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Testicular 3beta hydroxysteroid dehydrogenase in naringenin adjuvant under highly active antiretroviral therapy (HAART); preliminary data using Sprague-Dawley rats

Edidiong N. Akang[1,2], Misturah Y. Adana[1,3], Aniekan I. Peter[1,4], Edwin C.S. Naidu[1], Irene Mackraj[5], Onyemaechi O. Azu[1,6]

[1]Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa, [2]Department of Anatomy, College of Medicine, University of Lagos, Lagos, Nigeria.,[3]Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin, Nigeria., [4]Department of Anatomy, Faculty of Basic Medical Sciences, University of Uyo, Uyo, Nigeria, [5]Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, South Africa, [6]Department of Anatomy, School of Medicine, Windhoek, Namibia

SUMMARY

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 testes 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.

Key words: 3β HSD – Testis – Naringenin – Antiretroviral therapy – Leydig cells

Corresponding author: Dr Edidiong N. Akang. Department of Anatomy, College of Medicine, University of Lagos, Idi-Araba, P.M.B. 12003 Lagos, Nigeria. Phone: +2348065299278. E-mail: eltyeddy@gmail.com

INTRODUCTION

The advent of highly active antiretroviral therapy (HAART) in 1996 (Montaner et al., 1998; Nosyk et al., 2014) has brought much succour to persons living with HIV/AIDS (PLWHAS), increasing life expectancy and decreasing the incidence of opportunistic infections (Teeraananchai et al., 2017). It is expected that, by 2020, 90% of diagnosed HIV positive persons will be on HAART (UNAIDS, 2014). Global statistics shows that over 37 million persons are already infected with the virus (Platt et al., 2016), and about 40% are men in their 20s. It

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is quite unfortunate that, despite the relative life expectancy and other health benefits of HAART, it is associated with several comorbidities (Jacob, 2016; Croxford et al., 2017) including testicular toxicity, thereby impairing spermatogenesis (Kehl et al., 2011).

A study by Pilatz et al. (2014), 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 highlight 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 (Paiva et al., 2003).

Furthermore, studies by Ogedengbe et al. (2016) and Jegede et al. (2017) demonstrated very toxic effects of HAART on the testes 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 (Azu et al., 2014). 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 (O'Hara and Smith, 2015).

Testosterone biosynthesis, which begins from the transfer of cholesterol from the cytoplasm to the 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 (Raucci et al., 2014), 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 (Alamdar et al., 2017).

Excessive free radicals have been reported to hinder steroidogenesis (Chouan et al., 2015). In view of this, antioxidant supplementation is encouraged to stabilize the excesses of mitochondrial free radical synthesis (Azu, 2012). Flavonoids have a direct scavenging effect on free radicals, because they exhibit a wide variety of antioxidant properties (Xiao et al., 2011). Free radicals are oxidized by flavonoids, resulting in a more stable, less reactive molecule (Xiao et al., 2011). Naringenin bioflavonoid (4,5,7-trihydroxyflavon) which is found in abundance in fruits, especially grapefruit and tangerine (Erlund et al., 2001), is considered a safe natural product with reports of mediating decreased testicular oxidative stress by reducing hydrogen peroxidase activity (Sahin et al., 2017). It also ameliorated oxidative stress-induced hepatic and renal dysfunction (Mershiba et al., 2013), and has neuroprotective effects (Raza et al., 2013; Wu et al., 2016). However, a report on Naringenin-induced oxidative stress and spermatogenic toxicities (Ranawat and Bakshi, 2017) 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 Care and Use of Laboratory Animals.' The animals were housed in standard cages with dimensions of 20 cm long, 20 cm wide and 13 cm high. The rats were kept under controlled environmental conditions (25oC 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) (Meintjes et al., 2014) were 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 (Hegazy et al., 2016), Group Nar80: Naringenin, 80 mg/kg (Shi et al., 2009), 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 (Latendrese 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

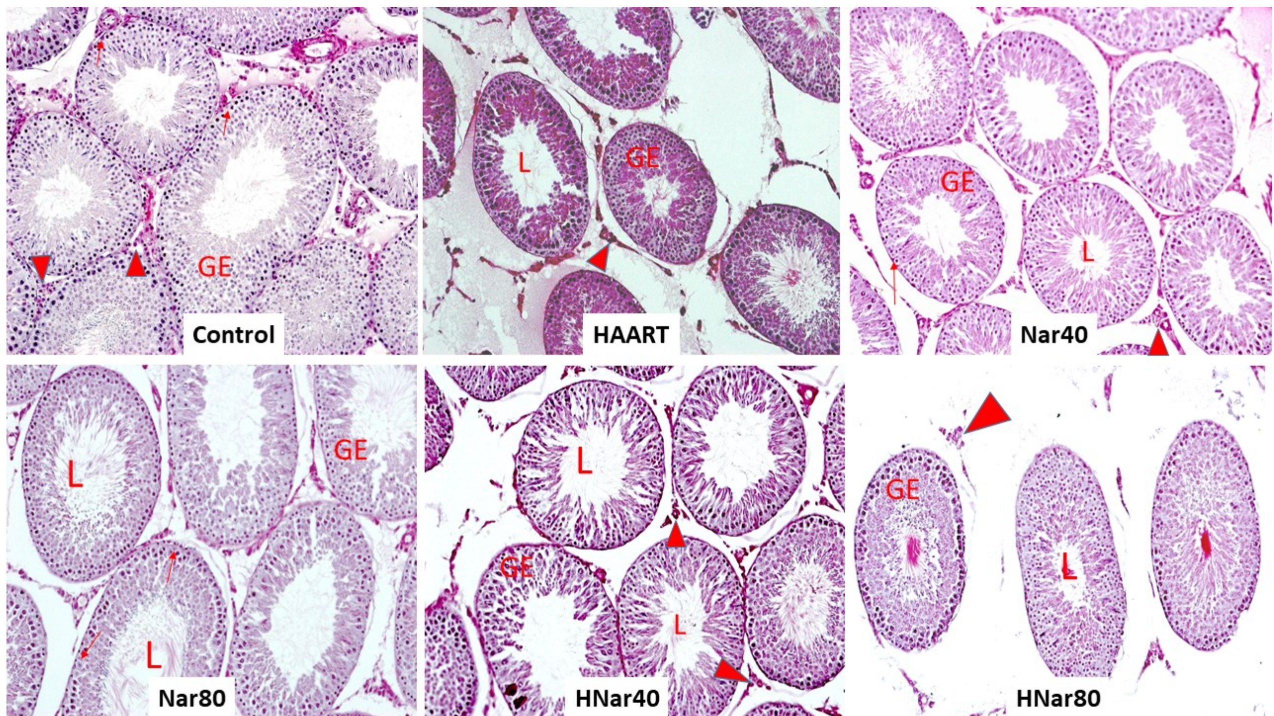

Fig 1. Photomicrographs of testis (H & E stainings), showing germinal epithelium (GE), interstitial cells of Leydig (arrow heads), Sertoli cells (arrows) and spermatozoa in lumen (L). Note the ballooning of the interstitium of HAART and HNar80 groups with sparse Leydig cells and narrowed/constricted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

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.

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. 2015). 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 (2004) and Akang et al. (2015). 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 photomi-

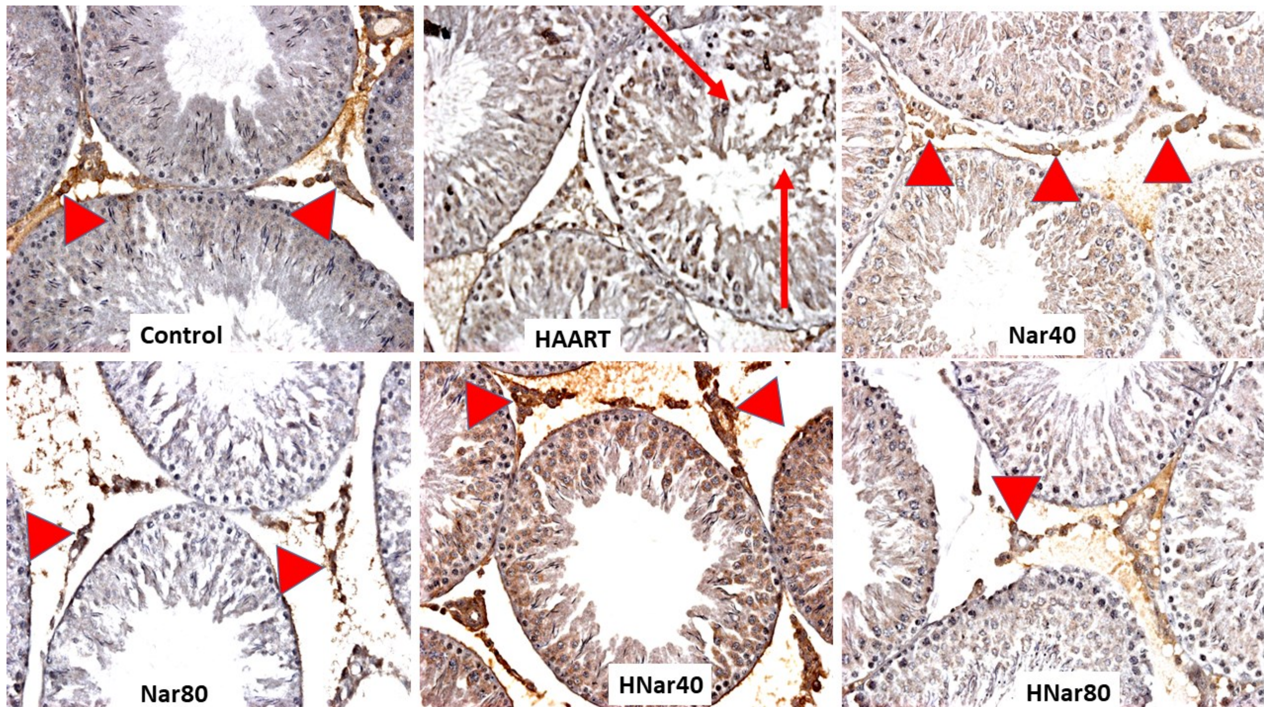

Fig 2. Photomicrographs of testis showing immunohistochemistry of 3 beta hydroxysteroid dehydrogenase (3β HSD) in the interstitium of testis (arrow heads) and perturbations (arrows) in the seminiferous tubule of HAART group. x400.

crographs 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 (Fig. 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 (Fig. 1), with interrupted spermatogenesis in the HAART group (Fig. 2).

Morphometric analysis

The relative volume fraction of the germinal epithelium, lumen and interstitial cells of animals that were treated with HAART decreased significantly, compared to control though not. 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 1).

Immunohistochemical analysis

All the photomicrographs showed expression of 3β HSD in the interstitial cells of Leydig (Fig. 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 (Fig. 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 (WHO, 2016), has been reported to increase the production of superoxide anions and morphological alterations of the mitochondria (Polo et al., 2015). A study by Ganta et al. (2017), showed that EFV-mediated toxicity is initiated via the permeabilization of the outer membrane of the mitochondria and subsequent change in the membrane potential (Δψm), which triggers a series of events like Cytochrome C release resulting 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 (Park et al., 2014). It is therefore plausible to infer that the downregulation testicular 3β HSD by HAART explored the oxidant-antioxidant pathway. These findings are in tandem with reports by Wang et al. (2015) 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

Table 1. Relative volume fraction of the germinal epithelium, lumen and interstitial cells

	Control	*HAART*	*Nar40*	*Nar80*	*HNar40*	*HNar80*
Germinal epithelium (%)	52.5 ± 7,2	48.6 ± 10.1	59.7 ± 5.2	63.5 ± 13.3*	59.5 ± 13.8$^{\alpha}$	47.6 ± 9.2
Lumen (%)	11.0 ± 1.8	10.0 ± 2.7	11.3 ± 2.2	15.6 ± 2.0*	14.3 ± 3.4$^{\alpha}$	11.6 ± 2.7
Interstitial cells (%)	9.6 ± 1.7	9.2 ± 2.2	10.4 ± 1.1	13.4 ± 1.8*	13.6 ± 2.0$^{\alpha}$	8.4 ± 1.1

Values are expressed as mean ± Standard deviation. *p<0.05 compared to control, αp<0.05 compared to HAART.

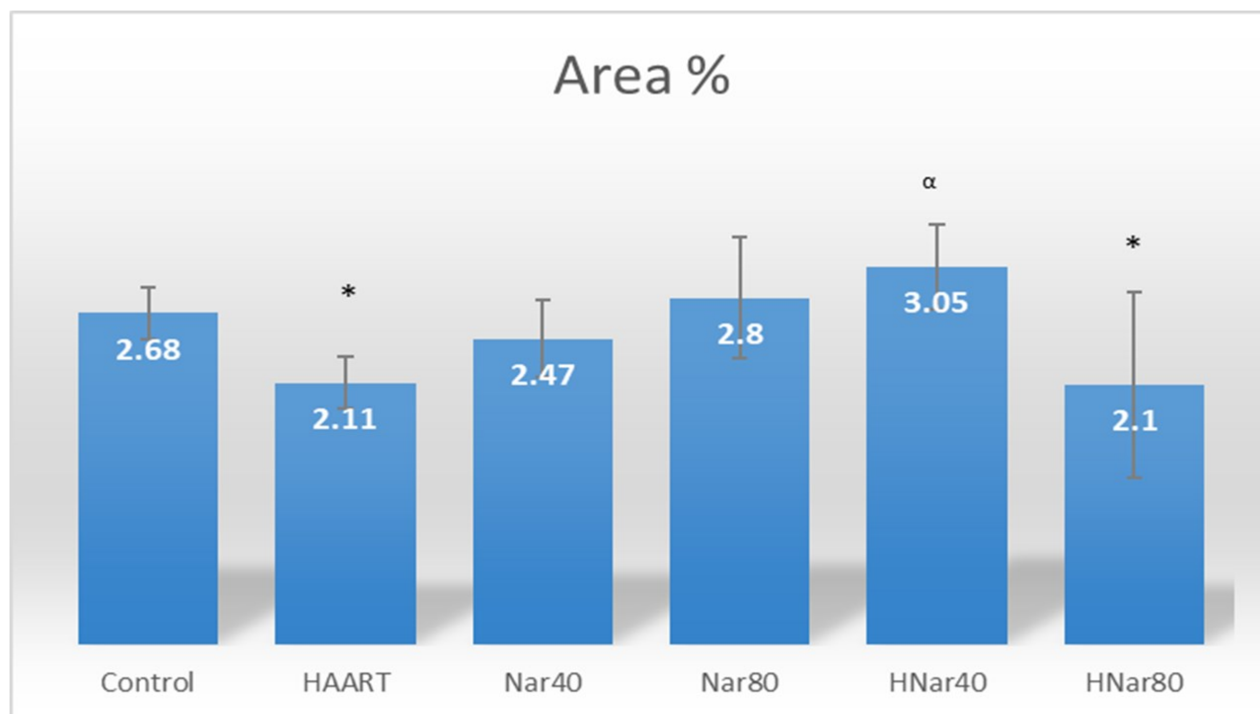

Fig 3. Bars represent mean area percentage of 3 beta hydroxysteroid dehydrogenase (3β HSD) immunohistochemical stains in the interstitium of the testis. *p<0.05 compared to control, αp<0.05 compared to HAART.

the proper functioning of the Sertoli-Sertoli tight junctions also known as the blood-testis- barrier (BTB), and normal spermatogenesis (Walker, 2010). Thus, a deficiency of this steroid hormone will be counterproductive on spermatogenesis, rarely progressing beyond diplotene spermatocytes stage (De Gent et al., 2004; Tsai et al., 2006). 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 (Adana et al., 2018).

In spite of these 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 (Mostafa et al., 2016). 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 (Mostafa 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).

Contrary to the report by Ranawat and Bakshi (2017), Naringenin improved the histomorphology and morphometry of the testes, which may be attributed to the differences in the route of administration. Whereas, Naringenin was administered intraperitoneally by Ranawat and Bakshi (2017), in this study Naringenin was administered orally, which probably reduced tissue absorption rate and the concentrations of total naringenin in the testes (Zou et al., 2012). 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 prop-

erties depending on the physiological milieu (Martirosyan et al., 2011).

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.

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