bars represent mean area percentage of 3 beta hydroxysteroid dehydrogenase (3β HSD) immunohistochemical stains in the interstitium of the testis. $^{*}p<0.05$ compared to control, $^{\alpha}p<0.05$ compared to HAART.

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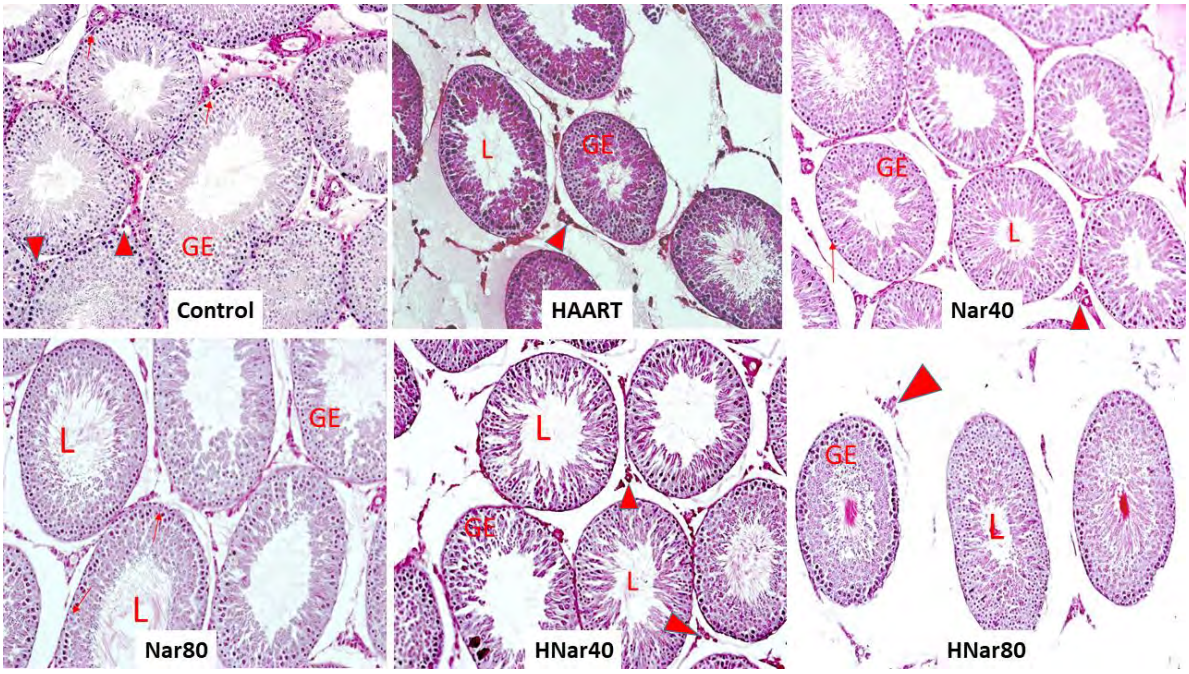
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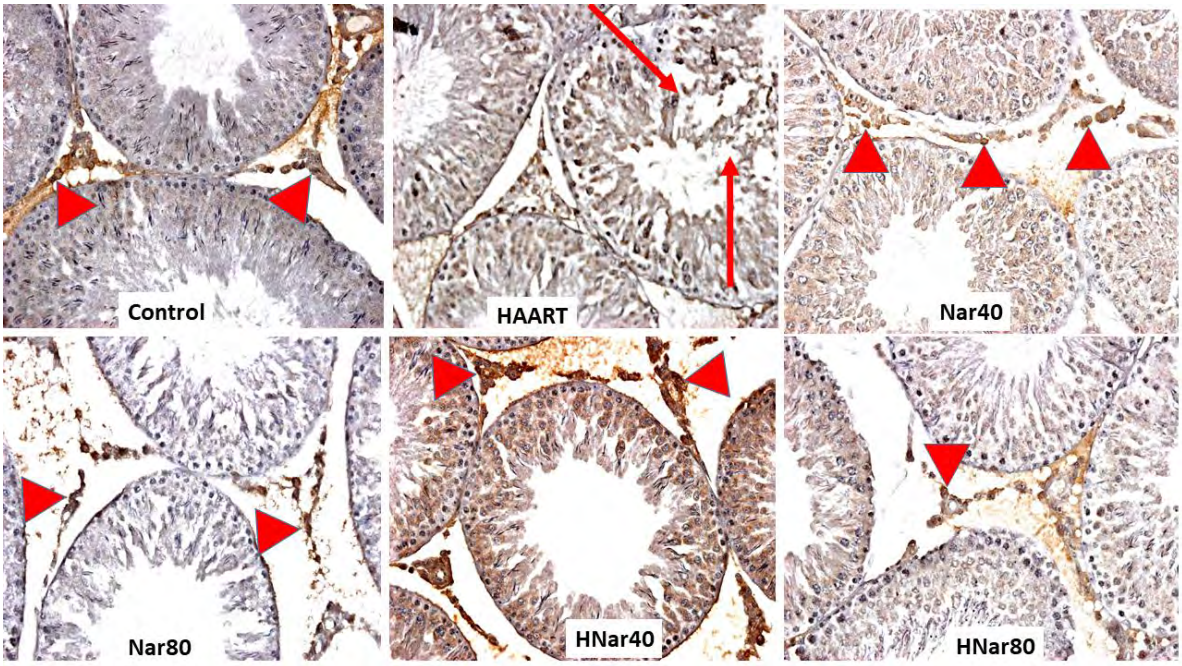
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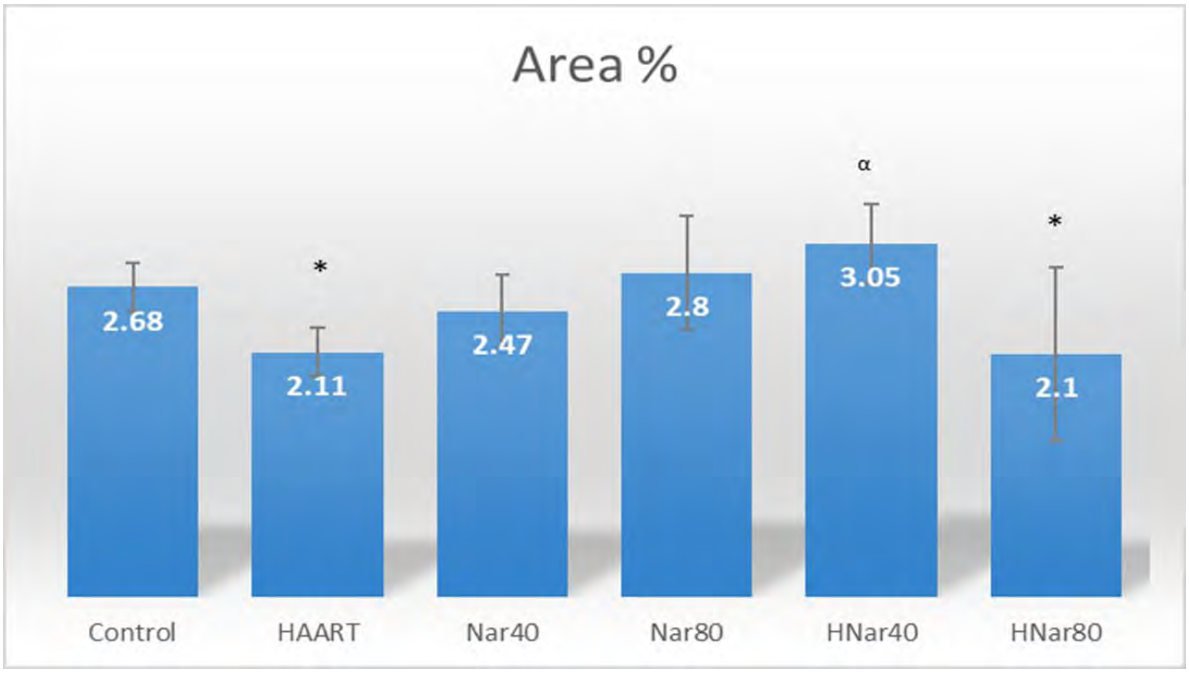
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BRIDGING

BETWEEN CHAPTERS FIVE AND SIX

The findings of earlier chapters have all revealed the beneficial properties of Naringenin, a bioactive flavonoid when co-administered with antiretroviral therapy. The next chapter summarises the findings and suggested recommendations.

6 CHAPTER SIX

SYNTHESIS, CONCLUSION, RECOMMENDATION

6.1 Synthesis

The overall aim of this study is to determine the impacts of the recently approved first line antiretroviral therapy for adults FDC, TDF/FTC/EFV on the testes and explore the role of Naringenin, a potent antioxidant in alleviating testicular perturbations induced by HAART. An analysis of the effects of Naringenin on HAART-induced alteration in semen parameters, hormonal changes, oxidative imbalance, histochemical changes, cytoarchitectural changes in the testis using histomorphometric indices including volume fraction of seminiferous epithelium, tubular lumen, intersitium as well as ultrastructural changes were considered. As noted in the existing body of literature, studies from our laboratory have suggested the deleterious effects of ARVs on the testes using the older generation antiretroviral combinations. Azu et al. (2014), described the deleterious histopathological changes in the testes; changes observed include tubular atrophy with altered morphometric indices. Also reported were significant decline in sperm motility, decline in epithelial height closely mirrored by extensive reticulin framework and positive PAS cells (Ogedengbe et al., 2016, Ismail et al., 2017). Even though this study employed the more recent drugs in the HIV management guideline that are considered safer, it was shown that they are not completely devoid of adverse effects. Thus corroborating the findings of previous research (Ogedengbe et al., 2016, Ismail et al., 2017). Tenofovir, Emtricitabine and Efavirenz (TDF/FTC/EFV) combination is the current first line therapy for persons being commenced on HAART. This informed the choice of HAART and the duration of treatment in this two-phase study. Despite the success in reduction of morbidity and mortality achieved during this HAART era, the

population of persons on HAART is increasing. Most of these individuals are in their reproductive years and desire their own biological children (Basu, 2007). Using this combination of HAART, there was distortion of the seminiferous tubular epithelium with dead immotile multinucleated cells in the lumen. The diameter of the seminiferous tubules were also severely decreased. These were observed following both short term (28 days) and long term (10 weeks) use of TDF/FTC/EFV combination. The evidence of spermatogonic cells without spermatozoa in the lumen of the seminiferous tubules is suggestive of unsuccessful spermiation (Beardsley and O'Donnell, 2003). These findings clearly support the literature insofar as there is a strong positive link between use of HAART and testicular microanatomical changes. An intact seminiferous epithelium and interstitial tissue in the testes is required for optimal steroidogenesis and hence spermatogenesis.

In order to delineate the mechanism of testicular injury, the study investigated the effects of HAART on Sperm mitochondrial DNA (mtDNA) and progressive sperm motility. The spermatozoa have been shown to contain numerous mitochondria necessary to provide energy required for progressive motility (van Leeuwen E, 2008). An increase in the sperm DNA tail length observed was suggestive of mtDNA fragmentations. The mitochondria powers sperm motility and underlies a successful spermiation. This was reflected in the reduction of percentage of progressively motile sperm in the semen analysis. Tenofovir has been reported to cause renal proximal tubular ultrastructural defects and depleted mtDNA in that specialized tissue (Kohler et al., 2009). White et al (2001), suggested that the proportion of patients with mitochondrial DNA (mtDNA) deletions was significantly greater in those patients who had taken HAART for more than 12 months compared with those who had taken HAART for less than 12 months (White et al., 2001). Some scholars have hinged the decrease

in progressive sperm motility on the relationship between HAART induced toxicity and mitochondrial damage (Soriano et al., 2008, Kohler et al., 2009, Patil et al., 2015).

The deleterious effects were extended to other semen parameters revealing a reduction in sperm count as well as an increase in the percentage of sperms with abnormal morphology. The sperm count of animals treated with HAART decreased significantly to less than 1% of those observed in controls, and the motile sperm cells had comparatively decreased by 70% in the HAART-treated group. Similarly, the sperm morphology showed a marked increase in abnormalities especially tail abnormalities. This is in tandem with the observations of researchers who used a different combination of HAART (Ogedengbe et al., 2017a, Ogedengbe et al., 2017b, Ismail et al., 2017, Jegede et al., 2017, Azu et al., 2014b). In 2011, Nicopoullos and his colleagues postulated that HAART significantly decreases total sperm count, progressive motility, and increases the number of abnormal forms (Nicopoullos et al., 2011). A clear pointer to the biochemical property of the class of ARV being used and not necessarily individual drugs as the culprit in this menace.

The mechanism by which HAART induces organ injury particularly testicular toxicity may likely be due to cascade of events involving the oxidant-antioxidant pathway (Azu et al., 2014a, Azu, 2012; Olaniyan, 2015). Earlier studies have reported that increased activities of reactive oxygen species (ROS) overwhelms tissue`s total antioxidant status leading to oxidative stress (Aitken 2011; Aitken, 2008; Aitken, 2007). Oxidative stress leads to increased lipid peroxidation indicated by malondialdehyde (MDA) markers, deleterious to the unsaturated fatty acid rich membrane of the spermatozoa (Oremosu and Akang, 2015). In order to further understand the ongoing events at cellular levels, an analysis of testicular antioxidant activity was carried out. HAART was associated with a reduction in the activity

of testicular antioxidant enzymes. The activities of glutathione peroxidase decreased while that of catalase displayed a marginal decline. Due to the delicate balance in the oxidant-antioxidant system in the testes and its role in spermatogenesis, an abnormal semen parameter is expected. This has been proposed to underlie the mitochondrial DNA fragmentation and hence decline in normal process of spermiation.

Excessive accumulation of free radical production follow a reduced activity of antioxidant enzymes. This obstructs the biosynthesis of testosterone essential for the proper functioning of the Sertoli-Sertoli tight junction which constitutes part of the blood-testis-barrier, and normal spermatogenesis (Aly et al., 2017). A shortage of this steroid hormone will be counterproductive on spermatogenesis, which is unlikely to progress beyond diplotene spermatocytes stage (Smith et al., 2015, De Gendt et al., 2004, Tsai et al., 2006). The implication thereof, corroborates with our previous study which showed that HAART decreased sperm count, sperm motility and sperm morphology and increased sperm DNA fragmentations.

Using transmission electron microscopic studies, this study reports alteration of the normal ultrastructure of the testes following HAART use. This is evidenced by degeneration of nuclear membranes, an altered and disordered cellular progression in the germinal epithelium as well as irregular contouring and thickening of the basement membrane. A result of cellular infiltration of the basement membrane. This has been shown to be associated with tubular degeneration and impedance of metabolic exchange between the germinal epithelium and the interstitium (Amat et al., 1985). The impaired metabolic exchange is frequently accompanied by subsequent gonadotrophin deficiencies, a typical finding in crypto-orchidism (Hadziselimović, 1977, Hadžiselimović, 1982). Similar pathology has been noted in other

atrophic testes with a different aetiology (Neaves, 1977). The altered exchange of materials within the testicular tissue with further induced basement membrane infiltration and hence a continuous cycle of basement membrane thickening. Also observed is the alteration in the normal progression of the cells in the seminiferous germinal epithelium. This was demonstrated by the presence of elongating spermatids being retained and resorbed into basilar Sertoli cell cytoplasm within the basal compartment. The spermatogonic cells along the basement membrane were observed to be scanty following the use of HAART. However the Sertoli cells seem unaffected, there was an associated reduction in the population of primary spermatocytes. The spermatocytes and round spermatids displayed incomplete nuclear membrane. These are indicative of an ongoing degenerative process within the cell. The morphological changes described has been associated with impaired Sertoli cell function (Vidal and Whitney, 2014). Other studies that described the morphologic changes in the rat testes such as spermatid retention and degeneration of cells linked it with decreased intratesticular testosterone levels (Beardsley and O'Donnell, 2003, Troiano et al., 1994). Our study revealed obstructed spermatogenesis and widening of the interstitium, with fewer Leydig cells in HAART treated animals. The implication thus corroborates with our previous study which showed that HAART decreased sperm count, sperm motility and sperm morphology and increased sperm DNA fragmentations.

The activity of this steroidogenic enzyme and regulatory protein 3 beta hydroxysteroid dehydrogenase (3β HSD) is critical for normal steroidogenesis. The findings from this study clearly demonstrates a reduction in the expression of testicular 3β HSD. Moreover, normal steroidogenesis is promoted by the maintenance of normal mitochondrial pH, membrane potential and ATP synthesis is necessary for steroidogenesis (Wang et al., 2015, Park et al., 2014). It is only reasonable to infer that the down regulation of testicular 3β HSD by

TDF/FTC/EFV therapy explored the oxidant-antioxidant pathway. These findings are in tandem with reports by some scholars who reported that the alteration in mitochondrial antioxidants adversely affected 3β HSD expression and Leydig cell steroidogenesis (Jana et al., 2010). Thus, a deficiency of this steroid hormone will be deleterious to optimal spermatogenesis.

The effect on male fertility was further revealed by the reduction in the pregnancy rates in male rats exposed to fertile females when compared to controls. This is the first study to determine the fertility rate in males following long term use of TDF/ETC/EFV using animal models. There are still a lot of puzzles to be unravelled concerning mammalian reproduction and infertility. In human infertility has been recorded in many cases where both partners are normal and healthy. So also, pregnancies have been reported in few cases with abnormal semen parameters and hormonal perturbations. The continual search for answers by researchers in the different related medical fields is the only hope for success.

The co-administration of Naringenin proved beneficial. The protective effects of Naringenin was observed with respect to semen parameters especially in the short term use of HAART. The normal cellular progression was restored and cells of the spermatogenic series were well represented however there persist occasional defects in the continuity of the nuclear membrane of the cells. Kwatra et al (2016) revealed that naringin usage, a closely related flavonoid ameliorated the oxidative stress, proinflammatory cytokines, mitochondrial dysfunction and improved monoamines contents in brain hippocampus of Doxorubicin treated rats (Kwatra et al., 2016). Addition of Naringenin to oseltamivir treatment improved the neurofunctions of the brain through improving the Y maze task and reductions in the pathophysiological effect of oseltamivir. This was attributed to increasing total antioxidant

capacity, brain fatty acid binding proteins FABP7 and Ca-ATPase and reducing oxidative (Hegazy et al., 2016). In the same study Naringenin was proposed to have reduced total nitric oxide, total oxidant capacity and total cytochrome P450 (CYP450) contents (Hegazy et al., 2016).

Some studies have proposed an improvement in the antioxidant activity. Supplementation of Naringenin and its synthetic derivative enhanced antioxidant enzyme activities of erythrocyte and liver in high cholesterol-fed rats by enhancing the antioxidant defence system (Lee et al., 2002). In cisplatin-Naringenin combined treated rats, the activities of superoxide dismutase, glutathione peroxidase, and catalase were significantly increased (Badary et al., 2005).

In spite of this deleterious effects of HAART, the Naringenin co-treated animals especially at a lower dose (40 mg/kg) had an increased expression of testicular 3β HSD compared to those treated with HAART only. Likewise, the histomorphology and morphometry showed complete spermatogenesis with a very conspicuous presence of spermatozoa in lumen of the seminiferous tubules. During oxidative stress, Naringenin chelates ions and scavenge ROS (Jamalan et al., 2016). 5-hydroxy and 4-carbonyl groups in the C-ring of Naringenin play a role in ROS scavenging, as well as copper (Cu) and iron (Fe) ions interaction. Naringenin also restores mitochondrial membrane potential mitigating mitochondrial dysfunction and subsequent apoptotic cascade (Jamalan et al., 2016). This finding confirms the fact that antioxidant supplementation may be useful adjuvants in the treatment of HAART-induced toxicities (Azu, 2012, Jegede et al., 2017).

The study proposed a number of mechanisms for the observed protection from Naringenin. Naringenin possess chelating properties capable of binding to toxic metal ions to form complex structures, which make it easier to eliminate from intracellular or extracellular

spaces. Cavia-Saiz et al (2010) also proposed that Naringenin acts as an active chelator of metallic ions. The ameliorative effects of Naringenin may also be attributed to its chemical structure. The hydroxyl groups present are able to donate electrons and mop up generated free radicals from the altered oxidative status thereby restoring the oxidant-antioxidant balance in the testes. This provides an insight into the mechanism of gonadotoxic effect of antiretroviral therapy.

Other proposed mechanism not investigated by this study include the oestrogen modulating activities and its ability to alter peroxisome proliferator-activated receptor (PPAR) alpha and PPAR gamma signalling by enhancing insulin-stimulated tyrosine phosphorylation and insulin tissue sensitivity (Kuiper et al., 1998, Kannappan and Anuradha, 2010, Goldwasser et al., 2010). Further probe into these properties may expand the present understanding of its adjuvant roles.

6.2 Conclusion

This study reveals that both the short-term and long-term use of HAART may predispose to degeneration of the seminiferous tubules, abnormality of sperm parameters, and increased sperm DNA fragmentations. This places PLWHA at an increased risk of infertility. The study proposes that TDF/FTC/EFV therapy impacts negatively on male fertility by distorting testicular ultrastructure thereby causing Sertoli cell dysfunction and impaired spermatogenesis and steroidogenesis which ultimately reduce fertility in the male rats. A balance of the oxidant-antioxidant status of the testes is necessary for optimal spermatogenesis. Maintaining this poses a challenge in the presence of antiretroviral therapy. This warrants the use of free radical scavenging agents to ensure maintenance of an

equilibrium. Be that as it may, the use of Naringenin promises moderate mitigation of HAART-induced perturbations.

Naringenin's ability to mitigate HAART (FDC- TDF/FTC, EFV) induced testicular changes in the ultrastructural architecture and function of the testes suggests that the observed protection was due to its antioxidant, metal chelating and possibly anti-inflammatory properties, which could present it as a useful adjuvant in the treatment of HIV patients especially those desirous of future fertility.

6.3 Limitations and Recommendations

- The study utilized healthy animal models to demonstrate effects of HAART on male fertility. It will however be more representative of a true life situation if an HIV model is used. This may reveal the possible interaction that may influence tissue response to injury.
- The principle of replacement, refinement and reduction is strictly implemented by the animal house of the Biomedical Resource Unit (BRU) and the Animal Research Ethics committee (AREC) of the University of KwaZulu-Natal. The number of animals per group was limited to five. A larger sample size particularly with regards to the Fertility index would be more statistical validity.
- More extensive research on the long-term use and a probe into the afore-discussed oxidant– antioxidant mechanism of action of HAART and Naringenin on the male fertility axis is encouraged to better understand the activity of HAART on male reproductive health.

- Safer alternatives to the presently used drug combination in the HAART therapy need to be researched and introduced since it is easier to prevent these toxicities than deal with its attendant adverse effect. Antioxidant therapy derived from phytonutrient that possess anti-inflammatory properties may be introduced into the management of HIV at an early stage of the disease to reduce or prevent observed toxicities.

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Appendix I

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

07 September 2016

Dr Misturah Adana (215082673)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Dr Adana,

Protocol reference number: AREC/046/016D
Project title: The Therapeutic potential of Flavonoids on Highly Active Antiretroviral Therapy (HAART)-Induced Testicular-Toxicity

Full Approval – Research Application

With regards to your revised application received on 05 August 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following condition:

CONDITION:

Animals must be weighed daily and doses administered per individual animal weights.

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 07 September 2017.

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

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Onyemaechi Okpara Azu
Cc Acting Academic Leader Research: Dr Michelle Gordon
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Sanil Singh

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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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Appendix II

OVERVIEW OF THE STEPS IN TISSUE PROCESSING FOR PARAFFIN SECTIONS

An Introduction to Tissue Processing by Geoffrey Rolls (Rolls, 2017)

1. Obtaining a fresh specimen

Fresh tissue specimens will come from various sources. It should be noted that they can very easily be damaged during removal from patient or experimental animal. It is important that they are handled carefully and appropriately fixed as soon as possible after dissection. Ideally fixation should take place at the site of removal, perhaps in the operating theatre, or, if this is not possible, immediately following transport to the laboratory.

2. Fixation

The specimen is placed in a liquid fixing agent (fixative) such as formaldehyde solution (formalin). This will slowly penetrate the tissue causing chemical and physical changes that will harden and preserve the tissue and protect it against subsequent processing steps.[2] There are a limited number of reagents that can be used for fixation as they must possess particular properties that make them suitable for this purpose. For example tissue components must retain some chemical reactivity so that specific staining techniques can be applied subsequently.[3] Formalin, usually as a phosphate-buffered solution, is the most popular fixative for preserving tissues that will be processed to prepare paraffin sections. Ideally specimens should remain in fixative for long enough for the fixative to penetrate into every part of the tissue and then for an additional period to allow the chemical reactions of fixation to reach equilibrium (fixation time). Generally this will mean that the specimen should fix for between 6 and 24 hours. Most laboratories will use a fixative step as the first station on their processor.

Following fixation the specimens may require further dissection to select appropriate areas for examination. Specimens that are to be processed will be placed in suitable labelled cassettes (small perforated baskets) to segregate them from other specimens. The duration of the processing schedule used to process the specimens will depend on the type and dimensions of the largest and smallest specimens, the particular processor employed, the solvents chosen, the solvent temperatures and other factors. The following example is based on a six hour schedule suitable for use on a Leica Peloris™ rapid tissue processor.

3. Dehydration

Because melted paraffin wax is hydrophobic (immiscible with water), most of the water in a specimen must be removed before it can be infiltrated with wax. This process is commonly carried out by immersing specimens in a series of ethanol (alcohol) solutions of increasing concentration until pure, water-free alcohol is reached. Ethanol is miscible with water in all

proportions so that the water in the specimen is progressively replaced by the alcohol. A series of increasing concentrations is used to avoid excessive distortion of the tissue.

A typical dehydration sequence for specimens not more than 4mm thick would be:

1. 70% ethanol 15 min
2. 90% ethanol 15 min
3. 100% ethanol 15 min
4. 100% ethanol 15 min
5. 100% ethanol 30 min
6. 100% ethanol 45 min

At this point all but a tiny residue of tightly bound (molecular) water should have been removed from the specimen.

4. Clearing

Unfortunately, although the tissue is now essentially water-free, we still cannot infiltrate it with wax because wax and ethanol are largely immiscible. We therefore have to use an intermediate solvent that is fully miscible with both ethanol and paraffin wax. This solvent will displace the ethanol in the tissue, then this in turn will be displaced by molten paraffin wax. This stage in the process is called "clearing" and the reagent used is called a "clearing agent". The term "clearing" was chosen because many (but not all) clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index. Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration.

A popular clearing agent is xylene and multiple changes are required to completely displace ethanol.

A typical clearing sequence for specimens not more than 4mm thick would be:

1. xylene 20 min
2. xylene 20 min
3. xylene 45 min

5. Wax infiltration

The tissue can now be infiltrated with a suitable histological wax. Although many different reagents have been evaluated and used for this purpose over many years, the paraffin wax-based histological waxes are the most popular. A typical wax is liquid at 60°C and can be infiltrated into tissue at this temperature then allowed to cool to 20°C where it solidifies to a consistency that allows sections to be consistently cut. These waxes are mixtures of purified paraffin wax and various additives that may include resins such as styrene or polyethylene. It should be appreciated that these wax formulations have very particular physical properties

which allow tissues infiltrated with the wax to be sectioned at a thickness down to at least 2 µm, to form ribbons as the sections are cut on the microtome, and to retain sufficient elasticity to flatten fully during flotation on a warm water bath.

Histological waxes such as Paraplast™ are popular and multiple changes are required to completely displace the clearing agent.

A typical infiltration sequence for specimens not more than 4mm thick would be:

1. wax 30 min
2. wax 30 min
3. wax 45 min

6. Embedding or blocking out

Now that the specimen is thoroughly infiltrated with wax it must be formed into a "block" which can be clamped into a microtome for section cutting. This step is carried out using an "embedding centre" where a mould is filled with molten wax and the specimen placed into it. The specimen is very carefully orientated in the mould because its placement will determine the "plane of section", an important consideration in both diagnostic and research histology. A cassette is placed on top of the mould, topped up with more wax and the whole thing is placed on a cold plate to solidify. When this is completed the block with its attached cassette can be removed from the mould and is ready for microtomy. It should be noted that, if tissue processing is properly carried out, the wax blocks containing the tissue specimens are very stable and represent an important source of archival material.

The combined effects of fixation and processing is to harden the tissue and it is inevitable that shrinkage will also occur. It has been estimated that tissues shrink as much as 20% or more by the time they are infiltrated with wax[1]. Notwithstanding these effects, sections prepared from optimally processed tissues will consistently show excellent morphological detail which allows comparisons to be made between specimens and accurate histopathological diagnoses to be determined.

In theory and in practice the paraffin blocks that will be easiest to section contain relatively homogenous tissue of uniform soft consistency (such as kidney), which, when infiltrated with wax, have a consistency similar to that of solidified wax alone (not containing tissue). Tissues of a dense or fibrous nature, or a specimen where both hard and soft tissue are present in discrete layers can pose more of a challenge because parts of them are not so well supported by the solidified wax. Differential shrinkage of the various elements in these blocks during fixation and processing contributes to the problems that might be experienced when they are being sectioned.

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Appendix III

RAT DISSECTION GUIDE

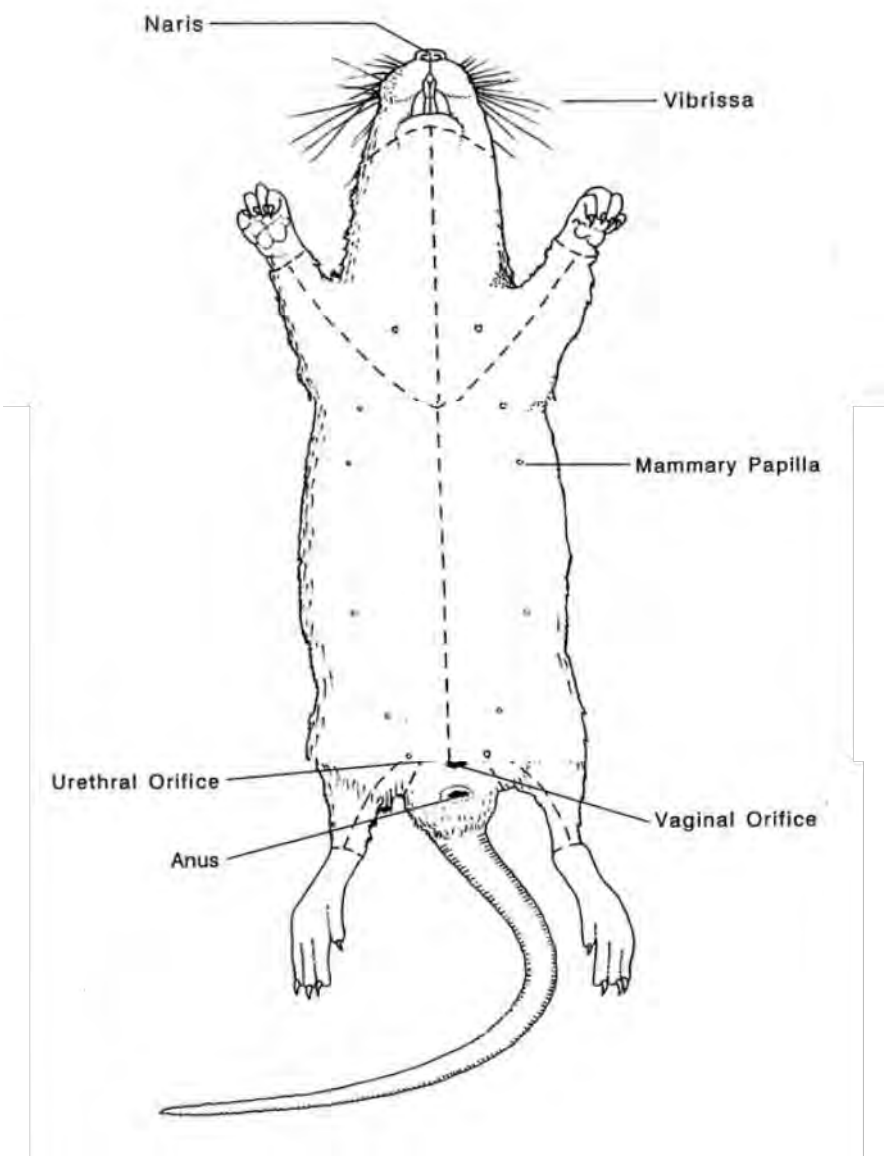
INTRODUCTION

Rats are often used in dissection classes because they are readily available and they possess the typical mammalian body plan. Most of what you learn on the rat is applicable to the anatomy of other mammals, such as humans.

RAT DISSECTION GUIDE

EXTERNAL FEATURES

Refer to the drawing below and identify the indicated structure on the rat prior to skinning.


Naris
Vibrissa
Mammary Papilla
Urethral Orifice
Vaginal Orifice
Anus

Vibrissae - also referred to as the "whiskers". They have a sensory function that allows the animal to

judge the size of an opening that it is about to pass through.

Nares - the nares (plural) or naris (singular) are the external openings into the nasal cavity.

Female urogenital structures

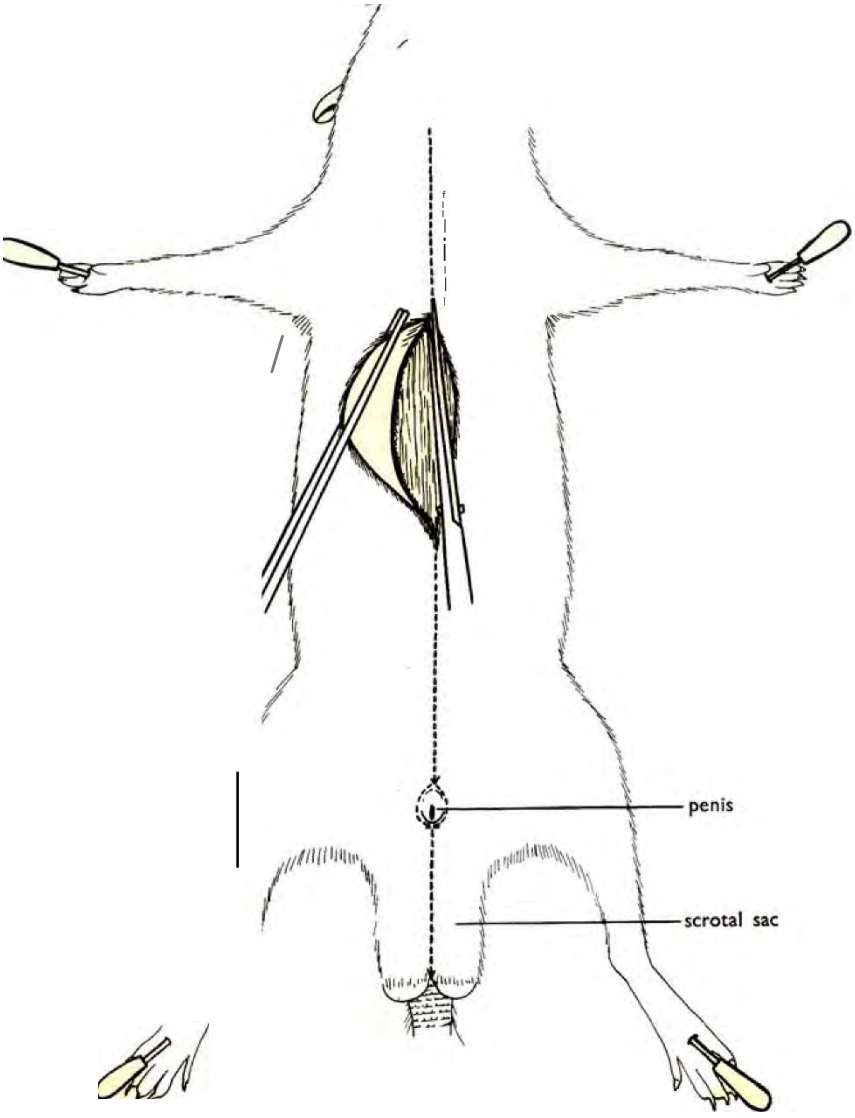
Urethral orifice - is the opening into the urethra (part of the urinary system).

Vaginal orifice - is the opening into the vagina (part of the reproductive system).

Male urogenital structures

Penis - is hidden on the male rat beneath a fold of skin (the foreskin or prepuce).

Scrotum - is a pouch that contains the testes (see the drawing below)



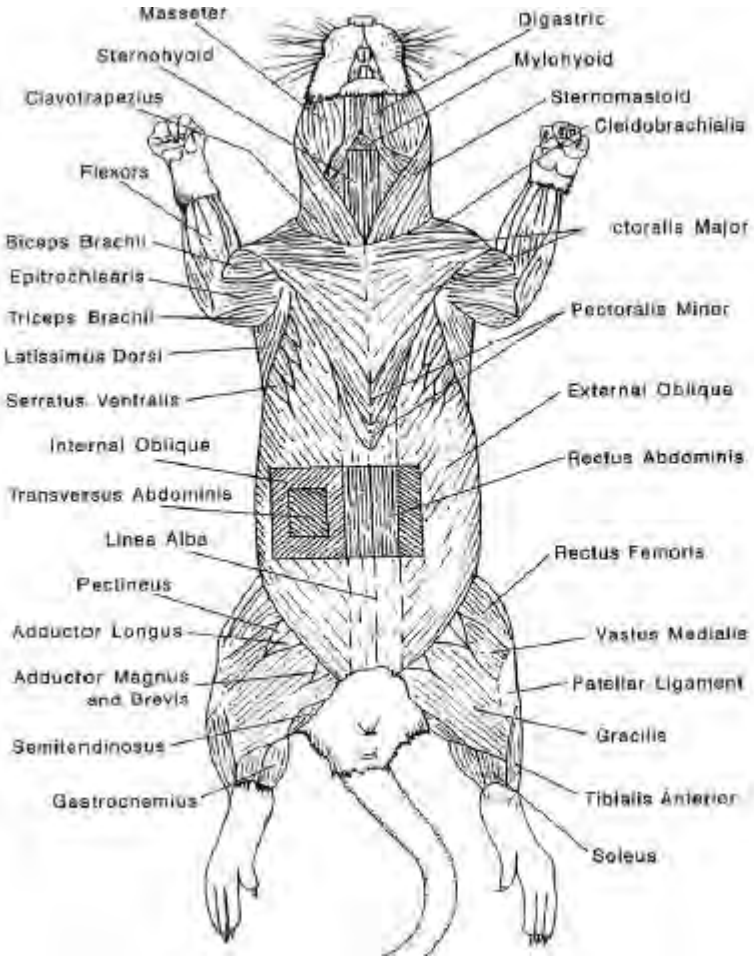
Skinning the rat

Make a midventral incision as illustrated on the drawing above. Use your probe, scissors and finger to free the skin from the underlying tissue. Follow the basic cut pattern illustrated on the first page of this handout.

You should encounter two brownish muscles attached to the skin (the cutaneous maximus in the trunk area, and the platysma in the neck area). You will probably have to cut these muscles close to the skin before the skin can be removed.

MUSCULAR SYSTEM

The diagram below illustrates the muscles of the ventral surface of the rat. Be able to identify those listed. Use the photographs on the following pages and your lab atlas to assist you.



Head and Throat muscles

Digastric - this V shaped muscle follows the lower jaw line. It functions to open the mouth.

Mylohyoid - runs at right angles to the longitudinal axis. You may have to gently raise up the edge of the digastric muscle on each side of the jaw in order to see this muscle. It functions to raise the floor of the mouth.

Sternohyoid - runs parallel to the longitudinal axis on either side of the midline. It functions to pull the hyoid towards the sternum.

Sternomastoid - runs diagonally from the sternum to a point behind the ear. It functions to rotate the head to one side (when one contracts), or rotates the head down (when both contract).

Masseter - this is the "cheek" muscle. It functions in mastication (chewing).

Chest and Front leg (medial) muscles

Pectoralis Major - the large triangular muscle covering the upper thorax. It functions to pull the arm towards the chest.

Pectoralis Minor - part of this muscle runs beneath the Pectoralis Major. It function is the same as the Pectoralis Major.

Biceps Brachii - the large muscle located on inside of the upper arm. It functions to flex (bend) the lower arm.

Epitrochlearis - this is a flat, thin muscle on the medial surface of the upper arm. It functions to extend the lower arm.

Flexors - the collection of small muscles on the medial surface of the lower arm. They function to flex the wrist and hand.

Shoulder and Lateral (outside) Muscles of the Front Leg

Be able to identify the muscles illustrated on the drawing on the next page.

Clavotrapezius - one of a group of three muscles that help to stabilize the scapula. Its function is to pull the clavicle forward.

Acromiotrapezius - pulls the scapula forward. Spinotrapezius - pulls the scapula downward. Latissimus dorsi - pulls the arm downward.

Serratus ventralis - is made up of fingerlike projections of muscle running between the armpit and the ribs. Its function is to depress the scapula.

Cleidobrachialis - runs between the clavicle and the humerus. Its function is to pull the arm forward.

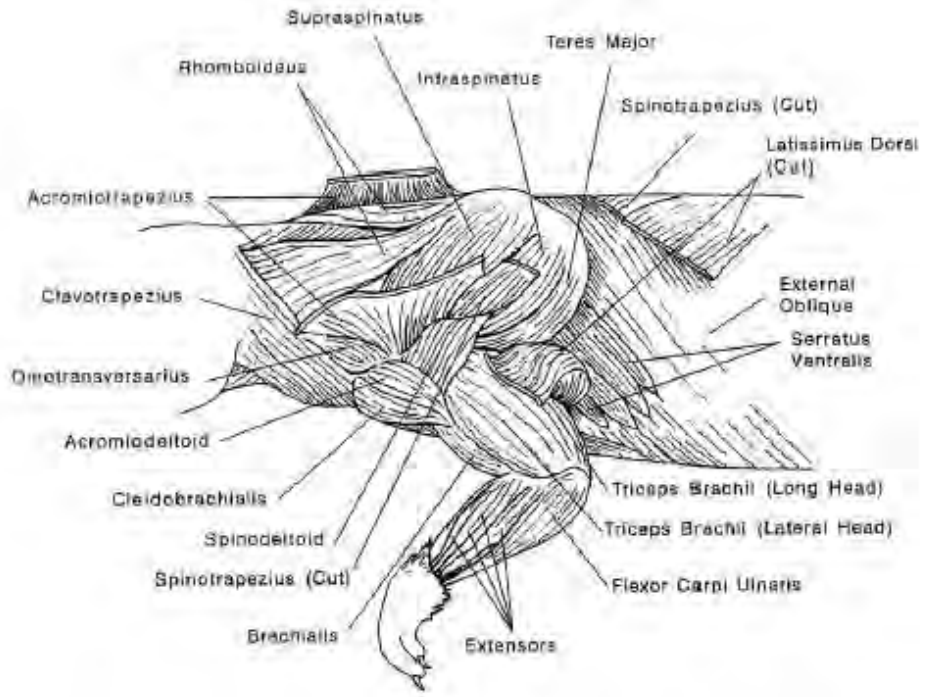
Acromiodeltoid - Runs between the outer clavicle and the scapula. Pulls the upper arm away from the ribs.

Spinodeltoid - runs between the scapular spine and the upper arm. Pulls the scapula away from the ribs.

Triceps brachii - this is a muscle with three heads. It runs between the scapula and humerus and the ulna. Its function is to extend the lower arm.

Brachialis - runs next to the Biceps Brachii. Its function is to flex the lower arm.

Extensors - a group of small muscles on the lateral side of the lower arm. They extend the wrist and hand.



Deep Muscles of the Shoulder

Identify the deep muscles illustrated on the drawing above.

Rhomboideus - runs between the dorsal border of the scapula and vertebral column. Its function is to pull the scapula in and forward.

Supraspinatus - covers the lateral side of the scapula, above the scapular spine and attaches to the upper arm. Its function is to pull the upper arm forward.

Infraspinatus - covers the lateral side of the scapula, below the scapular spine and attaches to the upper arm. Its functions is the rotate the upper arm outwardly.

Teres Major - runs along the outer edge of the scapula to attach to the upper arm. Its function is to rotate the humerus laterally.

Hip and Lateral Muscles of the Hind Leg

Identify the muscles illustrated on the drawing below.

Gluteus Superficialis - covers a large portion of the anterior hip. Its function is to pull the thigh outward.

Biceps Femoris - located posterior to the Gluteus. Its function is to pull the upper leg outward and flex the lower leg.

Semitendinosus - it is located on the posterior margin of the leg and is partly hidden by the Biceps Femoris. Its function is to flex the lower leg.

Gastrocnemius - the major "calf" muscle on the posterior surface of the lower hind leg. Its function is to extend the foot.

Soleus - located under the Gastrocnemius. Its function is to extend the foot.

Tibialis Anterior - located on the anterior surface of the lower hind leg. Its functions is to flex the foot.

Hip and Medial Muscles of the Hind Leg

Be able to identify the following muscles illustrated on the drawing on page 3 of this handout.

Gracilis - a flat muscle on the caudal (near the tail) portion of the inner thigh. Its function is to pull the thigh inward.

Rectus femoris - located on the anterior (front) surface of the thigh. Its function is to extend the lower hind leg.

Vastus medialis - runs parallel to the Rectus femoris on the caudal side. Its function is to extend the lower hind leg.

Adductor Magnus and Brevis - runs parallel to and beneath the Gracilis. Its function is

to pull the thigh towards the body.

Adductor Longus - runs parallel to and beneath the Vastus Medialis. Its function is to pull the thigh towards the body.

Pectineus - located next to the femoral vein. Its function is to pull the thigh towards the body.

Abdominal Muscles

Be able to identify the following muscles illustrated on the drawing on page 3 of this handout.

External Oblique - covers most of the ventral and lateral abdomen. Its function to compress and hold the internal organs in place.

Rectus Abdominis - runs parallel to the longitudinal axis of the body on either side of the linea alba. Its function is to compress and hold the internal organs.

Throat and Oral Cavity

Be able to identify the structures illustrated on the drawing below. The structures indicated are components of a variety of organ system.

Parotid Gland - the major salivary gland. Its function is to secrete saliva containing starch digesting enzymes.

Parotid duct - empties saliva from the Parotid Gland into the oral cavity.

Mandibular gland - a salivary gland that secretes a thick mucous.

Sublingual gland - a small salivary gland that empties into the oral cavity behind the lower incisors.

Lymph Nodes - part of the immune system.

Lacrimal Gland - secretes lacrimal fluid (tears) to lubricate the eye.

The Abdominal Cavity and the Digestive System

Be able to identify the structures illustrated on the drawing below.

Dissection - use scissors to make a midventral cut up the entire length of the abdominal cavity.

When you reach the diaphragm (a broad sheet of muscle that separates the thorax from the abdomen) make lateral cuts along the lower border of the rib cage.

Parietal Peritoneum - a very thin and shiny membrane that lines the inside of the abdominal wall.

Visceral Peritoneum - a very thin and shiny membrane that covers the internal organs of the abdominal cavity.

Mesenteries - folds of the Visceral Peritoneum that attach the small intestine and colon to the posterior abdominal wall.

Liver - is the large brown organ taking up most of the anterior portion of the cavity. I consists of four lobes. Its function is to manufacture bile, selectively remove and reintroduce nutrients into the blood, remove toxins, and manufacture needed proteins and carbohydrates.

Gall Bladder - the rat does not have a gall bladder.

Stomach - a curved bag-like organ right below the diaphragm. Its function is to mechanically grind up the food and start the digestion process.

Esophagus - muscular tube that passes through the diaphragm to empty food into the stomach.

Small Intestine - composed of three major parts. The stomach empties its contents into the first section of the intestine called the Duodemum. The Ileum is the terminal section of the small intestine that connects with the cecum. The middle section (between the duodenum and ileum) is the Jejunum. The small intestine is where most digestion and ingestion occurs.

Large Intestine (Colon) - is shorter and wider than the small intestine. The ileum of the small intestine empties into the first section of the colon, the Cecum. The Vermiform Appendix is a blind-ended sac attached to the cecum. The appendix functions as a lymphatic organ in immunity and often becomes infected in humans.

Rectum - the terminal portion of the colon leading to the anus.

Spleen - is located to the left of the stomach at the end of the pancreas. It functions in immunity.

Pancreas - has a glandular lobular appearance and is attached to the duodenum. It has both a digestive function (it secretes enzymes) and a hormonal function.

Respiratory System

Be able to identify the structures illustrated on the drawing below.

Trachea - air tube with conspicuous rings. Gently move the contents of the thorax around until it can be found.

Bronchus - the trachea divides into a left and right bronchus. Gently move the contents of the thorax around until they can be found. Like the trachea they have conspicuous rings.

Lungs - appear spongy and on either side of the heart. The right lung on the rat has four lobes and the left lung has three. Each lung is covered by a thin layer of tissue called the Visceral Pleura. The inside of the thoracic wall is covered by a similar layer called the Parietal Pleura.

Diaphragm - is a thin sheet of muscle separating the abdomen from the thorax. When contracted it draws air into the lungs.

The Circulatory System

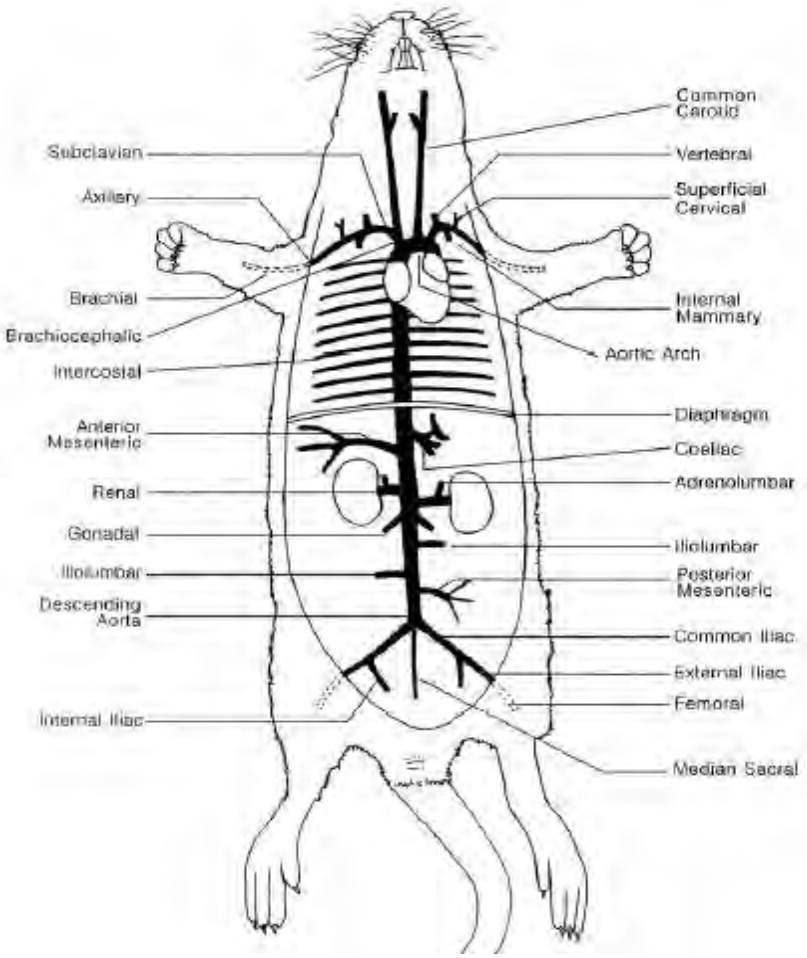
Be able to identify any of the blood vessels illustrated on the drawing above and below that you can find without cutting any internal organs out of your specimen.

Heart - sits in the space between the left and right lungs. Portions of the right and left atria can be seen as dark flaps on top of the heart. The two anterior vessels and one posterior vessel entering the right atrium are the Vena Cavae that return unoxygenated blood to the heart.

Pulmonary Veins can be seen leaving each lung and entering the Left Atrium.

The Ventricles (right and left) can not be differentiated without opening the heart. A Pulmonary Artery can be seen exiting the right ventricle and passing under the Aorta.

Pericardium - is the tough fibrous sack surrounding the heart for protection.



MAJOR ARTERIES

Aorta - is the major artery shown exiting the top of the heart and forming an arch that bends to the left. It empties the left ventricle and supplies oxygenated blood to the entire body.

Brachiocephalic Artery - is the first artery to branch off of the Aorta. It supplies

blood to the head and right arm. This artery splits to from the Right Subclavian Artery that takes blood to the right arm, and the Right Carotid Artery that takes blood to the head.

Left Common Carotid Artery - is the second artery to branch off of the Aorta. It supplies blood to the head.

Left Subclavian Artery - is the third artery to branch off of the Aorta. It supplies blood to the left arm.

Common Iliac Artery - divides to form the Internal Iliac Artery (supplying various organs in the pelvic area) and the External Iliac Artery (passing to the leg).

Femoral Artery - is an extension of the External Iliac Artery located on the medial surface of the thigh.

MAJOR VEINS

Subclavian Vein - as it returns blood from the arm in connects with the Lateral Thoracic Vein, External Iliac Vein, and, Internal Iliac Vein to form the Anterior Vena Cava.

Anterior Vena Cava - enters the right atrium on the dorsal side of the heart.

External Iliac Vein - runs parallel to the External Iliac Artery.

Internal Iliac Vein - runs parallel to the Internal Iliac Artery.

Posterior Vena Cava - formed when the right and left Common Iliac Veins unite. It empties into the right Atrium. The Renal Artery and Iliolumbar Artery drains into it.

Hepatic Portal Vein - takes blood from the digestive organs to the liver. Should be injected with yellow latex.

FEMALE URINARY AND REPRODUCTIVE STRUCTURES

Be able to identify the structures illustrated on the drawing of the Female Urogenital System below.

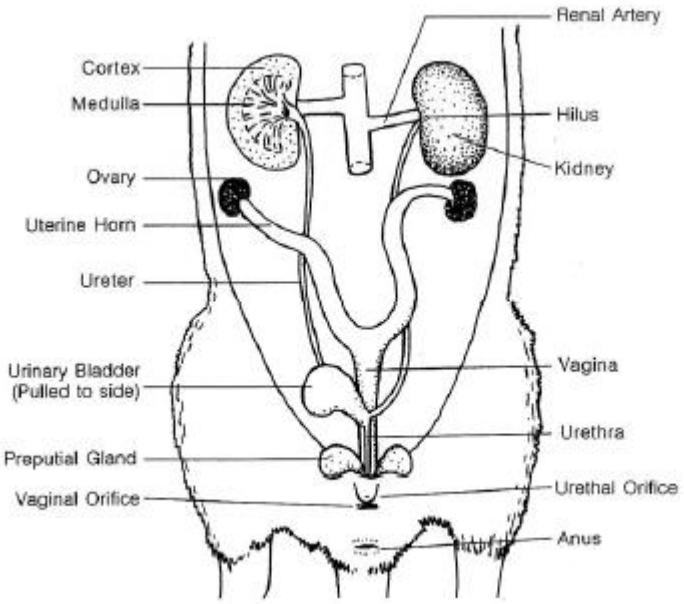
Kidney - can be found close to the dorsal wall of the abdomen surrounded by fat.

Ureter - is a whitish tube extending from each kidney and running to the Urinary Bladder

where urine is stored.

Ovary - is a small nodular gland often found buried in fat near each kidney.

Uterus - in female rats the uterus is split into two **Uterine Horns.** The distal end of each horn is located close to each ovary.



Appendix IV

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Catalase (CAT) Assay Kit

Catalog No: E-BC-K031
Method: Colorimetric method
Specification: 100 Assays

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help.

Phone: 240-252-7368(USA) Fax: 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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It is recommended to take 2~3 samples which expected large difference to do pre-experiment before formal experiment.

Application

This kit can be used to measure CAT activity in animal serum, plasma and tissue homogenate samples. This kit (100 Assays) can detect 50 samples.

Detection principle

The reaction that catalase (CAT) decomposes H_2O_2 can be quickly stopped by ammonium molybdate. The residual H_2O_2 reacts with ammonium molybdate to generate a yellowish complex. CAT activity can be calculated by production of the yellowish complex at 405 nm.

Kit components

Reagent 1: Buffer solution, 60 mL × 2 vials, store at 4℃ for 6 months.

Reagent 2: Substrate, 12 mL × 1 vial, store at 4℃ for 6 months.

Note: Incubate Reagent 1 and Reagent 2 at 37℃ for 10 min before use.

Reagent 3: Chromogenic agent, powder, 1 vial, store at 4℃ for 6 months. Dissolve the powder to 120 mL with double-distilled water. (If there is sediment in the bottom, please directly take the supernatant for test, it will not affect the result). The prepared reagent 3 can be stored at 4℃ for 3 months.

Reagent 4: Clarificant, 12 mL × 1 vial, store at 4℃ for 6 months. It is frozen when cold, please warm it with 37℃ water-bath until clear.

Experimental instrument

Test tube, Micropipettor, Vortex mixer, Centrifuge, Spectrophotometer (405 nm)

Sample preparation

1. **Serum/plasma or Cell supernatant:** Detect the sample directly. It need to centrifuge if serum precipitates existed, otherwise the result will be greatly affected. If the concentration is beyond the linear range, then dilute the sample with saline before detection. There is hemolysis in the serum sample, each sample should be needed a control, if not, you can use double-steamed water as the control.
2. **Tissue:** Mince the tissues to small pieces, then weighed and homogenized in PBS(0.01 M, pH 7.4) on ice, the volume of PBS (mL): the weight of the tissue (g) =9:1. The tissue homogenate is centrifuged at 3000 r/m for 15 min. Collect the supernatant for detect. Determine the concentration of supernatant with BCA or CBB (Coomassie brilliant blue). If the sample is high-fat, each sample should be needed a control, if not, you can use double-steamed water as the control.

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Operation steps

	Control tube	Sample tube
Sample (mL)		a*
Reagent 1(preheated at 37℃) (mL)	1.0	1.0
Reagent 2 (preheated at 37℃) (mL)	0.1	0.1
Mix fully and react accurately at 37℃ for 1 min.		
Reagent 3 (mL)	1.0	1.0
Reagent 4 (mL)	0.1	0.1
Sample (mL)*	a*	

Mix thoroughly, stand for 10 min at room temperature. Set to zero with double-distilled water and measure the OD values of each tube at 405 nm with 0.5 cm diameter cuvette.

Note: 1) If there are no obvious hemolysis and blood fat for serum (plasma) samples, or there is no high- fat in tissue homogenate samples, * can be replaced by double-distilled water for control tube. Control tubes only need 1-2 for each experiment.

2) For Serum or plasma samples, a* is 0.1 mL. For Tissue homogenates, a* is 0.05 mL.

Calculation of results

1. **Calculation formula of CAT activity in serum (plasma)**

Definition: The amount of CAT in 1 mL of serum or plasma that decompose 1 μmol H_2O_2 per second is defined as 1 unit.

CAT activity (*U/mL*)

$$= (OD_{Control} - OD_{Sample}) \times \frac{325^*}{60 \times \text{The volume of sample}}$$

× Dilution factor of sample before tested

Note: * 325 is the reciprocal of slope.

2. **Calculation formula of CAT activity in tissue homogenates**

Definition: The amount of CAT in 1 mg of tissue protein that decompose 1 μmol H_2O_2 per second is defined as 1 unit.

CAT activity (*U/mgprot*)

$$= (OD_{Control} - OD_{Sample}) \times \left(\frac{325^*}{60 \times \text{The volume of sample}}\right)$$

× Dilution factor of sample before tested

÷ Protein concentration of tested sample $(mgprot/mL)$

Note: *325 is reciprocal of slope.

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Technical parameter

1. The sensitivity of the kit is 0.045 U/mL.
2. The intra-assay CV is 3.1% and the inter-assay CV is 5. 1%.
3. The recovery of the kit is 96%.
4. The detection range of the kit is 0.045-25.9 U/mL.

Notes

1. Please operate strictly according to operation procedures.
2. The test tube can be prepared and labeled in advance. After incubating at 37 ℃ for 10 min, add samples and reagent 1, then incubate the test tube at 37 ℃ for 5 min.
3. Dilute the samples to the optimal concentration for detection if the CAT activity of samples exceed the detection range.
4. If serum and plasma samples are hemolysis, it should take control tube for each sample.
5. If tissue samples are high fat, it should take control tube for each sample.

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Appendix V

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Glutathione Peroxidase (GSH-PX) Assay Kit

Catalog No: E-BC-K096
Method: Colorimetric method
Specification: 100 Assays

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help.

Phone: 240-252-7368(USA) Fax: 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

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Application

This kit can be used to measure GSH-PX activity of animal serum, plasma, tissue, cells, cell culture supernatant and other samples. This kit (100 Assays) can detect 48 samples.

Detection significance

Glutathione Peroxidase (GSH-PX) is an important enzyme that catalyzes decomposition of hydrogen peroxide. GSH specifically catalyze the reaction between GSH and hydrogen peroxide, protecting cell membrane structure and keeping membrane function integrity. Se-cysteine is the active center of the GSH-PX. Determination of GSH-PX activity in organism can be an indicator of selenium level as Se is essential section of GSH-PX.

Detection principle

Glutathione Peroxidase (GSH-PX) can promote the reaction of hydrogen peroxide (H_2O_2) and reduced glutathione to produce H_2O and oxidized glutathione (GSSG). The activity of glutathione peroxidase can be expressed by the rate of enzymatic reaction. The activity of glutathione can be calculated by measuring the consumption of reduced glutathione. Hydrogen peroxide (H_2O_2) and reduced glutathione can react without catalysis of GSH-PX, so the portion of GSH reduction by non-enzymatic reaction should be subtracted. GSH can react with dinitrobenzoic acid to produce 5-thio-dinitrobenzoic acid anion, which showed a stable yellow color. Measure the absorbance at 412nm, and calculate the amount of GSH.

Experimental instrument

Test tube
Micropipettor
Vortex mixer
Centrifuge
37℃ water bath/gas bath
Spectrophotometer (412 nm)

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Kit components

Item	Component	Specifications	Storage
Reagent 1	Stock solution	2 mL × 1 vial	4℃, 6 months
Reagent 1 application solution: dilute the Reagent 1 with double distilled water at ratio of 1: 99. Prepare fresh solution before use. Store at 4℃.			
Reagent 2	Acid reagent	60 mL× 4 vials	4℃, 6 months
Reagent 3	Phosphate powder	Powder×1 vial	4℃, 6 months
Reagent 3 application solution: dissolve the powder with 120 mL double distilled water.			
Reagent 4	DTNB solution	30 mL× 1 vial	4℃, 6 months (shading light)
Reagent 5	Salt reagent	Powder×4 vials	4℃, 6 months (shading light)
Reagent 5 application solution: dissolve each vial of powder with 10 mL double distilled water. Store at 4℃ for 1 month with shading light.			
Reagent 6	GSH Standard	3.07 mg × 3 vials	4℃, 6 months
Reagent 7	GSH Standard stock diluent	6 mL × 1 vial	4℃, 6 months
Standard diluent: Reagent 7: Double distilled water=1:9. Prepare fresh solution before use.			
1 mmol/L GSH standard solution: dissolve a vial of GSH standard with Standard diluent to a final volume of 10 mL before use and mix fully. Prepare fresh solution before use.			
20 μmol/L GSH standard solution: dilute 1 mmol/L GSH standard solution with Standard diluent at ratio of 1: 49 and mix fully.			

Note: Bring all reagents to room temperature before use.

Sample preparation

1. **Serum (Plasma):**

   Detect the sample directly. If the concentration is beyond the linear range, then dilute the sample with normal saline before detection.

2. **10% tissue homogenate:**

   Accurately weigh the tissue weight, add 9 times the volume of homogenized medium according to the ratio of Weight (g): Volume (mL) =1:9. Mechanical homogenate the sample in ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant for detection. Meanwhile, determine the concentration of supernatant (E-BC-K318, E-BC-K168, E-BC-K165).

3. **Cell sample:**

   Collect the 1×10^6 cells and centrifuge at 1000~1500 g for 10 min. Discard the supernatant and keep the cell sediment. Add 300~500 μL of PBS (0.01 M, pH 7.4) to prepare cell suspension. Sonicate in ice water bath (power: 300 W, 3~5 second/time, interval for 30 seconds, repeat for 3~5 times) or grind with hand-operated. Centrifuge at 10000 g for 10 min, then take the supernatant for detection. Meanwhile, determine the concentration of supernatant (E-BC-K318, E-BC-K168, E-BC-K165).

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Pre-experiment

1. Determine optimal dilution factor of samples before formal experiment. Samples are diluted into difference concentration with ddH_2O, then take the pre-experiment according to the operation procedures. Calculate the inhibition ratio of serial diluent, and choose the optimal dilution factor when inhibition ratio in the range of 45%~55%.

   $$\text{Inhibition ratio}=\frac{OD_{\text{Non-enzyme tube}}-OD_{\text{Enzyme tube}}}{OD_{\text{Non-enzyme tube}}}\times 100\%$$

2. The optimal dilution factor of sample are different for different species, the GSH-PX also are different for different samples. So it is best to do a preliminary experiment to determining optimal dilution factor for a new sample.

3. It is best to reserve 3 paralleled tubes with different sampling volumes in preliminary experiment for determining the optimal sampling volume. For example,
   Tissue homogenate: Pipet 200 μL of 5%, 1%, 0.5%, 0.4%, 0.3%, 0.1% tissue homogenate supernatant respectively for the assay.
   Serum: Dilute serum samples with normal saline at ratio of 1:1, 1:4, 1:7, 1:9 respectively. Then pipet 100 μL of undiluted and diluted serum samples for the assay.

4. Adjust dilution factor: If inhibition ratio > 60%, need to further dilute the sample then process the test. If inhibition ratio < 10%, need to increase sample concentration.

Operation steps

1. **For tissue, cells and cell culture supernatant sample:**

1) **Enzymatic reaction** (Reagent 1 application solution are pre-warmed in advance at 37℃)

	Non-enzyme tube	Enzyme tube
1 mmol/L GSH (mL)	0.2	0.2
Sample (mL)		0.2
Pre-heat the tubes at 37℃ water bath for 5 min. Preheat Reagent 1 application solution at 37℃ for 5 min at the same time.		
Reagent 1 application solution (mL)	0.1	0.1
React at 37℃ water bath for 5 min accurately.		
Reagent 2 (mL)	2	2
Sample (mL)	0.2	

Mix fully and centrifuge at 3100 g for 10 min, then take 1 mL of supernatant for chromogenic reaction.

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2) Chromogenic reaction

	Blank tube	Standard tube	Non-enzyme tube	Enzyme tube
GSH standard application solution (mL)	1			
20 μmol/L GSH standard solution (mL)		1		
Supernatant (mL)			1	1
Reagent 3 application solution (mL)	1	1	1	1
Reagent 4 (mL)	0.25	0.25	0.25	0.25
Reagent 5 application solution (mL)	0.05	0.05	0.05	0.05

Mix fully and stand for 15 min at room temperature. Set to zero with double-distilled water and measure the OD values of each tube at 412 nm with 1 cm diameter cuvette.

2. For serum/plasma sample:

1) Enzymatic reaction (Reagent 1 application solution are pre-warmed in advance at 37℃)

	Non-enzyme tube	Enzyme tube
1mmol/L GSH (mL)	0.2	0.2
Sample (mL)		0.1
Pre-heat the tubes at 37℃ water bath for 5 min. Preheat Reagent 1 application solution at 37℃ for 5 min at the same time.		
Reagent 1 application solution (mL)	0.1	0.1
React at 37℃ water bath for 5 min accurately.		
Reagent 2 (mL)	2	2
Sample (mL)	0.1	

Mix fully and centrifuge at 3100 g for 10 min, then take 1 mL of supernatant for chromogenic reaction.

2) Chromogenic reaction

	Blank tube	Standard tube	Non-enzyme tube	Enzyme tube
GSH standard application solution (mL)	1			
20 μmol/L GSH standard solution (mL)		1		
Supernatant (mL)			1	1
Reagent 3 application solution (mL)	1	1	1	1
Reagent 4 (mL)	0.25	0.25	0.25	0.25
Reagent 5 application solution (mL)	0.05	0.05	0.05	0.05

Mix fully and stand for 15 min at room temperature. Set to zero with double-distilled water and measure the OD values of each tube at 412 nm with 1 cm diameter cuvette.

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Calculation of results

1. **For tissue sample:**

   Definition: at 37℃, decreasing 1 μmol/L GSH concentration with deducting the effect of non-enzyme reaction for each 1 mg of protein per minute is defined as 1 activity unit.

   GSH-PX activity in tissue

   $$= \frac{OD_{Non-enzyme\ tube} - OD_{Enzyme\ tube}}{OD_{Standard} - OD_{Blank}}$$

   × Concentration of standard (20 μ*mol/L*) × Dilution factor (5)

   ÷Reaction time÷ (Protein concentration of sample × The volume of sample)

2. **For serum/plasma sample:**

   Definition: at 37℃, decreasing 1 μmol/L GSH concentration with deducting the effect of non-enzyme reaction for each 0.1 mL of serum/plasma in 5 min is defined as 1 activity unit.

   GSH-PX activity in serum/plasma

   $$= \frac{OD_{Non-enzyme\ tube} - OD_{Enzyme\ tube}}{OD_{Standard} - OD_{Blank}}$$

   × Concentration of standard (20 μ*mol/L*) ×Dilution factor (6)

   × Dilution factor of sample before tested

3. **For cell sample:**

   Definition: at 37℃, decreasing 1 μmol/L GSH concentration with deducting the effect of non-enzyme reaction for 1 mg of protein per minute is defined as 1 activity unit.

   GSH-PX activity in cell sample

   $$= \frac{OD_{Non-enzyme\ tube} - OD_{Enzyme\ tube}}{OD_{Standard} - OD_{Blank}}$$

   × Concentration of standard (20 μ*mol/L*)× Dilution factor (5)

   ÷Reaction time÷ (Protein concentration of sample × The volume of sample)

4. **For cell culture supernatant sample:**

   Definition: decreasing 1 μmol/L GSH concentration with deducting the effect of non-enzyme reaction for each 0.1 mL of cell culture supernatant (react at 37℃ for 5 min) is defined as 1 activity unit.

   GSH-PX activity in cell culture supernatant

   $$= \frac{OD_{Non-enzyme\ tube} - OD_{Enzyme\ tube}}{OD_{Standard} - OD_{Blank}}$$

   × Concentration of standard (20 μ*mol/L*)× Dilution factor (5)

   × Dilution factor of sample before tested÷2*

   2*: The volume of cell culture supernatant in the definition is 0.1 mL and the volume of cell culture supernatant in operation step is 0. 2 ml

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Technical parameter

1. The sensitivity of the kit is 12.65 U.
2. The intra-assay CV is 4.9% and the inter-assay CV is 9.3%.
3. The recovery of the kit is 105%.
4. The detection range of the kit is 12.65-387 U.

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Appendix VI

Kit components & Storage

An unopened kit can be stored at 4°C for 1 month. If the kit is not used within 1 month, store the items separately according to the following conditions once the kit is received.

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	8 wells ×12 strips	-20°C, 6 months
Reference Standard	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 120 μL	
Concentrated HRP Conjugate (100×)	1 vial, 120 μL	-20°C(shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	4°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	4°C(shading light)
Stop Solution	1 vial, 10 mL	4°C
Plate Sealer	5 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.
The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

Microplate reader with 450 nm wavelength filter

High-precision transfer pipette, EP tubes and disposable pipette tips

Incubator capable of maintaining 37°C

Deionized or distilled water

Absorbent paper

Loading slot for Wash Buffer

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(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Rat LH(Luteinizing Hormone) ELISA Kit

Synonyms: Lutropin, Lutrophin

Catalog No : E-EL-R0026
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

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Intended use

This ELISA kit applies to the in vitro quantitative determination of Rat LH concentrations in serum, plasma and other biological fluids.

Specification

- Sensitivity: 0.94 mIU/mL
- Detection Range: 1.56-100 mIU/mL
- Specificity: This kit recognizes Rat LH in samples. No Significant cross-reactivity or interference between Rat LH and analogues was observed.
- Repeatability: Coefficient of variation is < 10%.

Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat LH. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat LH and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat LH, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat LH. You can calculate the concentration of Rat LH in the samples by comparing the OD of the samples to the standard curve.

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Note

1. Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
2. A freshly opened ELISA Plate may appear to have a water-like substance, which is normal and will not have any impact on the experimental results.
3. Do not reuse the diluted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.
4. The microplate reader should have a 450(±10 nm) filter installed and a detector that can detect the wavelength. The optical density should be within 0~3.5.
5. Do not mix or use components from other lots.
6. Change pipette tips in between adding standards, in between sample additions, and in between reagent additions. Also, use separate reservoirs for each reagent.

Sample collection

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 min at 1000×g at 2~8°C. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2~8°C within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for ELISA assay!

Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 10min at 1500×g at 4°C. Remove the cell fragments, collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000×g to get the supernatant.

Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2~ 8°C. Collect the supernatant to carry out the assay.

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Note for sample:

1. Samples should be assayed within 7 days when stored at 4°C, otherwise samples must be divided up and stored at -20°C (≤1 month) or -80°C (≤3 months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility of causing a deviation due to the introduced chemical substance.
5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

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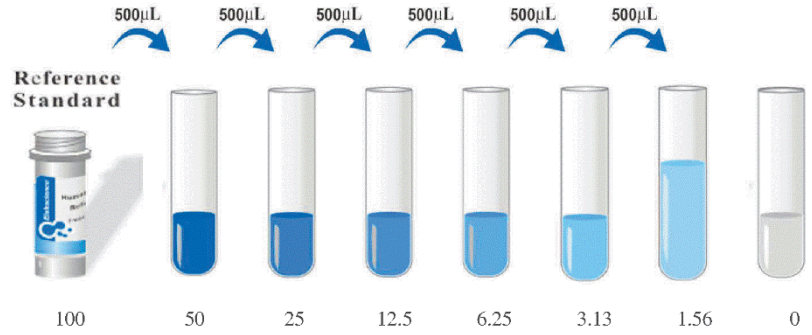
Reagent preparation

1. Bring all reagents to room temperature (18~25°C) before use. Follow the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.Note: if crystals have formed in the concentrate, warm it in a 40°C water bath and mix it gently until the crystals have completely dissolved
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 100 mIU/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0 mIU/mL.
   Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 100 mIU/mL working solution to the first tube and mix up to produce a 50 mIU/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

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Assay procedure (A brief assay procedure is on the 11th page)

1. Add the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Remove the liquid out of each well, do not wash. Immediately add 100 μL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.
3. Aspirate or decant the solution from each well, add 350 uL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 μL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 90 μL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
7. Add 50 μL of **Stop Solution** to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

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Calculation of results

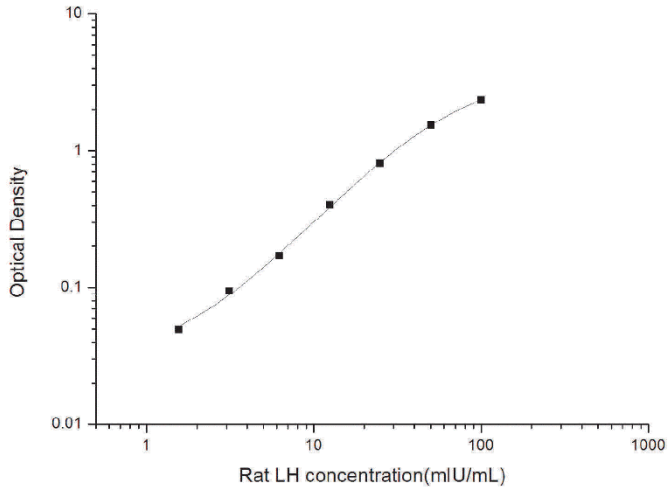
Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

Concentration(mIU/mL)	100	50	25	12.5	6.25	3.13	1.56	0
OD	2.429	1.618	0.888	0.479	0.246	0.17	0.125	0.076
Corrected OD	2.353	1.542	0.812	0.403	0.17	0.094	0.049	-



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Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid range and high level Rat LH were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid range and high level Rat LH were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean(mIU/mL)	4.70	15.40	40.80	5.00	15.10	38.80
Standard deviation	0.20	0.70	1.70	0.30	0.90	1.60
C V (%)	4.26	4.55	4.17	6.00	5.96	4.12

Recovery

The recovery of Rat LH spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	91-105	99
EDTA plasma (n=5)	93-105	98

Linearity

Samples were spiked with high concentrations of Rat LH and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma(n=5)
1:2	Range (%)	85-97	95-108
	Average (%)	91	101
1:4	Range (%)	98-116	82-96
	Average (%)	106	88
1:8	Range (%)	101-113	82-93
	Average (%)	107	87
1:16	Range (%)	95-108	86-97
	Average (%)	102	91

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Troubleshooting

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by gentle mixing.
	Wells are not completely aspirated	Completely aspirate wells in between steps.
Low signal	Insufficient incubation time	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB, rapid coloring.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader.
		Open the Microplate Reader ahead to pre-heat.
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of target protein is too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution is not added	Stop solution should be added to each well before measurement.

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SUMMARY

1. Add 100 μL standard or sample to each well. Incubate for 90 min at 37°C.
2. Remove the liquid. Add 100 μL Biotinylated Detection Ab. Incubate for 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 μL HRP Conjugate. Incubate for 30 min at 37°C.
5. Aspirate and wash 5 times.
6. Add 90 μL Substrate Reagent. Incubate for 15 min at 37°C.
7. Add 50 μL Stop Solution. Read at 450 nm immediately.
8. Calculation of results.

Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operational skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions!
4. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from the above reasons, too.

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Appendix VII

Elabscience®

7th Edition, revised in April, 2017

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Rat FSH(Follicle Stimulating Hormone) ELISA Kit

Synonyms: FSH

Catalog No : E-EL-R0391
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label on the side of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

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Intended use

This ELISA kit applies to the in vitro quantitative determination of Rat FSH concentrations in serum, plasma and other biological fluids.

Specification

●Sensitivity: 1.88 ng/mL

●Detection Range: 3.13-200 ng/mL

●Specificity: This kit recognizes Rat FSH in samples. No Significant cross-reactivity or interference between Rat FSH and analogues was observed.

●Repeatability: Coefficient of variation is < 10%.

Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat FSH. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat FSH and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat FSH, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat FSH. You can calculate the concentration of Rat FSH in the samples by comparing the OD of the samples to the standard curve.

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Kit components & Storage

An unopened kit can be stored at 4°C for 1 month. If the kit is not used within 1 month, store the items separately according to the following conditions once the kit is received.

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	8 wells ×12 strips	-20°C, 6 months
Reference Standard	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 120 μL	
Concentrated HRP Conjugate (100×)	1 vial, 120 μL	-20°C(shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	4°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	4°C(shading light)
Stop Solution	1 vial, 10 mL	4°C
Plate Sealer	5 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.
The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

Microplate reader with 450 nm wavelength filter

High-precision transfer pipette, EP tubes and disposable pipette tips

Incubator capable of maintaining 37°C

Deionized or distilled water

Absorbent paper

Loading slot for Wash Buffer

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Note

1. Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
2. A freshly opened ELISA Plate may appear to have a water-like substance, which is normal and will not have any impact on the experimental results.
3. Do not reuse the diluted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.
4. The microplate reader should have a 450(±10 nm) filter installed and a detector that can detect the wavelength. The optical density should be within 0~3.5.
5. Do not mix or use components from other lots.
6. Change pipette tips in between adding standards, in between sample additions, and in between reagent additions. Also, use separate reservoirs for each reagent.

Sample collection

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 15 min at 1000×g at 2~8°C. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2~8°C within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for ELISA assay!

Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 10min at 1500×g at 4°C. Remove the cell fragments, collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogenizer on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000×g to get the supernatant.

Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2~ 8°C. Collect the supernatant to carry out the assay.

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Note for sample:

1. Samples should be assayed within 7 days when stored at 4°C, otherwise samples must be divided up and stored at -20°C (≤1 month) or -80°C (≤3 months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility of causing a deviation due to the introduced chemical substance.
5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

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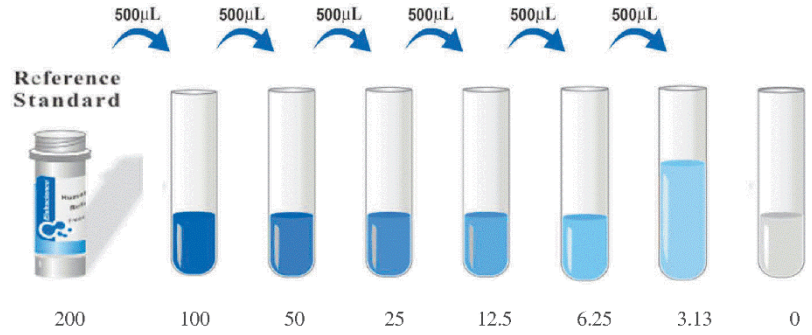
Reagent preparation

1. Bring all reagents to room temperature (18~25℃) before use. Follow the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.Note: if crystals have formed in the concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 200 ng/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 200, 100, 50, 25, 12.5, 6.25, 3.13, 0 ng/mL.
   Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 200 ng/mL working solution to the first tube and mix up to produce a 100 ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

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Assay procedure (A brief assay procedure is on the 11th page)

1. Add the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Remove the liquid out of each well, do not wash. Immediately add 100 μL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.
3. Aspirate or decant the solution from each well, add 350 uL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 μL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 90 μL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
7. Add 50 μL of **Stop Solution** to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

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Calculation of results

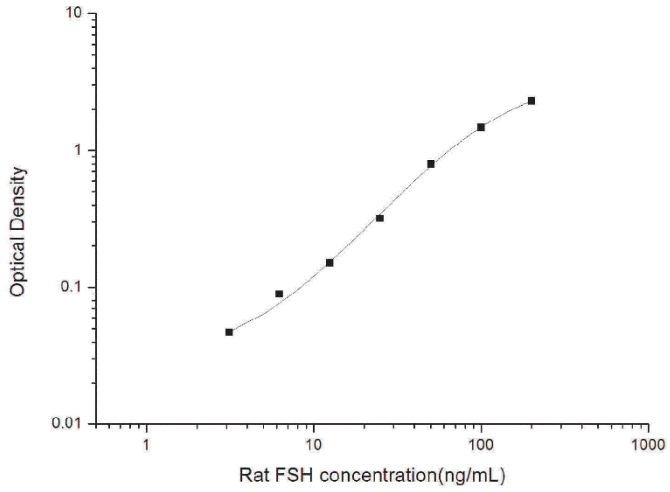
Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

Concentration(ng/mL)	200	100	50	25	12.5	6.25	3.13	0
OD	2.385	1.557	0.882	0.406	0.236	0.175	0.133	0.086
Corrected OD	2.299	1.471	0.796	0.32	0.15	0.089	0.047	-



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Precision

Intra-assay Precision (Precision within an assay): 3 samples with low, mid range and high level Rat FSH were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, mid range and high level Rat FSH were tested on 3 different plates, 20 replicates in each plate.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean(ng/mL)	10.50	22.00	75.70	10.90	21.50	75.20
Standard deviation	0.70	1.10	3.30	0.70	1.30	3.80
C V (%)	6.67	5.00	4.36	6.42	6.05	5.05

Recovery

The recovery of Rat FSH spiked at three different levels in samples throughout the range of the assay was evaluated in various matrices.

Sample Type	Range (%)	Average Recovery (%)
Serum (n=5)	92-102	97
EDTA plasma (n=5)	90-106	97

Linearity

Samples were spiked with high concentrations of Rat FSH and diluted with Reference Standard & Sample Diluent to produce samples with values within the range of the assay.

		Serum (n=5)	EDTA plasma(n=5)
1:2	Range (%)	96-110	87-103
	Average (%)	101	94
1:4	Range (%)	101-117	81-94
	Average (%)	107	87
1:8	Range (%)	100-115	86-98
	Average (%)	106	91
1:16	Range (%)	98-113	82-95
	Average (%)	105	87

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Troubleshooting

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes.
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by gentle mixing.
	Wells are not completely aspirated	Completely aspirate wells in between steps.
Low signal	Insufficient incubation time	Ensure sufficient incubation time.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB, rapid coloring.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader.
		Open the Microplate Reader ahead to pre-heat.
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of target protein is too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Prepare fresh wash buffer.
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions.
	Stop solution is not added	Stop solution should be added to each well before measurement.

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SUMMARY

1. Add 100 μL standard or sample to each well. Incubate for 90 min at 37°C.
2. Remove the liquid. Add 100 μL Biotinylated Detection Ab. Incubate for 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 μL HRP Conjugate. Incubate for 30 min at 37°C.
5. Aspirate and wash 5 times.
6. Add 90 μL Substrate Reagent. Incubate for 15 min at 37°C.
7. Add 50 μL Stop Solution. Read at 450 nm immediately.
8. Calculation of results.

Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operational skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions!
4. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from the above reasons, too.

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Appendix VIII

Elabscience
www.elabscience.com

5th Edition, revised in June, 2014

（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGONOSIS !）

T (Testosterone) ELISA Kit
Catalog No: E-EL-0072
96T

This manual must be read attentively and completely before using this product.

May you have any problems, please contact our Technical Service Center for help.

Phone: 86-27-87385095
Email: techsupport@elabscience.com
techelabscience@gmail.com
Website: www.elabscience.com

Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

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Intended use

This ELISA kit applies to the in vitro quantitative determination of T concentrations in serum, plasma and other biological fluids.

Sensitivity

The minimum detectable dose of T is 0.188ng/mL (The sensitivity of this assay, or lowest detectable limit (LDL) was defined as the lowest protein concentration that could be differentiated from zero).

Detection Range

0.313-20ng/mL

Specificity

This kit recognizes natural and recombinant T. No significant cross-reactivity or interference between T and analogues was observed.

Note:

Limited by existing techniques, cross reaction may still exist, as it is impossible for us to complete the cross-reactivity detection between T and all the analogues.

Repeatability

Coefficient of variation were<10%.

Statement: Thank you for choosing our products. This product is produced by using raw material from world-renowned manufacturer and professional manufacturing technology of ELISA kits. Please read the instructions carefully before use and check all the reagent compositions! If in doubt, please contact Elabscience Biotechnology Co., Ltd.

Storage: All the reagents in the kit should be stored according to the labels on vials. Unused wells should be returned to the foil pouch with the desiccant pack and resealed along entire edge of zip-seal. Substrate Reagent shouldn't be kept at -20℃ (Check!). Exposure of reagents to strong light should be avoided in the process of incubation and storage. All the taps of reagents should be tightened to prevent evaporation and microbial contamination. If not to store reagents according to above suggestions, erroneous results may occur.

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Kit Components：

Item	Specifications	Storage
Micro ELISA Plate	8 wells ×12 strips	4℃
Reference Standard	2 vials	4℃
Reference Standard & Sample Diluent	1vial 20mL	4℃
Concentrated Biotinylated Detection Ab	1vial 120μL	4℃
Biotinylated Detection Ab Diluent	1vial 10mL	4℃
Concentrated HRP Conjugate	1vial 120μL	4℃(shading light)
HRP Conjugate Diluent	1vial 10mL	4℃
Concentrated Wash Buffer (25×)	1vial 30mL	4℃
Substrate Reagent	1vial 10mL	4℃(shading light)
Stop Solution	1vial 10mL	4℃
Plate Sealer	5pieces	
Manual	1 copy	
Certificate of Analysis	1 copy	

Test principle

This ELISA kit uses Competitive-ELISA as the method. The microtiter plate provided in this kit has been pre-coated with T. During the reaction, T in the sample or standard competes with a fixed amount of T on the solid phase supporter for sites on the Biotinylated Detection Ab specific to T. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of T in the samples is then determined by comparing the OD of the samples to the standard curve.

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Sample collection and storage

Samples should be clear and transparent and be centrifuged to remove suspended solids.

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4℃ before centrifugation for 15 minutes at 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8℃ within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Hemolysis samples are not suitable for ELISA assay!

Cell culture supernate: Centrifuge supernate for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2 - 8°C. Collect the clear supernate and carry out the assay immediately.

Tissue homogenates: You'd better get detailed references from other literatures before assay aiming at different tissue types. For general information, hemolysis blood may affect the result, so you should mince the tissues to small pieces and rinse them in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (the volume depends on the weight of the tissue) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifugated for 5minutes at 5000×g to get the supernate.

Other biological fluids: Centrifuge samples for 20 minutes at 1000×g at 2 - 8℃. Collect the supernatant and carry out the assay immediately.

Note:

1. Samples should be used within 7 days when stored at 2-8℃, otherwise samples must be divided and stored at -20℃ (≤1month) or -80℃ (≤6months) to avoid the loss of bioactivity and contamination. Avoid repeated freeze-thaw cycles.
2. Please take the samples to room temperature (18-25℃) without extra heating before performing the assay.
3. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

Sample preparation

1. Elabscience is only responsible for the kit itself, but not for the samples consumed during the experiment. The user should calculate the possible amount of the samples needed in the whole test. Reserving sufficient samples in advance is recommended.
2. If the samples are not mentioned in this manual, a pre-experiment to determine the validity of the kit is necessary.
3. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected Elisa results due to the impacts of certain chemicals.

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4. Due to the possibility of mismatching between antigen from other origins and antibodies used in our kits, some native or recombinant proteins from other manufacturers may not be detected by our kits.
5. Influenced by factors including cell viability, cell number or sampling time, molecular from cells culture supernatant may not be detected by the kit.
6. Grossly hemolyzed samples are not suitable for use in the assay.
7. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

Other supplies required

Microplate reader with 450nm wavelength filter
High-precision transferpettor, EP tubes and disposable pipette tips
37℃ Incubator
Deionized or distilled water
Absorbent paper
Loading slot for Wash Buffer

Reagent preparation

Bring all reagents to room temperature (18-25℃) before use.

Wash Buffer - Dilute 30 mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water. Put unused solution back at 4℃. If crystals have formed in the concentrate, you can warm it with 40℃ water bath (Heating temperature should not exceed 50℃) and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use.

Standard – Prepare standard within 15 minutes before use. Centrifuge at 10,000×g for 1 minute, and reconstitute the Standard with **1.0mL** of Reference Standard &Sample Diluent. Tighten the lid, let it stand for 10 minutes and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution of 20ng/mL. Then make serial dilutions as needed (making serial dilution in the wells directly is not permitted). The recommended concentrations are as follows:**20、10、5、2.5、1.25、0.625、0.313、0 ng/mL**. If you want to make standard solution at the concentration of 10ng/mL, you should take0.5mL standard at 20ng/mL, add it to an EP tube with 0.5mL Reference Standard &Sample Diluent, and mix it. Procedures to prepare the remained concentrations are all the same. The undiluted standard serves as the highest standard (20ng/mL). The Reference Standard &Sample Diluent serves as the zero (0 ng/mL).

(Standards can also be diluted according to the actual amount， such as 200μL/tube)

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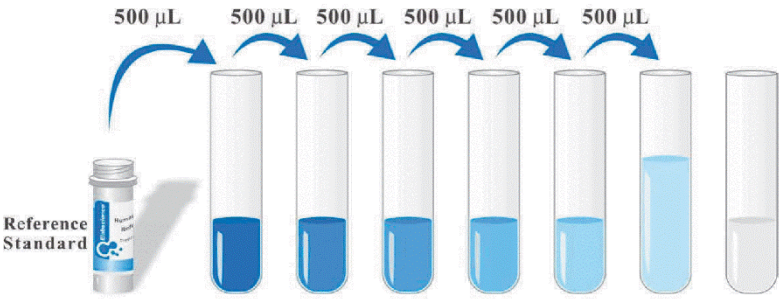

1	2	3	4	5	6	7	8	tube
20	10	5	2.5	1.25	0.625	0.313	0	ng/mL

Biotinylated Detection Ab – Calculate the required amount before experiment (50µL/well). In actual preparation, you should prepare 100~200µL more. Centrifuge the stock tube before use, dilute the concentrated Biotinylated Detection Ab to the working concentration using Biotinylated Detection Ab Diluent (1:100).

Concentrated HRP Conjugate – Calculate the required amount before experiment (100µL/well). In actual preparation, you should prepare 100~200µL more. Dilute the Concentrated HRP Conjugate to the working concentration using HRP Conjugate Diluent (1:100).

Substrate Reagent: As it is sensitive to light and contaminants, so you shouldn't open the vial until you need it! The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent shouldn't be dumped back into the vial again.

Note: Please don't prepare the reagent directly in the Diluent vials provided in the kit. Contaminated water or container for reagent preparation will influence the result.

Washing Procedure:

1. **Automated Washer:** Add 350µL wash buffer into each well, the interval between injection and suction should be set about 60s.
2. **Manual wash:** Add 350µLWash Buffer into each well, soak it for 1~2minutes. After the last wash, decant any remaining Wash Buffer by inverting the plate and blotting it dry by rapping it firmly against clean and towcling absorbent paper on a hard surface.

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Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** It's recommended that all samples and standards be assayed in duplicate.

1. **Add Sample and Biotinylated Detection Ab:** Add 50µl of Standard, Blank, or Sample per well. The blank well is added with Reference Standard &Sample Diluent. Immediately add 50 µl of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer we provided. Gently tap the plate to ensure thorough mixing. Incubate for 45minutes at 37°C. (Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible.)
2. **Wash:** Aspirate each well and wash, repeating the process three times Wash by filling each well with Wash Buffer (approximately 350µl) using a squirt bottle, multi-channel pipette, manifold dispenser or automated washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
3. **HRP Conjugate:** Add 100µl of HRP Conjugate working solution to each well. Cover with a new Plate sealer. Incubate for 30 minutes at 37°C.
4. **Wash:** Repeat the aspiration/wash process for five times as conducted in step 4.
5. **Substrate:** Add 90µl of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, you can terminate the reaction.
6. **Stop**: Add 50µl of Stop Solution to each well. Color turn to yellow immediately. The adding order of stop solution should be as the same as the substrate solution.
7. **OD Measurement**: Determine the optical density (OD value) of each well at once, using a microplate reader set to 450 nm. You should open the microplate reader ahead, preheat the instrument, and set the testing parameters.
8. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Important Note:

1. **ELISA Plate**: The just opened ELISA Plate may appear water-like substance, which is normal and will not have any impact on the experimental results.
2. **Add Sample**: The interval of sample adding between the first well and the last well should not be too long, otherwise will cause different pre-incubation time, which will significantly affect the

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experiment's accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. Parallel measurement is recommended.

3. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Do not let the strips dry at any time during the assay. Strict compliance with the given incubation time and temperature.
4. **Washing**: The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper in the washing process. But don't put absorbent paper into reaction wells directly. Note that clear the residual liquid and fingerprint in the bottom before measurement, so as not to affect the micro-titer plate reader.
5. **Reagent Preparation**: As the volume of Concentrated Biotinylated Detection Ab and Concentrated HRP Conjugate is very small, liquid may adhere to the tube wall or tube cap when being transported. You better centrifugal it for 1 minute at 1000rpm. Please pipette the solution for 4-5 times before pippeting. Please carefully reconstitute Standards, working solutions of Detection Ab and HRP Conjugate according to the instructions. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting. Do not reuse standard solution, working solution of Detection Ab and HRP Conjugate, which have been diluted. If you need to use standard repeatedly, you can divide the standard into a small pack according to the amount of each assay, keep them at -20～-80℃ and avoid repeated freezing and thawing.
6. **Reaction Time Control:** Please control reaction time strictly following this product description!
7. **Substrate:** Substrate Solution is easily contaminated. Please protect it from light.
8. **Stop Solution:** As it is an acid solution, please pay attention to the protection of your eyes, hands, face and clothes when using this solution.
9. **Mixing:** You'd better use micro-oscillator at the lowest frequency, as sufficient and gentle mixing is particularly important to reaction result. If there is no micro-oscillator available, you can knock the ELISA plate frame gently with your finger before reaction.
10. **Security:** Please wear lab coats and latex gloves for protection. Especially detecting samples of blood or other body fluid, please perform following the national security columns of biological laboratories.
11. Do not use components from different batches of kit (washing buffer and stop solution can be an exception).

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12. To avoid cross-contamination, change pipette tips between adding of each standard level, between sample adding, and between reagent adding. Also, use separate reservoirs for each reagent. **Otherwise, the results will be inaccurate!**

Calculation of results

Average the duplicate readings for each standard and samples. Create a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. It is recommended to use some professional software to do this calculation, such as curve expert 1.3. In the software interface, a best fitting equation of standard curve will be calculated using OD values and concentrations of standard sample. The software will calculate the concentration of samples after entering the OD value of samples. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution. The actual concentration is the calculated concentration multiplied dilution factor.

SUMMARY

1. Add 50μL standard or sample to each well.
2. Immediately add 50μL Biotinylated Detection Ab to each well.
3. Incubate for 45 minutes at 37℃
4. Aspirate and wash 3 times
5. Add 100μL HRP Conjugate to each well. Incubate for 30 minutes at 37℃
6. Aspirate and wash 5 times
7. Add 90μL Substrate Reagent. Incubate 15 minutes at 37℃
8. Add 50μL Stop Solution. Read at 450nm immediately.
9. Calculation of results

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Troubleshooting

Problem	Causes	Solutions
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by a gentle mix.
	Wells not completely aspirated	Completely aspirate wells between steps.
Low signal	Too brief incubation times	Ensure sufficient incubation time;
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation
	Improper dilution	
Deep color but low value	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
		Open the Plate Reader ahead to pre-heat
Large CV	Inaccurate pipetting	Check pipettes
High background	Concentration of detector too high	Use recommended dilution factor.
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions
	Stop solution not added	Stop solution should be added to each well before measurement

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5th Edition, revised in June, 2014

Declaration:

1. Limited by current conditions and scientific technology, we can't completely conduct the comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operation skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions in the description!
4. Incorrect results may led by wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for Micro-plate reader. Please read the instruction carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get better reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some unexpected reasons such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from above reasons, too.
7. Valid period: 6 months.

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