THE EFFECT OF *NIGELLA SATIVA* OIL ON MALE REPRODUCTIVE FUNCTION IN MALE WISTAR RATS EXPOSED TO AN OBESOGENIC DIET

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Magister Scientiae

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DECLARATION

Hereby I, the undersigned, declare that the thesis *The effect of Nigella sativa oil on male reproductive function in male Wistar rats exposed to an obesogenic diet* is my own work, that it has not been submitted previously for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Mr Walid Almaghrawi

Signed: _____

Date: _____

DEDICATION

This thesis is dedicated to my parents for their love, endless support and their encouragement, and my wife and my beautiful children Mohamed, Khairi, and Hashem. Further dedication is to my homeland.

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KEYWORDS

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CHAPTER 1

INTRODUCTION

1.1. Obesity

Obesity is a growing public health concern globally, particularly in developed countries such as the United States (US). More than 30% of Americans are considered obese. In the past 20 years in America, incidence of obesity has increased significantly (Mokdad *et al.*, 2003). As a consequence, the Centers for Disease Control (CDC) anticipates that 1 in 3 American adults will be diabetic by 2050 (Boyle *et al.*, 2001; Hedley *et al.*, 2004). The most important cause of obesity is poor nutrition, absence of physical exercise and unfavourable lifestyle changes (James *et al.*, 2001).

Obesity is associated with co-morbidities such as insulin resistance, which is mediated through the metabolic syndrome and is a risk factor for type 2 diabetes mellitus. Associated hyperlipidemia and hypertension increase the risk for cardiovascular and renal disease. Complications of obesity also included the risk of developing various types of cancer, including esophageal, breast, endometrial, colorectal, kidney, pancreatic, gall bladder, and thyroid cancer (Falagas & Kompoti, 2006; Wolin *et al.*, 2010; Schottenfeld *et al.*, 2013). Obesity contributes to systemic inflammation, particulary through increased visceral adipose tissue (VAT) and the release of inflammatory cytokines, which further contribute to insulin resistance and oxidative stress (Gregor & Hotamisligil, 2011; Lukens *et al.*, 2011; Gerriets & MacIver, 2014; Harpsøe *et al.*, 2014).

Obesity is traditionally defined, diagosedand classified by the body mass index (BMI). However, the waist circumference (WC) assessment is established as a more sensitive predictor of risk. Magnetic resonance imaging (MRI) technology has been used for distribution of fat deposits, as visceral adiposity and not the subcutaneous fat is a medical concern(Eckel *et al*., 2005). Near-Infrared Spectroscopy (NIRS) can

also be used to measure the depth of the adipose tissue (Johnson *et al*., 2015). Morbid obesity is currently treated effectively by bariatric surgery, although this surgical procedure can cause post-surgical complications and side effects and it is advisable to use appropriate exercise and diet instead of surgery (Gerriets & MacIver, 2014).

1.1.1. Definition And Classification

The BMI is used as a simple method to assess how much deviation there is from so-called normal or desirable body weight, using height as a reference and represented as Kg/m^2 (James *et al.*, 2001). Normal weight, underweigh, overweight and obesity are defined as illustrated in Table 1 (Kuczmarski *et al.*, 2007).

Table 1: Classification of weight according to the BMI (World Health Organization, 2011).

Category	BMI Range
Underweight	<18.5
Normal range	18.5 – 24.9
Overweight	25.0 – 29.9
Obesity class 1	30.0 – 34.9
Obesity class 2	35.0 – 39.9
Obesity class 3	≥ 40.0

1.1.2. Epidemiology

Obesity has been increasing in recent decades among adults and teenagers.Obesity increased by 32.9% in adults between the age of 20–74 years old, and this was more than 17% recorded for teenagers between the ages of 12–19 years (Ogden *et al*., 2007). In 2012, the South African National Health and Nutrition Examination showed that 30.7% of adult men and 64.0% of women are overweight or obese. More than 80% of people in South Africa die due to heart failure or myocardial infarction, and

approximately 60% of them die due to stroke, often as complications of obesity (Klug *et al.*, 2013). Global distribution of BMI categories is illustrated in Fig. 1.

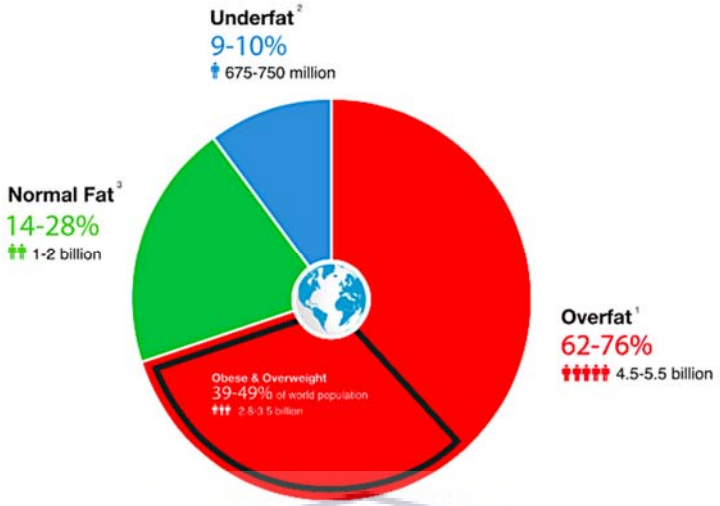

Figure 1: Overweight rates and distribution amongst adults and children around the world based on the world population in 2014 showed the percentage of normal fat , underfat ,obesity (Maffetone *et al.*, 2016).

Obesity co-morbodities have been recognized, associated with the metabolic syndrome. Metabolic disorders such as hypertension, dyslipidaemia and hyperglycaemia with insulin resistance and chronic inflammation are frequently associated with obesity, increasing the risk for non-communicable diseases (NCDs). According to the WHO studies, death rates caused by NCDs are increasing globally, particularly in the African region. The WHO predicted an increased rate in the incidence nd death of NCDs in the region by 2030. In 2010, the NCD rate in South Africa increased by 36% in individuals below the age of 60, which is similar to that of AIDS, HIV, and tuberculosis (TB). The mortality rates in South Africa have increased from diabetes, kidney, endocrine and blood diseases, probably as a result of lifestyle changes and urbanisation, especially in people who fall into the overweight and obese category (Nojilan *et al.*, 2016).

1.1.3. Risk Factor And Underlying Causes

1.1.3.1. Lifestyle

A positive correlation has been reported between poor lifestyle and increased adiposity. There is significant evidence linking a high fat and refined carbohydrate diet as a risk factor for obesity (Schulze & Hu, 2005). Sedentary lifestyles, like increase watching of TV more than 40 hours (h) per week, can lead to obesity compared to less than 1 h per week watching TV (Hu, 2003). In addition, pregnant mothers who smoke can lead to infants obesity and hypertension (Gao, *et al*., 2005; Defo *et al.,* 2017). In middle-aged adults, some studies indicated that there is no significant association between smoking status and body mass index (BMI). On the other hand, it has equally been reported that smoking may be associated with lower BMI and the stoppage can lead to increase of BMI. Obesity was also reported to be high in former smokers and the spread was highest between those who stopped smoking within the last ten years and lowest among those who had stopped more than thirty years before (Dare *et al*., 2015). Other behaviours such as stress, loneliness and frustration could lead to excessive food intake and in turn weight gain (Collins & Bentz, 2009). Additionally, studies have shown that people who consume a low carbohydrate diet expend low energy than those whose use high carbohydrate (Prieto & Kales, 2016). Lifestyle modification is the first option for the treatment of obesity, and it includes weight control, dieting and exercising (Wadden *et al.,* 2012).

1.1.3.2. Genetics And Single Nucleotide Polymorphisms (SNP'S)

Genetic studies have shown that there is a relationship between various single-nucleotide polymorphisms (SNPs) and obesity. These genetic factors encode proteins involved in biological processes to influence body conformation, including fat metabolism and adipocyte differentiation. SNPs were found to be involved in abdorml fat metabolism and inflammation (Povel *et al.*, 2011; Povel *et al.,* 2012). A study on Caucasian people shows a guide to obesity phenotype is linked with various genes including, mitochondrial carrier homolog 2 (MTCH2), Niemann-Pick disease, type C1 (NPC1), neuronal growth regulator 1 (NEGR1), brain-derived neurotrophic factor (BDNF), SEC16 homolog B (SCE16B) and ets variant 5 (ETV5). Others include

SH2B adaptor protein 1 (SH2B1), transmembrane protein 18 (TMEM18), v-maf musculoaponeurotic fibrosarcoma oncogene homolog (MAF), glucosamine-6-phosphate deaminase 2 (GNPDA2), prolactin (PRL), Fas apoptotic inhibitory molecule 2 (FAIM2), potassium channel tetramerisation domain containing 15 (KCTD15) and melanocortin 4 receptor (MC4R) (Hotta K *et al*., 2009). In addition, there exist a link between those genes and the hypothalamus thus indicating their importance in the regulation of food intake (Hotta K *et al*., 2009).

1.1.3.3. Socio Economic Risk

Socio-economic position (SEP) is closely related to increased obesity risk (Link & McKinlay, 2009; Alaba & Chola, 2014). It is also known that the socio-economic status of underdeveloped, developing and developed countries correlates with increased obesity amongst citizens of these countries. In South Africa, obesity in the adult population is more prevalent in women than in men and more common amongst the rich more than the poor (Alaba & Chola, 2014). It was also noticed that obese women are more likely to be unemployed and earn 5% less than other women. Hence, obesity has an impact on employment (Alaba & Chola, 2014). All of these social and economic factors are considered to be the main determinants of the occurrence of obesity (Link & McKinlay, 2009; Alaba & Chola, 2014).

1.1.3.4. Psychological Risk

Obesity-associated with a psychological disturbance is poorly understood and psychical health, according to weight status that leads to a few effects on the social functioning of teenagers'. If there is an effect of obesity on functioning, it may operate through mediators such as the body structure of those teenagers (Roberts & Hao, 2013).

1.1.4. Pathophysiology

The pathophysiology of obesity includes inflammation, leptin dysregulation and vascular injury, mediated through various cytokines and adpiokines (Redinger, 2007; Marinou *et al.*, 2010). Obesity also can lead to an increase in oxidative stress through

increased reactive oxygen species (ROS) and reduced antioxidant capacity. Inflammation and oxidative stress in turn can lead to co-morbidities and complications including atherosclerosis, diabetes mellitus and cancer (Burits & Bucar, 2000). Insulin resistance correlates with waist circumference, further significantly correlated with inflammation (Park *et al*., 2013). In males, hypogonadism is also associated with obesity, metabolic syndrome and type 2 diabetes mellitus as a consequence and a risk factors (Mulligan *et al.*, 2006). Male hypogonadism triggers other adverse conditions such as libido impairment, erectile dysfunction and reduced ejaculate volumes and impaired spermatogenesis (Mulligan *et al.*, 2006; Dohle *et al.*, 2012; Ferro *et al.*, 2017).

1.1.5. Consequences of Obesity

Obesity has a major effect on cardiovascular disease (CVD), particularly ischaemic heart disease, heart failure and venous thromboembolism (Gelber *et al.,* 2008). Obesity can lead to type II diabetes mellitus, which some researchers use the term epidemic 'diabesity' (Yaturu, 2011). In addition, sleep apnea is a common comorbidity linked with the epidemic of obesity (Schwartz *et al*., 2008). Certain cancers are associated with obesity, including common cancers such as colorectal, breast,ovary,endometrium and prostate, amogst others (Percik & Stumvoll, 2009).

1.1.6. Diagnostics And Risk Assessment

Obesity is clinically defined and determined using the BMI. A person who has a BMI of ≥ 30 is considered obese, while a person with a BMI ≥ 25 is considered overweight, and the person with the BMI <18 considered as underweight (Table 1) (WHO, 2011*)* (Table 1). However, BMI s a limited tool in the predictor of obeity outcomes, as it fails to distinguish between subcutaneous and visceral obesity. This can be assessed by waist-circumference (WC) or the waist:hip ratio (WHR). The use of body fat percentage is also used in the assessment of adiposity if was a high percentage (Neovius *et al*., 2005; Okorodudu *et al,* 2010).

1.1.7. Treatment And Management

1.1.7.1. Nutrition And Lifestyle

Nutritional modification remains a critical management option, where a decrease of unhealthy snacks such as refined high energy sugar foods and drinks, saturated and hydrolysed fats and red meat is recommended (Arterburn & Courcoulas, 2014;Klein *et al*., 2004; Paes *et al.*, 2015). Many different diets have been proposed for the treatment of obesity, many involving low calorie diets which leads to weight loss (Klein *et al*., 2004). Optimal weight loss over the long-term could help slow the onset of diabetes, where weight loss of 5% in obese people can significantly reduce the risk of cardiovascular disease and diabetes. However, the higher percentage of weight loss is linked with a high percentage of weight regain. Evidence suggests that a loss of more than 20% of weight has an increased risk of regaining the weight compared with those who lost 10–15% of their weight (Barte *et al*., 2010 ; Olson *et al*., 2017). Physical activity can also help to control promote weight loss and reduce co-morbidities (Levri *et al*., 2005; Seifarth *et al*., 2013).

1.1.7.2. Medical Treatment

Metformin is considered a central medication for diabetes and pre-diabetes, because improving hyperglycaemia, insulin sensitivity, hyperlipidemia and hyperglycemia, mediated through anti-inflammatory and lipolytic effects mediated through lowering increasing the expression of peroxisome proliferator-activated receptor γ (PPARγ) (Srinivasan *et al*., 2006; Ferreira *et al.,* 2015). Importantly, metformin is associated with weight reduction in diabetic and prediabetic patients and animal models (Seifarth *et al.,* 2013). Metformin therapy is effective for all organs and tissues, including the reproductive system (Guo *et al*., 2017; Kendall *et al.,* 2006), with reports of improvement in the reproductive functions of diabetic patients in both men and women (Seifarth *et al.,* 2013). Furthermore, some other medications can be used as treatment for obesity. They include Amfepramone (diethylpropion) and Phentermine, which can used in short-term, and Orlistat and Sibutramine used in the long-term.

The use of Sibutramine and Orlistat gives a more positive outcome (Ioannides-Demos *et al.,* 2006).

Recently, bariatric surgery techniques are in common use and are increasing as a result of obesity. This surgery is only for people who have a BMI more than 35. The first type of bariatric surgery is the removal of intestinal jejunum which leads to weight loss. A side effect of this surgery is that weight returns back in a few years. The second type of surgery is gastroplasty which involves both horizontal, vertical and banded gastroplasty. Other methods involve the use silicone belt with an inflatable balloon in the lining of the stomach that is fastened around the upper stomach (Arterburn & Courcoulas, 2014).

1.1.7.3. Alternative Approaches

As a result of the increase in obesity globally, and with many medications as a factor and cause of obesity, complementary and alternative management for modern treatments is increasing. This includes acupuncture, low level laser therapy (LLLT) and cryolipolysis as some of the approaches, leading to a successful reduction of fat in different areas, such as the waist, hips, thighs and upper arms (Esteghamati *et al.,* 2015). More importantly is the use of medicinal herbs as a alternative or complementary approach in obesity. Examples of herbs used effectively in obesity include *Nigella sativa*, *Commiphora mukul*, *Pterocarpus marsupium*, *Garcinia cambogia*, *Camellia sinensis*, *Fucus pediculosis*, *Gymnema sylvestre* and *Salacia reticulate* (Burits & Bucar,2000; Goswami *et al*., 2011; Valizadeh *et al*., 2016).

1.2. Male Reproduction And Infertility

The male reproductive system consists of external organs which includes the testes, epididymis and vas deferens within the scrotum, and the penis containing erectile tissue and the distal portion of the urethra (Figure 2). Internal reproductive organs incude male accessory glands, specifically the seminal vesicles, prostate and bulbourethral glands (Martini *et al*., 2015). The testes function for spermatogenesis, with the prostate, seminal vesicles and accessory glands involved in the formation of seminal fluid which protects the spermatozoa from the acidic environment of the

female genital system (De Kretser *et al.,* 1998). The male reproductive system is dependent on the hormone testosterone, which is produced by Leydig cells in the testes aided by the hypothalamus and the pituitary gland (Maruska & Fernald, 2011).

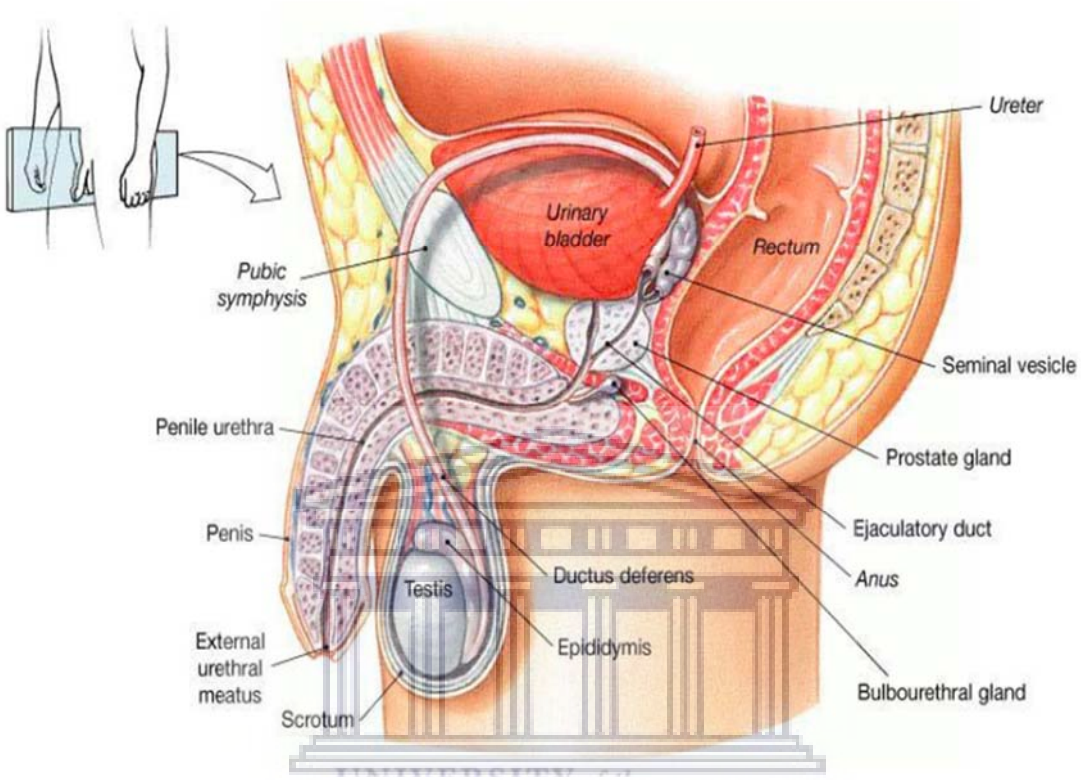

Figure 2: Cross section of the male reproductive system (Martini *et al*., 2015).

1.2.1. Anatomy And Physiology

1.2.1.1. Testes

The testes are located in the scrotum, covered by the tunica albuginea and tunica vaginalis. This communicates with the abdominal cavity through the inguinal canals, which stay open throughout life. Each testis contains efferent ducts of a series of tubes that join the rete testis to the epididymis. The epididymis stores and matures spermatozoa for ejaculation epididymis and epididymal adipose tissue. The testis consists of many pyramidal chambers called the testicular lobules. Each lobule contains 1 - 4 seminiferous tubules, enveloped in wide connective tissues, containing the Leydig cells and sertoli cells. The seminiferous tubules form the tubuli recti, which

lead to the rete testis in the middle, and then to the collecting cavity to ducts, and then to the head of the epididymis (Figure 3). The tubuli recti are confluent with the rete testis which is a network of delicate tubules, located in the hilum of the testicle, that transfers sperm from the seminiferous tubules to the efferent ducts as shown in Figure 4 (Knoblaugh & True, 2018).

Important male reproductive cells in seminiferous tubules include germ cells, Sertoli cells and Leydig cells. Sertoli cells support spermatogenesis, where Leydig cells produce androgens which is a critical aspect of spermatogenesis and male secondary sexual characteristics. Seminiferous tubules are padded with a complex stratified epithelium that consists of fibrous connective tissue, as well as lamina and a complex germinal epithelium (Dehghani, 2010). The spermatozoa take up space in the tubular compartments, forming about 60 - 80% of the total testicular volume. (Ilacqua *et al.*, 2018).

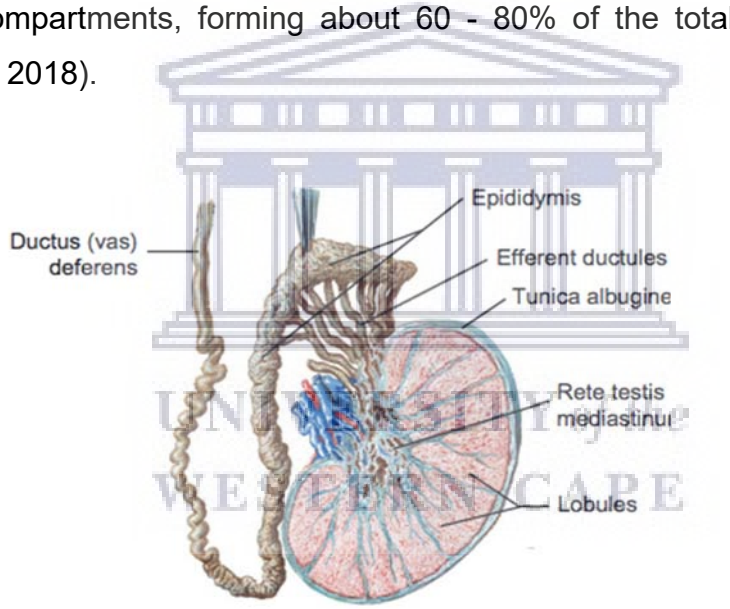

Figure 3*:* Testis, epididymis and ductus (vas) deferens (Knoblaugh & True, 2018).

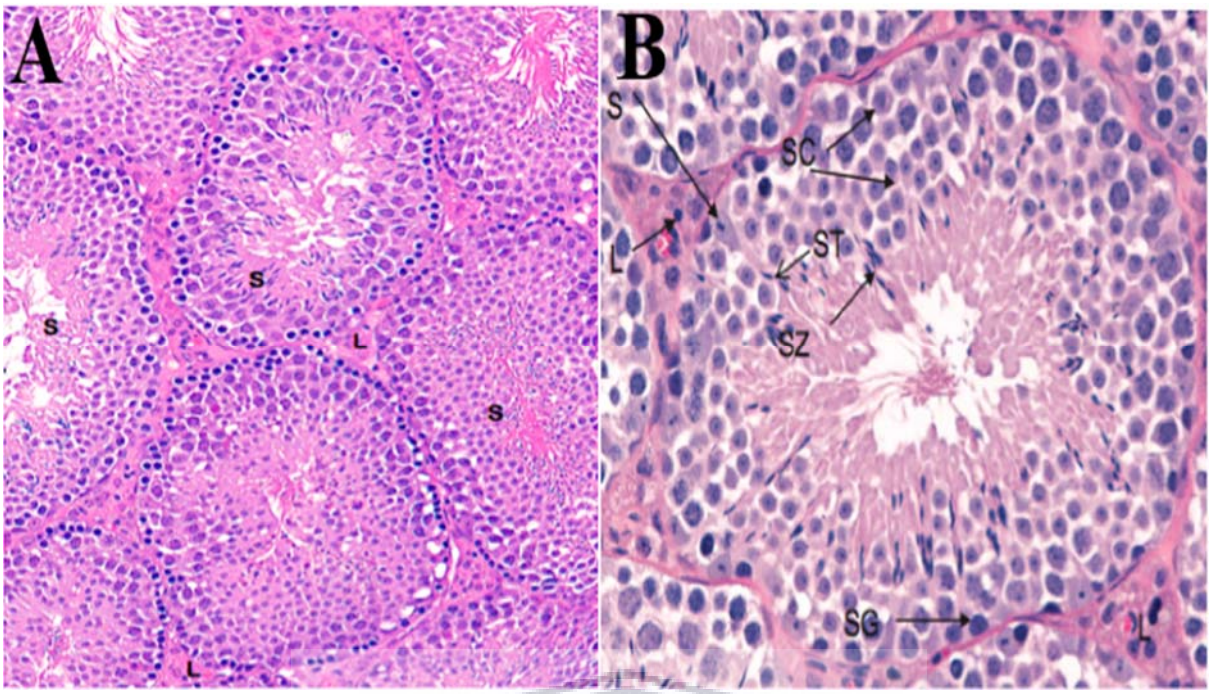

Figure 4: Histology of the testis in rats: Low magnification (A) High magnification (B)*.* S = seminiferous tubules L = interstitial or Leydig cells; SG = seminiferous tubules; SG = spermatogenic cells (spermatogonia), SC = spermatocytes; ST = spermatids, SZ = spermatozoa, S = Sertoli cells; L = Leydig cells (Knoblaugh & True, 2018*).*

1.2.1.2. Epididymis

The epididymis helps in the maturation and storage of spermatozoa. It is composed of three parts, namely the head, the body and the tail (Figure 5). The position of the epididymis is on the posterior side of the testes, with the head at the superior pole and tail at the inferior pole. In the rat, the head of the epididymis is enclosed by the epididymal fat body, epididymal ducts which are collections of smooth muscle walls, and a column of cuboidal epithelium (Knoblaugh & True, 2018; Ilacqua *et al.*, 2018; Murashima *et al*., 2015).

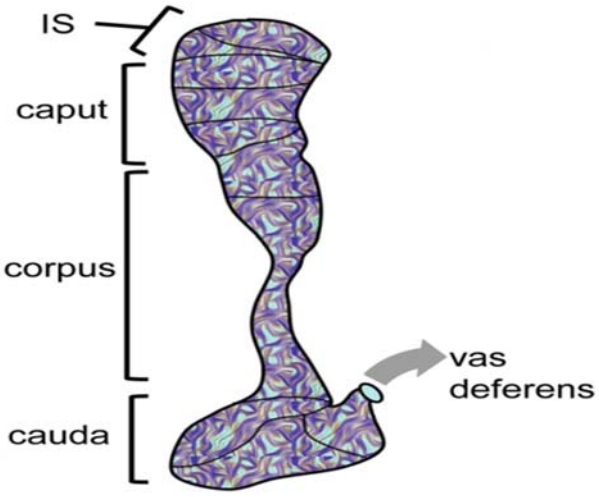

Figure 5: Structure of the epididymis showing the caput, corpus and cauda. IS = initial segment (Murashima *et al*., 2015).

1.2.1.3. Prostate

The prostate produces a milky-white alkaline fluid, which contributes approximately 30% of the total volume of the seminal fluid. The spermatozoa and the seminal vesicles account make up the remaining 70%. The prostate is divided anatomically into four different lobes, namely the anterior, dorsal, ventral and lateral lobes (Figure 6). The dorsal and lateral lobe is buttefly-shaped and surrounds the urethra (Figure 6) (Knoblaugh & True 2018; Ilacqua *et al.,* 2018). The anterior prostate is also known as the coagulating gland (Knoblaugh & True, 2018). The prostate contains a muscle fibre, which plays a role in the ejaculation process. On the ventral side, the prostate is lined by cuboidal epithelium, with sparse basal infolding and focal tufting, liminal spaces contain homogeneous, pale, small secretions, uniform and a basic nucleus (Figure 7) Shappell *et al*., 2004.

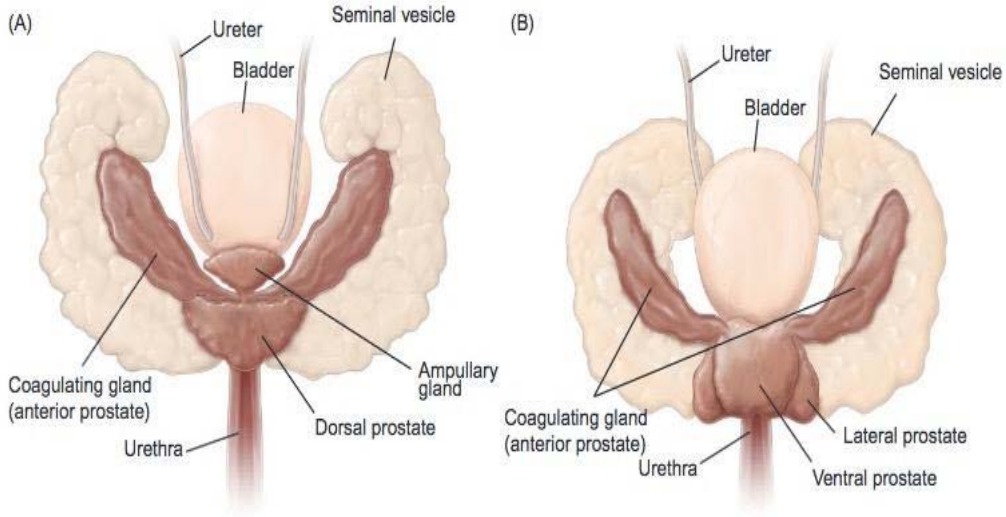

Figure 6: Prostate shape, the prostate is divided into four distinct lobes: anterior prostate (coagulating gland), dorsal prostate, lateral prostate, and ventral prostate. (Knoblaugh & True, 2018).

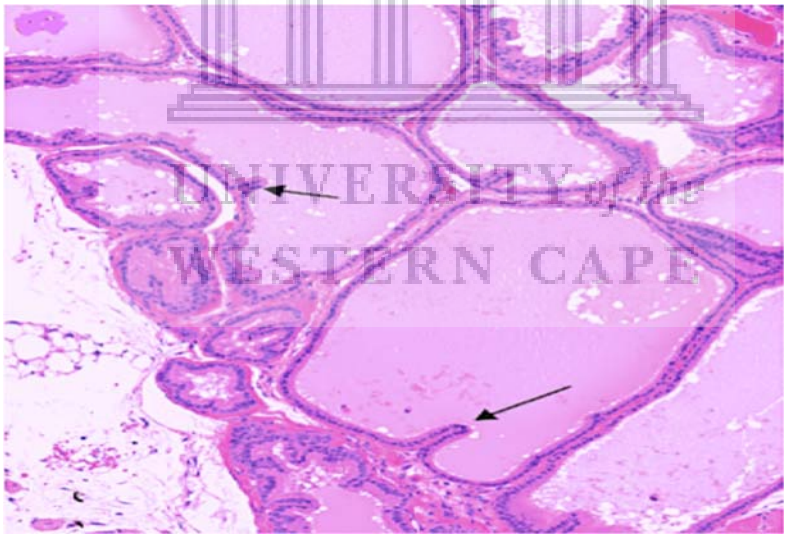

Figure 7: The ventral prostate lined by cuboidal epithelium, with sparse basally infolding and focal tufting (arrows), liminal spaces contain homogeneous, pale, small secretions, uniform and a basic nucleus (Shappell *et al*., 2004).

1.2.2. Steroidogenesis And Spermatogenesis

Steroidogenesis is a process that involves the enzymes necessary for the biosynthesis of steroid hormones from cholesterol in the gonads, adrenal glands and placenta. These enzymes are connected with the adrenal steroid hormones including corticosterone, cortisol and aldosterone. They are also involved in the synthesis of the gonadal steroid hormones including progesterone, estradiol and testosterone. The enzymes can be classifid into two groups of proteins, the first group is the hydroxysteroid dehydrogenases which is classified into four subtypes, including 3β-hydroxysteroid dehydrogenase (3β-HSD), isomerase and hydroxysteroid dehydrogenases. The second group consist of cytochrome P450 heme-containing proteins (CYP11A, CYP17, CYP19, CYP21, CYP11B1 and B2) (Payne & Hales, 2004). Androgen synthesis through steroidogenesis in males is essential for sexual differentiation, fertility, reproduction, lean body mass, physiological homeostasis and blood pressure (Miller & Auchus, 2010).

Spermatogenesis is a process by which spermatozoa develop from germ cells within the male reproductive system. It starts the mitotic division of the spermatogonial stem cells located near the basement membrane of the seminiferous tubules. This division gives rise to two cell types: the type A cells which helps to replenish the stem cells and type B cells that finally differentiate into spermatocytes. The primary spermatocyte divides meiotically (Meiosis I) into two secondary spermatocytes; each secondary spermatocyte divides into two equal haploid spermatids by Meiosis II (Sharma *et al*., 2009).The spermatids are then transformed into spermatozoa through spermiogenesis. However, spermatogenesis does not continue in the mitosis stages. A primary goal of testosterone is to signal the Sertoli cells and germ cells to stimulate spermatogenesis (Walker, 2011).

1.2.3. Male Infertility

Infertility is defined as the inability for a couple to achieve pregnancy after 12 months of regular sexual intercourse without contraception. This may be the result from male related infertility, female reproductive system, or sometimes from the combination of factors (Poongothai *et al*., 2009; Gurunath *et al.,* 2011). Infertility is a medical

problem that has a significant psychological and social impact (Gurunath *et al.,* 2011). The WHO indicated that approximately 15% of couples have an infertility problem (WHO, 2010). Infertility prevalence varies from one geographical region to the othe. It has been reported that in some West African communities, the infertility rate is about 50%, and in European communities around 12%. Also infertility prevlalance varies form developed countries to undereveloped countries (Roupa *et al*., 2009).

Male infertility is associated with reduced sperm production, abnormal sperm function or reproductive tract obstructions that prevent the transmission of sperm. It can also be caused by injuries and chronic health problems, lifestyle choices and age factors which lead to a decrease of ejaculate volume, concentration, motility, morphology, and/or increased levels of polymorphonuclear elastase (PMN) in elderly men (Henkel *et al*., 2007). Oxidative stress (OS), which is as a result of imbalance between ROS and antioxidants in the body, has also been implicated to play a role in the cause of male infertility (Makker *et al.*, 2009). Though ROS is important for cellular physiological processes, when generated excessively, it could hamper cell function. Common endogenous ROS include the hydroxyl ion, superoxide, hydrogen peroxide, radical peroxyl sand hypochlorite ions (Makker *et al.*, 2009).

Infertility poses a serious reproductive health challenge to couples as they suffer from from involuntary childlessness, and this is increasing in Africa. Studies have shown that men are susceptible to infertility in South Africa, as cases of low sperm count have been reported. Sexually transmitted diseases are the most common causes of male infertility in South Africa (Dyer *et al.*, 2004).

Infertility is a complex and serious disease that can affect both males and females. Causes of infertility can be from anatomical, endocrinological, immunological, infectious, thrombophilic disorders as well as other unknown causes. In addition, common risk factors of both female and male infertility include alcohol consumption, diabetes, smoking habits and urethral infections. Certain medications, such as antibiotics, antihypertensives or anabolic steroids can also affect fertility. Frequent exposure to heat, such as in saunas or hot tubs, can raise the core body temperature and may affect spermatogenesis (Henkel *et al*., 2007; Katib, 2015).

Infertility can also be classified depending on treatment, which can be classified as including congenital anatomic anomalies, that caused by surgery as varicocele, erectile dysfunction therapy, and obstruction of the ductal system in the reproductive tract. Medical symptoms such as immunological conditions, like anti-sperm antibodies, an infectious disease which linked with leukocytopenia, anatomical obstruction, endocrinopathy, exposure to gonadotoxin, systemic illness, spermatogenic dysfunction, and cryptorchidism also czn lead to infertility problems (Meacham *et al*., 2007).

1.2.4. Obesity And Male Infertility

Obesity in men is associated with infertility through a decline in semen parameters, damage in semen quality and hypogonadism (Wake *et al* .,2007; Katib, 2015). This includes negative impact on motility, vitality, morphology, DNA damage and Mitochondrial Membrane Potential (MMP) (Henkel *et al*., 2007; Katib, 2015). In addition, reduced fertility among obese men may be mediated by sexual dysfunction, endocrinopathy, aromatization activity, scrotal thermal effects, toxins and inflammation (Katib, 2015).

1.2.4.1. Pathophysiology Of Obesity Related Male Infertility

Obesity is associated with testiclar toxicity, which negatively affects spermatogenesis and steroidogensis (Sharpe, 2010). This is most apparent in seminiferous tubules structural changes, increased apoptosis, increased OS and hypogonadism (Jia *et al*.,2018).In addition, obesity can negatively impact male fertility via damaged spermatogenesis and increased DNA fragmentation (Chambers & Anderson, 2015).

1.2.4.1.1. Germ Cells And Spermatogenesis

Spermatogenesis conservation in vivo is dependent on adequate glucose metabolism.The blood-testis barrier BTB tightly controls the transport of glucose to germ cells and Sertoli cells. Glucose transport across the BTB is mediated by several

glucose transmit molecules (GLUT's) (Alves *et al.,* 2013). There is increasing sensibility that male obesity reduces sperm quality, through the modification of the physical and molecular structure of germ cells in the testes and mature sperm (Palmer *et al*., 2012).

1.2.4.1.2. Testicular Histology

Histopathological changes in testes of obese males showed suave degeneration of seminiferous tubules and epididymis, the absence of spermatogenic series and spermatozoa in tubular lumen and a significant decrease in the height of the germinal epithelium in the testes and epididymis. These changes induce a negative impact on spermatogenesis, sperm quality and testosterone levels (Harishankar *et al.,* 2011; Hadi *et al*., 2013). Obesity may disrupt both sperm quality and testis histology by deteriorating the testicular structure associated with a high-fat diet (Demirci & Sahin, 2019).

1.2.4.1.3. Semen Quality, Sperm Function And Fertilization

Male obesity is associated with fewer chromatin intact spermatozoa, with fewer normal motile spermatozoa and redcued normal morphology (Kort *et al*., 2006). Male obesity can affect sperm function and physiology through oxidative stress increased sperm DNA damage (Bakos *et al*., 2011). Furthermore, cysteine-rich secretory protein 4 (CRISP4) can be decreased in the testis and epididymis in obese rats, and this can lead to an induced decline in sperm motility and fertilization capacity (Borges *et al*., 2017).

1.2.4.1.4. Hypogonadism

Obesity causes a low concentration of total testosterone concentrations which leads to unfavourable modification of the hypothalamic-pituitary-testicular axis (HPT) (Grossmann *et al*., 2018). Hypogonadism is a biological mechanism that can mediate the relationship between obesity and male infertility, as well as induction of leptin/insulin resistance (Phillips & Tanphaichitr, 2010).

1.2.5. Clinical Investigation Of Male Fertility And Infertility

The sperm concentration, motility, viability and morphology are important for the spermatozoa as a fertility indication, including quality and formation of the seminal fluid, semen volume, liquefaction, viscosity, pH and leukocyte concentration according to World Health Organization (WHO) (WHO, 2010). Hormonal assessment may include testosterone, FSH and LH (Ford *et al*., 2016). The standard semen analysis according to WHO (2010) includes semen volume (> 1.5 ml), total sperm count (39 x 10^6), sperm concentration (15 x 10^6/ml), progressive motility (> 32%), total motility (> 40%), vitality (> 58%), morphology (> 4% normal forms), leukocyte concentration (< 1 x 10^6/ml) (WHO, 2010).

The mitochondrial membrane potential (MMP) describes mitochondrial energy reversal in the form of ATP, by abnormalities in the structure of the sperm mitochondria which are linked to sperm motility and infertility. Furthermore, a study of sperm energetic metabolism in animals fed with a high-fat diet shows weakness in the recreation of pyruvate, lactate dehydrogenase, citrate synthase, and respiratory chain complexes, reducing the cellular levels of adenosine triphosphate (ATP) and an increasing oxidative damage (Ferramosca *et al*., 2016). Asthenozoospermic males usually possess low enzymatic activities of the electron transport sequences. MMP is connected with other sperm functions, as a reduction of MMP and acrosome reaction causes an increase of levels of ROS, and decreased fertilisation rates. Spermatozoa that have intact MMP usually have intact acrosome reaction, high fertilisation potential and normal morphology (Grunewald *et al.,* 2008; Agnihotri *et al*., 2016).

1.2.6. Treatment And Management

Education abuut infertility and any associated diagnosis is the first approach, including relevant risk factors and lifestyle variables (Esteves *et al.,* 2012). Infertility treatment is classified into specific medical therapy (known causes) and non-specific medical therapy (unknown causes). The use of specific medical therapy involves treatment of urogenital infections, hypogonadism and erectile dysfunction, non-

steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, gonadotropin-releasing hormone and phosphodiesterase-5 inhibitors. All these drugs are only effective for approximately 20% of patients (Hamada *et al*., 2012). Non-specific medical treatments include hormonal treatment such as aromatase inhibitors, 5α-reductase inhibitors and testosterone, and anti-inflammatory, antidiabetic, and antioxidant therapies (Liu *et al*., 2017). However, the use of antioxidants to increase fertility is still not clear, hence the need fo further studies (Hamada *et al*., 2012). Herbal and alternative approaches indicting efficacy in male infertility includes *Nigella sativa*, *Camellia Sinensis*, *Rhizoma Coptidis* (Huang Lian), *Panax ginseng*, *Ren shen*, *Radix Lithospermi*, *Ephedra sinica* (MaHuang), *Rheumpalmatum*, *Da-Huang*, *Astragalus membranaceus* and *Ganoderma lucidum* (Lingzhi or Reishi) (Liu *et al*., 2017).

1.3. ***Nigella Sativa***

1.3.1. Scientific Classification

Medical plants have been a major source of traditional therapy since ancient times to treat human diseases. According to the WHO, 80% of people still depend on herbal medicine for their health care (Al-Attar *et al.*, 2010; Asgary *et al.,* 2012). *Nigella sativa* (Ns) is extensively used in recipes in the kitchen, and has been used as a medicine for thousands of years (Tembhurne *et al.*, 2014), and is commonly known as black seed or Kalonji seed, and habba albaraka (Arabic phrase). The name of Ns comes from the Latin word, *nigellus* meaning black. The seeds are dark gray-to-black, with a white wrinkled inside aromaticity (Tembhurne *et al.*, 2014) as represented in Figure 8 below. Ns seeds contain numerous oils in which health and medicinal benefit are derived (Hajar *et al.*, 1996; Gilani *et al.*, 2004).

Figure 8: *Nigella sativa* as (A) a whole plant, (B) the characteristic flowers and (C) seeds (Sharma *et al*., 2009).

Ns belongs to the Ranunculaceae family, within the Ranunculales order of the Platae division of Magnoliophyta (Ahmad & Gafoor, 2004). It is widely grown in different parts of world and is an annual herb, cultivated mostly in India and Pakistan, Southern Europe, Northern Africa and Asia Minor. Ns contains fixed oils, protein, alkaloids, saponins and essential oils, and has been described as a potent antioxidant, hepatoprotective, anticancer, antidiabetic, antimicrobial, anti-parasitic, analgesic, anti-inflammatory, anti-nociceptive, anti-ulcer and antihistaminic plant. The fruit has several follicles producing many seeds, and is brownish when it is ripe. Ns has been traditionally used for different conditions and related therapies, for respiratory, stomach and intestinal health, as well as kidney and liver function, circulatory and immune system support. The seeds have been traditionally used in the Middle East and Southeast Asian countries to treat ailments such as asthma, bronchitis, rheumatism and related inflammatory diseases. The seeds are also used to boost milk production in nursing mothers, improve digestion and to fight parasitic infections. Oil from the seeds were used to treat skin conditions, such as eczema and

boils, and to treat cold symptoms. Recent findings has shown that Ns can also be used to treat patients with type 2 diabetes (Hussain & Hussain, 2016). In addition, Ns can be used to improve abnormal semen quality via the improvement of sperm count, motility, morphology, semen volume, pH and round cells (Kolahdooz *et al.,* 2014). The seeds are also used to produce warmth, especially in cold environments, as they can induce sweating (Sultan *et al.*, 2009).

Ns seeds have been analysed and shown to have a high nutritional potential. This includes fat (~40%), carbohydrates (~32%) and protein (~22%) (Abdel-Aal & Attia, 1993; Datta *et al.*, 1987; Hajar *et al.*,1996), as well as micronutrients including iron (105 mg/Kg), copper (18 mg/Kg), zinc (60 mg/Kg), phosphorus (527 mg/Kg), calcium (1860 mg/Kg), thiamine (15.4 mg/Kg), niacin (57 mg/Kg), pyridoxine (5.0 mg/Kg) and folic acid (160 µg/Kg) (Takruri & Dameh, 1998; Bilal, 2008).

1.3.2. Characteristics

Ns has a strong agreeable aromatic and spicy odour, similar to nutmeg, and smells like strawberries (Sultan *et al.*, 2009; Maffei *et al.,* 2011). The plant is an annual herb of about 45 cm in height, with slender leaves of 2-4 cm, sallow blue flowers with 5–10 petals and a long stalk, as well as trigonous seeds that are black colour (Sharma *et al*., 2009) (Figure 8). Ns oil is yellowish in colour, very stable and can be preserved for long period of time due to its large polyphenolic content (Cheikh-Rouhou *et al.*, 2007). The ground seeds are a solid powder, greyish black in colour and with a hot odour. Ns seeds contain a total water isoluble ash of 4.5% and acid-insoluble ash of 0.33% (Hajhashemi *et al*., 2004).

1.3.3. Pharmacognasy Of *Nigella Sativa*

Ns is characterised by many chemical components. The pharmacologically active constituents of the volatile oil from the seeds are thymoquinone (TQ), dithymoquinone, thymol and thymohydroquinone (Figure 9) (Al-majed *et al.*, 2006). Dithymoquinone is a two-fold thymoquinone. The other constituents of the volatile oil of the seed are p-cymene carvacrol, t-anethole, 4-terpineol and longifolene (Ghosheh *et al.*, 1999; Hajhashemi *et al.*, 2004). Four alkaloids have been reported as

constituents of Ns seeds, namely nigellicine, nigellidine, nigellicimine and nigellicimine-N-oxide. A triterpene saponin, alfa heredin, has been isolated from the seeds of Ns. Alfa heredin has been identified to have anti-tumor activity (Ali & Blunden, 2003; Yessuf *et al* 2015).

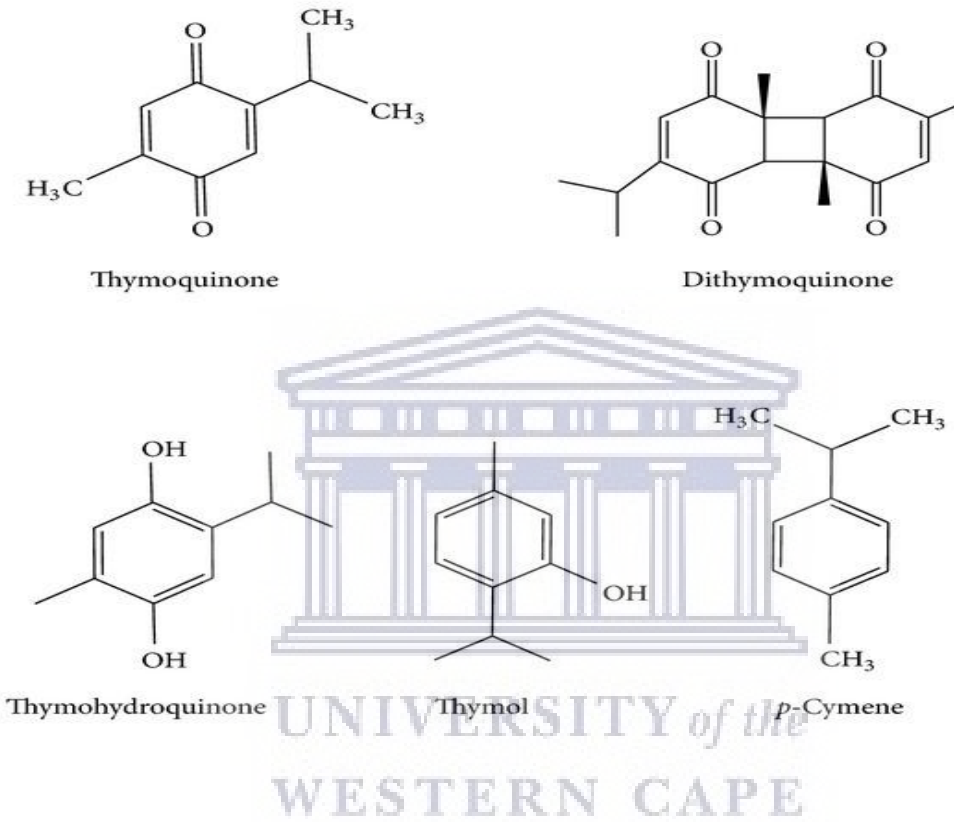

Figure 9:Chemical structure of active ingredient of *Nigella sativa* essential oil (Al-majed *et al*., 2006)

1.3.4. Medical Uses (Scientific Review)

Ns oil appears to have a strong antimicrobial activity against *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Vibrio cholerae*. In an *in-vitro* study on the volatile oil, Ns showed a similar activity to ampicillin, and was found to have a synergistic action with streptomycin and gentamicin (Sharma *et al.*, 2009). Its impact on cancer has also been reported, as compounds such as thymoquinone show potential in cancer treatments (Kolahdooz *et al.,* 2014;Hussain & Hussain, 2016). Ns has been shown to impact on neurological and psychiatric problems, such

as pain contriol, Parkinson's disease, epilepsy, anxiety and reduces the risk of drug dependence, as well as aids in improvement in learning and memory, alertness, elevation of mood, is renal protective and gastro protective (Alenazi, 2016). In addition, Ns is found to have anti-malaria properties, by acting against *Plasmodium falciparum* which causes cerebral malaria (Mathur *et al.*, 2011; Onifade *et al*., 2013). Antihypertensive, diuretic, liver tonic, anti-diarrheal, digestive, appetite stimulant, analgesic and antibacterial potentials have been reprted (Ahmad *et al.,* 2013).

Ns can be used for obesity by decreasing total body weight and waist-to-hip ratio (Hasani-Ranjbar *et al.,* 2013). Ns can be used as an effective therapy for patients who suffer from metabolic syndrome (Najmi *et al*., 2008). Management of metabolic syndrome in the context of menopause, through the lipid profile including an improvement in triglycerides (TG), low-density lipoprotein cholesterol (LDL), total cholesterol (TC), high-density lipoprotein cholesterol (HDL) and blood glucose levels, has been reported (Ibrahim *et al*., 2014). Traditionally, the seeds are used against diabetes, by affecting glucose tolerance, and lowering the glucose levels and by stimulating insulin secretion from pancreatic β-cells. Ns can positively modify the blood glucose level and immune system in diabetics (Hussain & Hussain, 2016).

The role of Ns in improving male reproductive parameters has also been reported. It was shown that administration of Ns improved sperm vitality by an increase in seminal vesicle weight and testosterone levels, and sperm parameters including sperm motility and sperm count, and a lowering of sperm morphological abnormalities (Bashandy, 2007). Ns plays an important role as an antioxidant. TQ in the seed oil protects against oxidative damage which is induced by a variety of free radical generating agents, such as carbon tetrachloride including the alkylating agents, cisplatin, and doxorubicin. Past studies show that the TQ can protect sperm and semen against testicular toxins, and increase the number of Leydig cells. Ns has also reported to increase testosterone levels and testicular somatic index, germinal cells, with the testis and epididymis showing increasing weight (Aithal *et al.,* 2016; Tembhurne *et al*., 2014). In addition, there was a significant increase in sperm count, sperm motility and sperm morphology in infertile men during three month treatments with Ns (Marbat *et al.,* 2013).

1.4. Problem Statement

Obesity is defined as presence of excess adipose tissue, increasing risk of cardiovascular risk and type 2 diabetes mellitus. This also leads to decreased reproductive function in males, negatively affecting spermatozoa concentration, motility, vitality, morphology and mitochondrial membrane potential, as well as inducing hypogonadism. Ns seed extracts (oil) have traditional and empirical evidence to suggest an improvement in obesity related derrangements and male fertility parameters. However, further research in obesity models are required to further nveitigate the potential of NS seed oil on male besity induced reproductive dysfunction and subfertility parameters.

1.5. Research Question

How can *Nigella sativa* oil affect on male reproductive function in male Wistar rats exposed to an obesogenic diet.

1.6. Hypothesis

A high sugar diet will increased body weight, organ weights, fertility parameters, tesiticular and prostate morhohology and serum testostone compared to normal chow. The administration of Ns oil and metformin will positively improve these parameters.

1.7. Research Aims And Objectives

The aim of the study is to investigate the *in vivo* effects of *Nigella sativa* seed extract (oil) on body weight, omentum and reproductive organ weights, semen parameters, serum testosterone and reproductive organ histology in male rats exposed to an obesogenic diet.

This aim is to be achieved with the following objectives:

1. To determine the impact of a high suger diet on male rats compared to rats fed a normal chow.
2. To determine the potential of metformin to improve parameters in rats exposed to a high suger diet compared to a saline control.
3. To determine the potential of Ns oil to improve parameters in rats exposed to a high suger diet compared to a saline control.

CHAPTER 2

MATERIALS AND METHODS

2.1. Overview Of Experimental Design

Male Wister rats (n = 48) weighing 180 - 220 g were obtained from Tygerberg Hospital (Tygerberg, South Africa) and housed in standard cages within the animal facilities unit of the Department of Medical Bioscience (MBS), University of Western Cape (UWC) (Bellville, South Africa). The animals were housed under controlled temperature between 21 - 23°C, and an artificial 12-hour light/dark cycle. Principles of animal care were conducted in accordance with the European Community guidelines (EEC Directive 1986; 86/609/EEC) following ethical clearance from the Institutional Review Board at UWC (Ethics Clearance Registration no: 15/4/102).

The rats were randomly allocated into 6 groups as follows: (i) a negative control (Saline) group (n = 8); (ii) a positive control (Metformin 75mg/Kg/day) group (n = 8); (iii) the *Nigella sativa* 200 mg/Kg/day (NS200) group (n = 8); (iv) the *Nigella sativa* 400 mg/Kg/day (NS400) group (n = 8); (v) the high sugar diet (HSD) group (n = 8); and (vi) the normal chow (NC) group (n = 8). The animals were housed in standard cages to a maximum of 3 animals per cage.

In brief, the experimental design was based on an obesogenic HSD model (Huisamen *et al*., 2013). All groups, except the NC group, received the obesogenic diet detailed below for 14 weeks. No intervention was provided for the first 8 weeks. This was followed by a 6-week intervention period conducted on animals in groups i - iv, as well as the negative and positive control groups (v – vi). The HSD group (v) received no intervention in this 14-week period. The NC (vi) group received normal rat chow for the entire 14-week period. The rats were monitored daily for signs of distress, as well as weighed and inspected physically each day for the duration of the 14-week period. Following the completion of the experimental phase, the animals were

euthanized by rapid cervical dislocation (Carbone *et al.*, 2012) and tissues obtained for data analysis. This broad study design is summarised in Table 2 below.

Table 2: Experimental design and treatment regimen.

Group	Group Code	n	Diet Exposure (Week 1 – 14)	Daily Exposure (Week 9 – 14)	Sacrifice
I.	Saline	8	High Sugar Diet	Saline 100 μl/day	14 week
II.	Metformin	8	High Sugar Diet	Metformin 75 mg/Kg/day	14 week
III.	NS200	8	High Sugar Diet	*Nigella sativa* 200 mg/Kg/day	14 week
IV.	NS400	8	High Sugar Diet	*Nigella sativa* 400 mg/Kg/day	14 week
V.	HSD	8	High Sugar Diet	None	14 week
VI.	NC	8	Normal Rat Chow	None	14 week

In order to induce obesity in the rats over the initial 8 week period, as well as throughout the experimental phase, an obesogenic HSD model was used (Naderali *et al.*, 2001; Huisamen *et al*., 2013; Lima-Martínez *et al*., 2016). The HSD consisted of rat chow (Petco, Bellville, South Africa) soaked in water, into which sucrose was dissolved. This was then mixed with condensed milk (Ndlovu Corp Supply, Montague Gardens, South Africa) to produce soft food. The final ratio was 33% rat chow, 33% condensed milk, 7% sucrose and 27% water. The NC group received only normal rat chow. Food and water was freely available to the rats throughout the study.

2.2. Experimental Exposures

2.2.1. Saline

The saline group was given 100 µl of 0.9% saline solution per animal per day for 6 weeks during the exposure phase. This was prepared by dissolving 9 g of NaCl (Sigma-Aldrich, Steinheim, Germany) to 1 L of deionised water. During

the 6-week exposure period, the 100 µl was force fed via oral gavage, as illustrated in Figure 10. This group served as the negative control for the experimental exposures of *Nigella sativa* oil.

Figure 10: Illustrating oral gavage technique for force feeding saline and experimental solutions.

2.2.2. Metformin

The Metformin group served as the positive control for the experimental exposures of *Nigella sativa* oil. During the 6-week exposure period, each animal in this group was administered 75 mg/Kg/day metformin (Kuhlmann& Puls, 2013). The metformin (Adcock Ingram, Johannesburg, South Africa) was administered in 100 µl of saline and force fed via oral gavage. Each tablet, containing 500 mg metformin, was weighed and subsequently ground by hand to a fine powder and dissolved in saline for the preparation of daily dosages to be administered. Calculations of individual dosages were done daily based on the animal weights. The individual rats weight (Kg) was multiplied by 75 (mg/Kg) to obtain the total metformin needed for each rat

each day. For example, a rat weighing 325g (0.325 Kg) would require 22.5 mg (75 mg/Kg x .03 Kg) metformin. Appropriate ground tablet weight was calculated to determine the total amount of tablet powder to dilute in saline for forced oral feeding daily.

2.2.3. *Nigella sativa* oil

Nigella sativa (Ns) oil, cold pressed extraction from the seeds, was obtained from Crede Natural Oils (Asla Park, South Africa). The oil is particluly rich in fatty acides (90.1 g), predominantly polyunsaturated fatty acids(51.7 g) (Table 3). For this study, two different concentrations were administered to the rats (200 mg/Kg/day and 400 mg/Kg/day; NS200 and NS400 groups, respectively) over the 6-week exposure period (Fararh *et al.*, 2004; Abbasnezhad *et al.,* 2015). Calculations of individual dosages were done daily based on the animal weights. This was done by dividing the rat weight (g) by 1000 to deterimine Kg weight, and then multiplying by 200 mg/Kg or 400 mg/Kg dosage to obtain the amount of Ns dosage (Hajhashemi *et al*., 2004; Szulinska *et al*., 2014; Ayuob *et al*., 2016).

Table 3: The contents of 100 ml crude *Nigella sativa* oil (Crede Oils, Alsa Park, South africa).

Average values	Contents of 100ml
Energy	3.331 kilojoules (Kj)
Protein	0 g
Glycaemmic carbohydrate	0 g
Total sugar	0 g
Total fat	90.1 g
Saturated fat	12.6 g
Polyunsaturated fat	51.7 g
Monounsaturated fat	23,7 g
Cholesterol	< 1mg
Dietary fibre	0 g
Total	< 1mg

2.3. Experimental Outcomes and Tissue Preparation

2.3.1. Summary Of Study Outcomes

In this study, the animals were sacrificed after 14-week experimental period followed by cervical dislocation, followed immediately by retrieval of of biological tissues and semen. Total body weight was recorded, alongside organ weights of omentum, testes, epididymis, prostate and gastrocnemius muscle, semen analysis (concentration, motility, vitality and mitochondrial membrane potential), serum testosterone, and histological analysis of the testes, epididymis and prostate. A summary of the outcomes variables and is provided in Table 4.

Table 4: Summary of outcomes for the examined tissues

Tissue	Variable	Method
Prostate Testes Epididymis	Weight; Histology;	Histology processsing (H&E stain)
Gastrocnemius muscle, Omentum	Weight	Scale
Spermatozoa	Sperm Motlity; Sperm concentration; Vitality; Mitochondrial membrane potential	CASA software; Eosin-nigrosin stain; DePsipher staining
Serum	Testosterone	ELISA Analysis

2.3.2. Tissue Preparation

After sacrificing the animal by cervical dislocation, the thorax and abdomen was opened along the longitudinal line. The testes, epididymis and prostate were excised and fixed in Bouin's solution. After fixation, cross section of tissues were cut and placed in casettes for routine histological tissue processing using the automatic tissue processor. A blood sample was obtained from cardiac puncture, and allowed to clot for 30 min in 1.5 ml microcentrifuge tubes (Corning, New York, USA). This was then centrifuged at 3000 x g for 15 min and serum was separated and stored at -20˚C biochemical evaluation. Spermatozoa was obtained from the cauda by cutting open the end of the cauda and using the sperm swim up technique in the Petri dish (35 x 10 mm) containing 1 ml Dulbecco's Modified Eagle's Medium DMEM (Gibco, Auckland, New Zealand), and supplemented with 1% bovine serum albumin (BSA) (Sigma, St. Louis, USA). Motility examination was done immediately, and preparation of slides for vitality and MMP was conducted.

2.4. Total Body And Organ Weights

The animals were weighed in grams (g) by a reliable scale (Amonstar, Guangdong, China) before euthanasia and tissue processing to determin Total Body Weight for data analysis.

2.4.1. Omentum

The omentum, the membranous dual layer of adipose tissue that covers the intestines and organs in the lower abdominal region, was gently removed by forceps, weighed and fixed in Bouin's solution (Broeckhoven *et al.*, 2017). An example of the rat isolated omentum is provided in Figure 11 below.

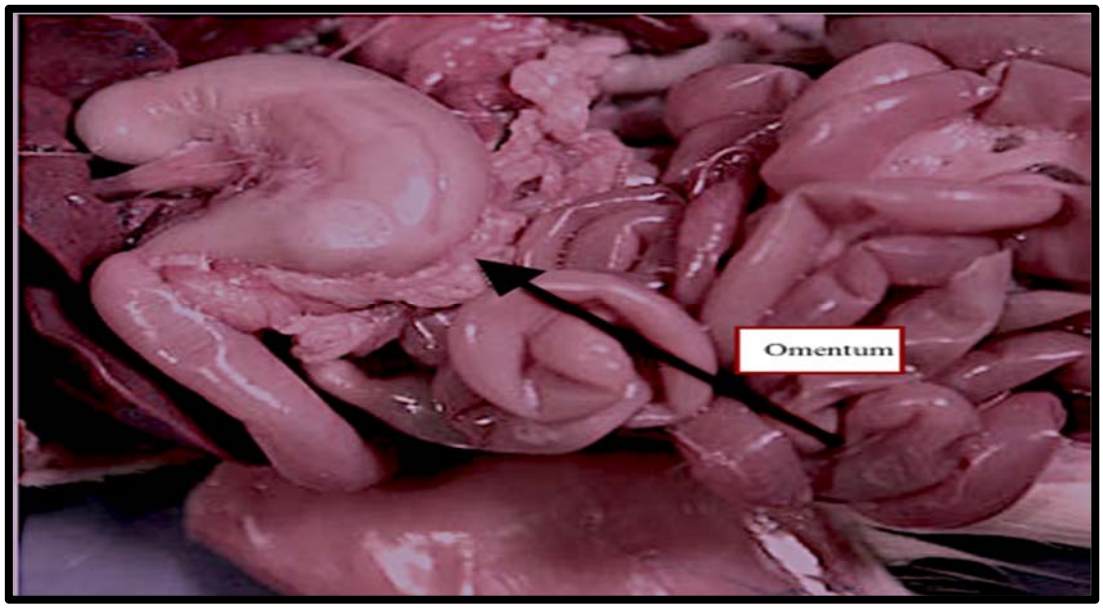

Figure 11: An image of the omentum during dissection. The arrow shows the omentum fat secluded from the greater warp of the stomach.

2.4.2. Gastrocnemius Muscle

The gastrocnemius muscle, located on the posterior aspect of the lower hind leg and functioning to extend the leg, was removed, weighed and fixed in Bouin's solution for subsequent use. This is used as a reflection of lean body mass (Weide *et al.*, 2015). An example of the isolated rat gastrocnemius muscle is provided in Figure 12 below.

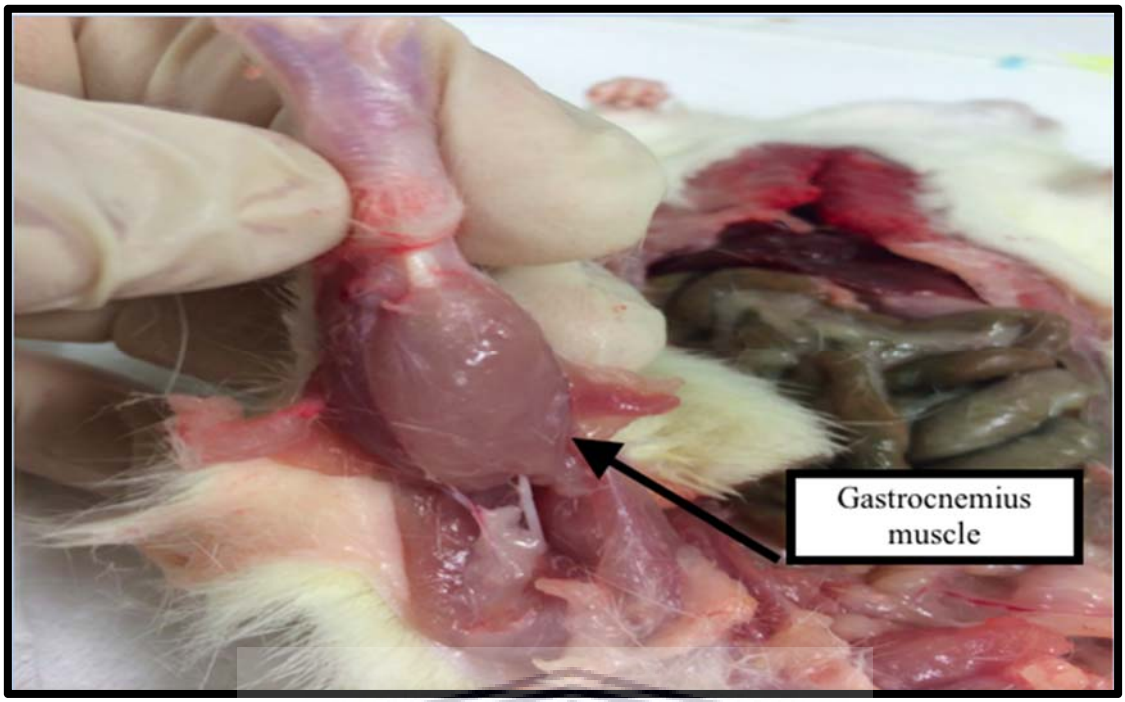

Figure 12: An image of the gastrocnemius muscle from experimental rat during dissection. The arrow shows the exposed gastrocnemius muscle.

2.4.3. Testes

The testis is the male genital gland in mammels is situated within the scrotum, (Figure 13). It is surrounded by the epididymis and adipose tissue (Abdul-Majeed *et al.*, 2014). The testes were removed using anatomical scissors, weighed and placed in Bouin's solution for subsequent investigation. An example of the isolated rat testes is provided in Figure 13 below.

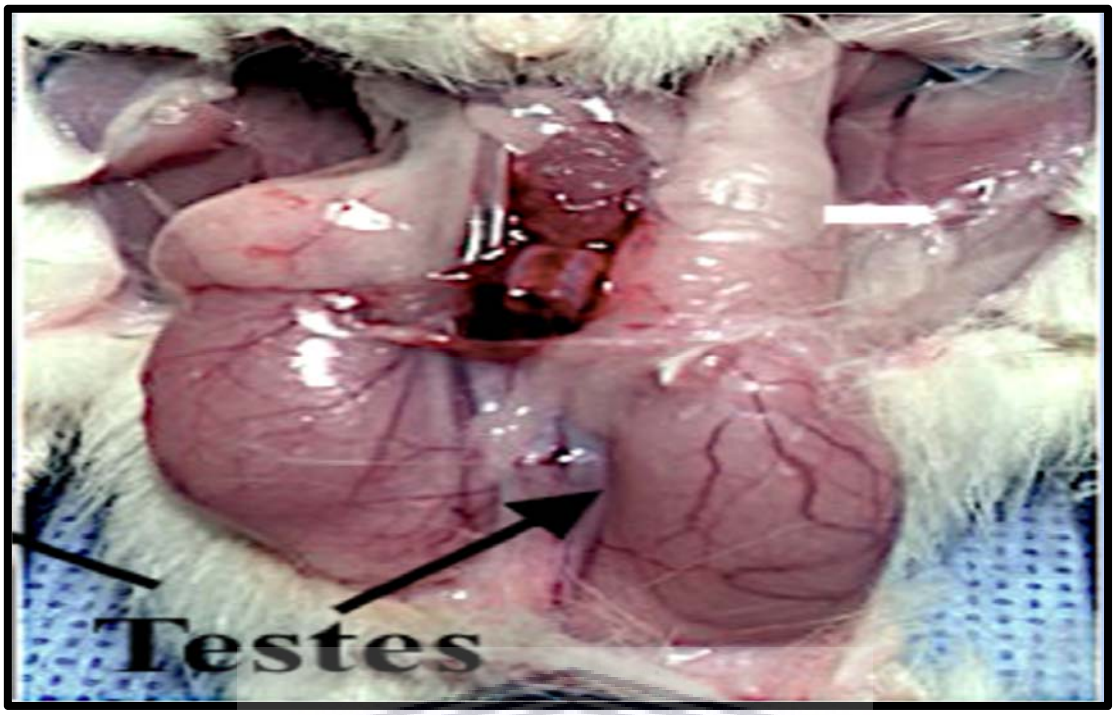

Figure 13: An image of the testes from an example experimental rat during dissection.

2.4.4. Epididymis

The epididymis is anatomically divided into three parts; the caput (head), the corpus (body), and the cauda (tail). The cauda is further sub-divided into a proximal and distal end. The cauda has a more solid myoepithelium than the head area, as it is involved in absorbing fluid to make the sperm more concentrated (Knoblaugh &True ,2018). The epididymis was separated gently from the testis, using dissecting scissors, and then weighted and placed in Bouin solution for subsequent use. Figure 14 illustrated the body of the epididymis and its position after the dissection process.

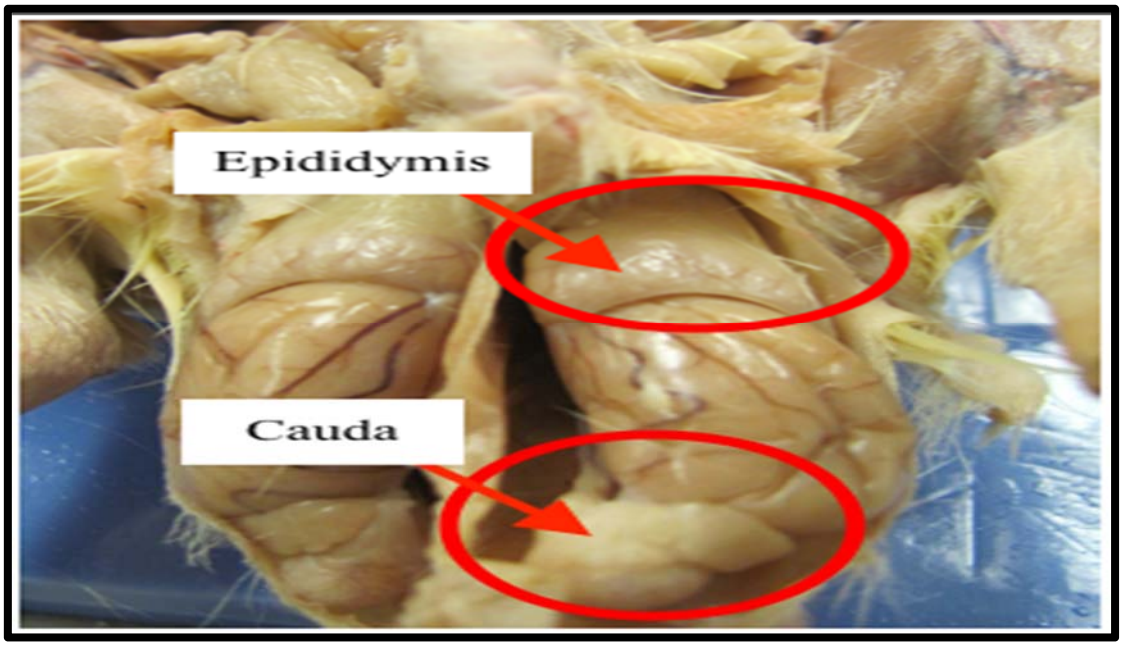

Figure 14: An image of the epididymis and cauda, as labelled, from an example experimental rat during dissection.

2.4.5. Prostate

The prostate gland functions primarly to contribute prostatic fluid to the ejaculate, which contributes 20 - 30% of the semen volume for nutrition and protection of spermatozoa (Knoblaugh &True, 2018). The prostate is attached to the bladder and there are two seminal vesicles located posterolaterally around it. This was removed via dissecting scissors, weighed and placed in Bouin solution for subsequent use. An example of the isolated rat prostate is provided in Figure 15.

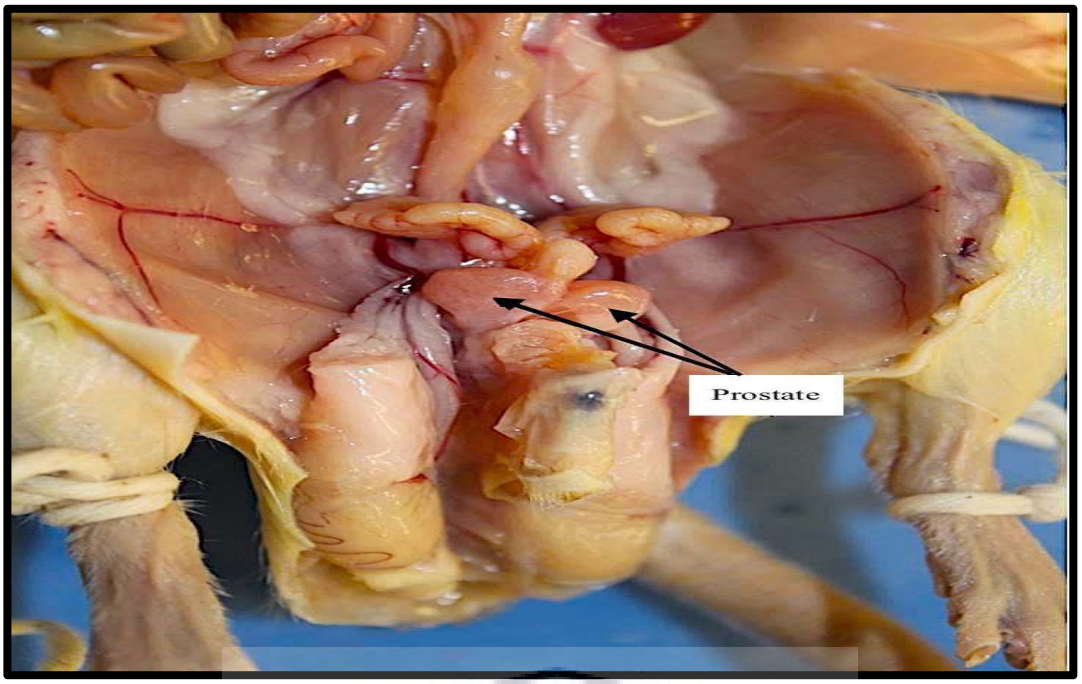

Figure 15: An image of the prostate, labelled with the prostate, from the experimental rat during dissection.

2.5. Histology

A suitable sample of the tissues were carefully removed as previously described and placed in appropriately labelled cassettes. Once the tissues were placed in the cassettes, the cassettes were placed into the automatic tissue processor in the rack Figure 16.

The processor was programmed for 18 hours as follows: 70% Alcohol (2h); 80% Ethanol (2h); 90% Ethanol (2h); 100% Ethanol 1(2h); 100% Ethanol 2 (2h); xylene (2h); xylene (2h); paraffin wax (2h); and paraffin wax (2h).Once the 18 hours cycle was complete, the cassettes were removed from the tissue processing rack (Table 5 shows the tissue processing steps).

Table 5: The 18 hour cycle of processing tissues for histology examination**.**

1	70% alcohol	2hour
2	80% alcohol	2hour
3	90% alcohol	2hour
4	Absolute alcohol 100% 1	2hour
5	Absolute alcohol 100% 2	2hour
6	Xylene 1	2hour
7	Xylene 2	2hour
8	Wax bath 1	2hour
9	Wax bath 2	2hour

A small amount of wax (Kimix, Eppingdust, South Africa) was placed inside the mould and care was taken to ensure that it covered the base of the mould evenly. The tissue was then delicately removed from the cassette and placed into the mould. More wax was added to the mould in order to completely cover the tissue specimen. The mould was then placed in a laboratory freezer (-20 °C) for about 10 minutes on ice. This was done to make the wax harden and make it easy to cut with a microtome. The wax was removed from the mould via a dissecting knife.

A microtome was used to cut the tissue into sections, the common angle used was 6° and 5µm for the study sections. The cut sections were carefully placed in a water bath heated to 45°C and sections were then picked up using clean microscope slides and left to dry. Following this, the slides were placed on a staining rack and then placed into a hot air oven at 60 °C for approximately 30 minutes to fix the tissue on the slide and to melt the wax.

The sections were then ready for staining with haematoxylin & eosin (H&E) (Meddah *et al.*, 2009). The H&E stain was prepared by dissolving 1 g of hematoxylin (Sigma-Aldrich, Steinheim, Germany) and 50 g of potassium aluminium (Sigma-Aldrich, Steinheim, Germany) in a litre of distilled water

dH_2O containing 0.2 g of sodium iodate (Sigma-Aldrich, Steinheim, Germany). This was left to stand overnight, and then 50 g of chloral hydrate 50 g (Sigma-Aldrich, Steinheim, Germany) and 1 g citric acid (Kimix Chemicals, Eppingdust, South Africa) was added to the solution which was boiled for 30 minutes. After it cooled down to room temperature, it was filtered using a filter paper (Sigma-Aldrich, Steinheim, Germany) (Munktell size 110mm, 100g/m3, grade 393).

Preparation of Eosin was as follows: Eosin 1% (10mg) (Sigma-Aldrich, Steinheim, Germany) was added with phloxine 1% (10mg) (Sigma-Aldrich, Steinheim, Germany) dissolved in 100 ml dH2O. The slides containing the tissues were placed on to a staining rack for staining, starting with the Xylene and ending with Eosin Table 6.

Table 6: Procedure for Haematoxylin and Eosin stain**.**

1	Xylene	5 minutes
2	100% Ethanol	5 minutes
3	Concentration 90% and 80% Ethanol	5 minutes
4	Haematoxylin	15 minutes
5	Rinse in tap water	1 minutes
6	Scott's tap water	2 minutes
7	1% Acid Alcohol	2 minutes
8	Eosin	3 minutes
9	Rinse in tap water	1 minutes
10	Concentration 80%,90%.100%,Ethanol	2 minutes
11	Xylene	2minutes
12	DPX (Distyrene Plasticizer xylene) one drop , then microscopic examination (Figure 16)	

After staining, the slides were allowed to dry. Therafter, a drop of the DPX (Kimix Chemicals, Eppingdust, South Africa) was placed onto each slide and mounted on a coverslip, as illustrated in, Figure 16.

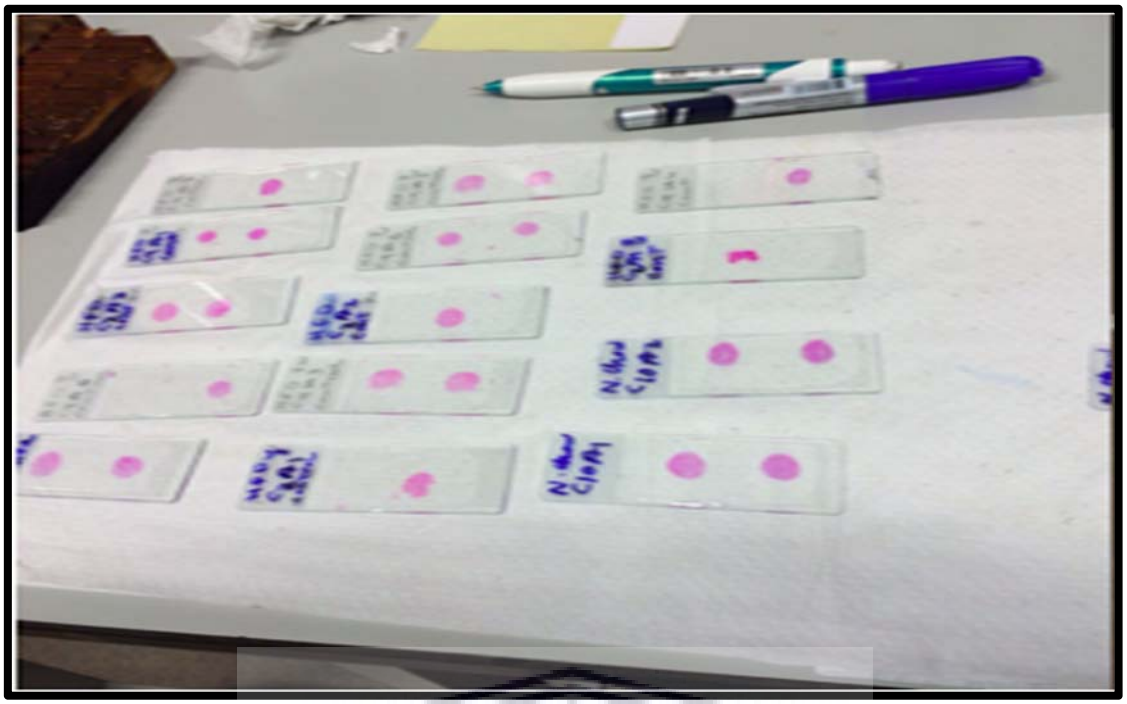

Figure 16: Slides format after using DPX.

After that, the tissue examination was performed by ScopeTek Scope Photo, Version x86, 3.1.386 software (Hangzhou Opto-Electric Co, Ltd., Zhejiang Province China). Parameters assessed for data analysis included lumen diameter, length and width, and the epithelial cell height of the seminiferous tubule and epididymis at 100x magnification. At least 30 tubular profiles (five fields) were randomly chosen and measured per animal.

2.6. Spermatozoa Isolation And Assessment

The head of caput epididymis was isolated and placed in a petri dish (35 x 10 mm) containing 1 ml Dulbecco's Modified Eagle's Medium DMEM (Gibco, Auckland, New Zealand), and supplemented with 1% bovine serum albumin (BSA) (Sigma, St. Louis, USA). A total of 1 ml of the medium was placed in a second petri dish, and the distal caudal epididymis was isolated and placed in the dish in an incubator at 37℃ (Gwayi & Bernard, 2002). A split was made in the cauda to allow the sperm to 'swim out' into the medium for about 10 seconds. Sperma concentration and motility analysis, including static,

progressive, non-progressive, was determined using the analyser system illustrated in Figure 17.

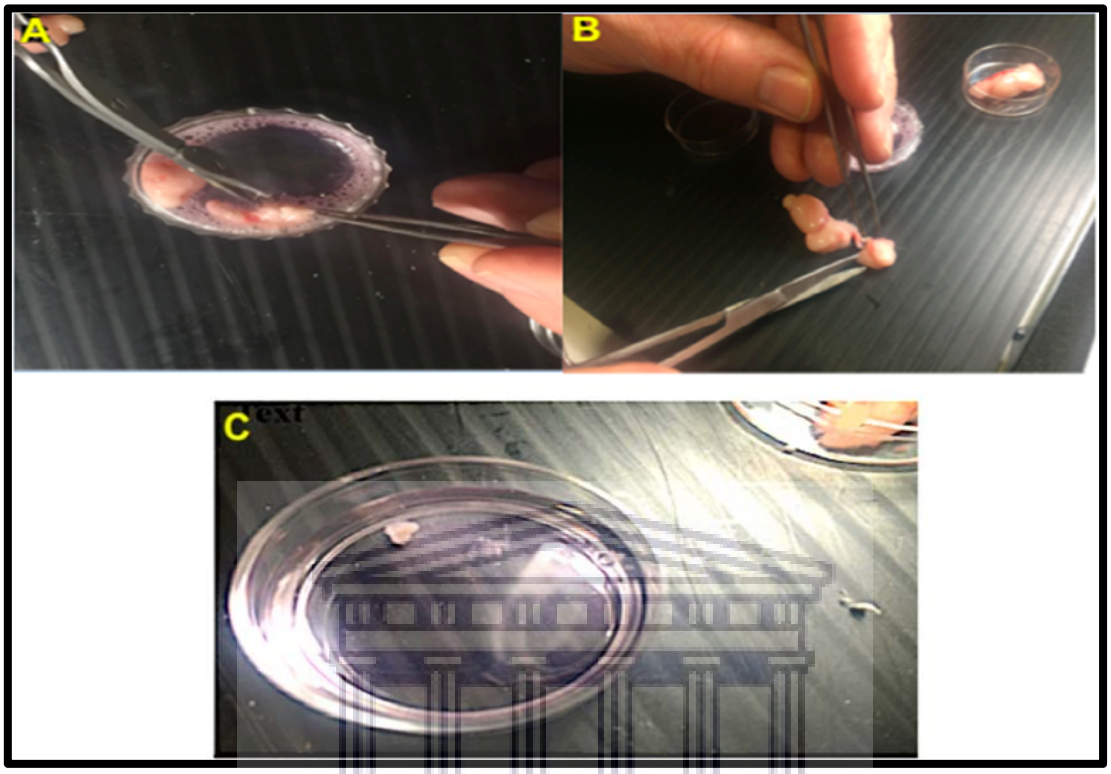

Figure 17: Illustrations of the techniques used to isolate spermatozoa using the swim out method. As well as the isolation of the cauda (A, B) as shown, featured in this figure are examples of the petri dish (35 mm x 10 mm) containing media (DMEM and BSA) (C).

2.6.1. Determination Of Sperm Concentration Motility

Spermatozoa concentration and motility assessment was conducted using the Computer Assisted Semen Analysis (CASA) (Rothschild & Gray, 2015) equipment and software (SCA® version 6.0) (Microptic S.L, Barcelona, Spain). A Basler A312FC digital camera, mounted onto a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa), was used for all CASA analyses. The microscope was provided with a 10X objective, phase contrast optics and a pre-heated stage of 37°C. In order to analyse the semen sample, 2 µl of the spermatozoa, which was obtained from the swim out from a cauda

epididymidis, was prepared in medium at the temperature of 37 °C and then placed onto a Leja slide (Leja, Nieuw Vernep, The Netherlands). Thereafter, the slide was viewed under the microscope. These steps were done within 10 minutes from via out spermatozoa to swim until complete the analysis.

A minimum of 200 motile spermatozoa over a minimum of two different frames from every three or four microscopic field were analysed for each sample, and an average of the two different analyses were taken for the data to be included for statistical analysis. Figure 18 provides a photomicrograph example of the sperm concentration and motility assessment as they appear in CASA.

Figure 18: Sperm concentration and motility assessment as they appeared in the CASA. This figure specifically indicates motility, with red and green indicating progressively motile sperm, blue representing non-progressively motile sperm and yellow representing immotile sperm.

2.6.2. Sperm Vitality

In order to determine spermatozoa vitality, the eosin-nigrosin staining technique was used (Mortimer *et al*, 1990; Björndahl *et al.*, 2004). This eosin-nigrosin stain was prepared in the laboratory by dissolving 0.67 g eosin Y (Sigma-Aldrich, Steinheim, Germany) and 0.9 g of sodium chloride (Merck, Wadeville Gauteng, South Africa) in 100 mL of deionised water in a glass beaker. The solution was placed on a stirring hot plate, heated gently, and stirred until completely dissolved. Following this, 10 g of nigrosin (Sigma-Aldrich, Steinheim, Germany) was added to the solution, dissolved completely and boiled for 10 minutes. This was then removed from the hot plate, cooled to room temperature and filtered through grade 393 filter paper (Lasec, Cape Town, South Africa). This was stored at 4°C in a light protected glass bottle.

To assess each spermatozoa sample, 50 µl of eosin-nigrosin stain was mixed gently with 50 µl of a spermatozoa sample separately, and 20 µl of the mixture was then smeared to a microscopy slide. After drying, the slide was analysed under the microscope (Zeiss, Oberkochen, Germany) at 100x magnification. A total of 200 spermatozoa were counted for each slide, and the percentage of live (viable) sperm was calculated from spermatozoa that remained white (indicates an intact cell membrane with the stain). Dead spermatozoa appeared pink or red (stain taken up into the cell due to a damaged cell membrane) (Chemes *et al.*, 2003). Examples of these staining differences showing a dead and a live (vital) spermatozoa are provided in Figure 19 below. An average of two separate samples was taken for the statistical analysis for each animal.

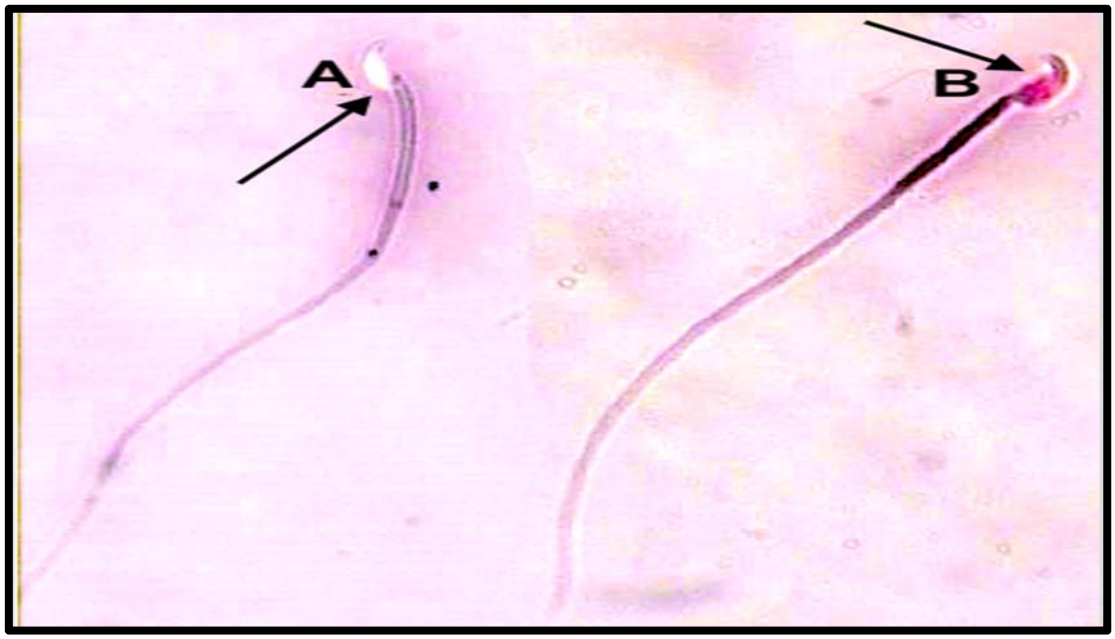

Figure 19: Eosin-Negrosin stain showing live spermatozoa as white (A), and dead spermatozoa as pinkish or red (B) (X100 magnification).

2.6.3. Mitochondrial Membrane Potential (MMP)

MMP was assayed using the DePsipher staining kit (Trevigen, Gaithersburg, USA). The reaction buffer was diluted with distilled water 1:10, and 20 µl stabilizer was added per 1 ml reaction buffer with 1 µl of DePsipher dye. This solution was added to 500 µl prepared reaction buffer, vortexed rigorously and centrifuged for 1 min at 10 000 g. The supernatant was discarded, and then placed into a test tube ready for immediate use. Subsequently, the spermatozoa were re-suspended in 50 µl of DePsipher solution and incubated for 20 min at 37°C in the dark. After incubation, 10 µl of each sample was viewed with 488 nm excitation and 590 emission filters at 1000 x magnification with oil immersion on an epifluorescence microscope (Zeiss, Oberkochen, Germany).

A total of 200 spermatozoa were manually counted. Cells with disturbed MMP showed in green fluorescence with an emission of 520nm, whilst spermatozoa with intact MMP showed red fluorescence at 590 nm. An average of two samples was obtained for each animal for data analysis, and data were

expressed as a percentage of sperm with intact MMP (red). Figure 20 below provides an example of the staining techniques under fluorescent microscopy.

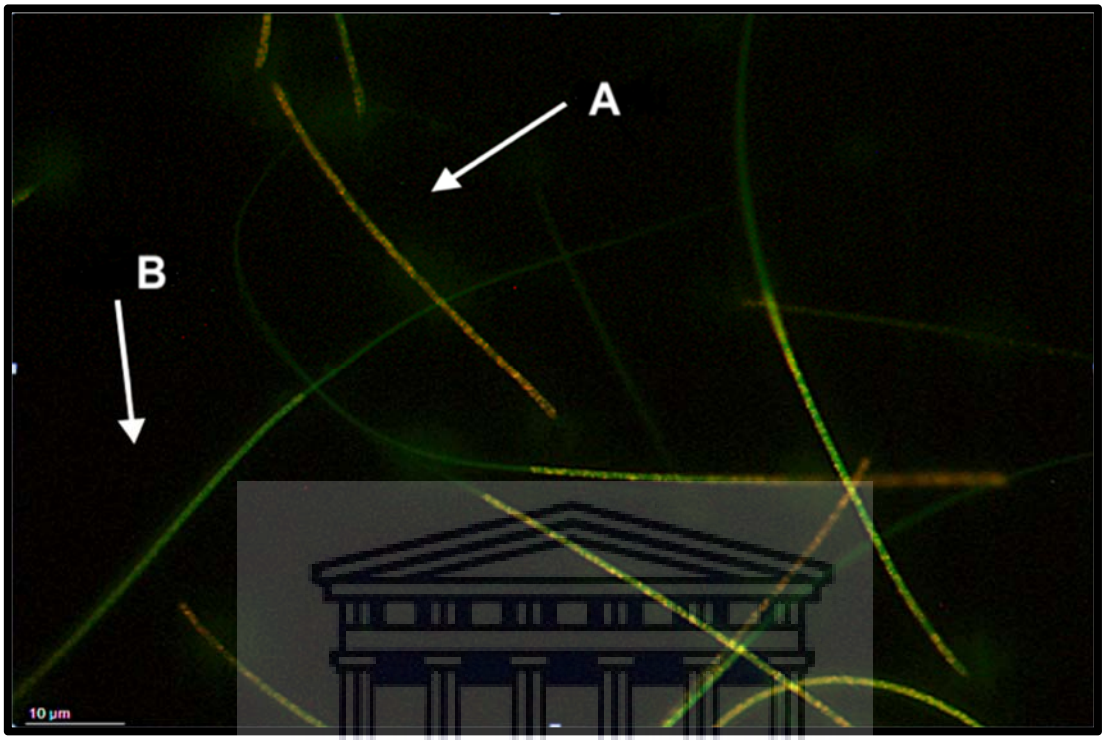

Figure20: Photomicrograph of Mitochondrial Membrane Potential. intact Spermatozoa colour red (A). Fluorescence, and disrupted one have green fluorescence (B). By using DePsipher kit (X10 magnification).

2.7. Determination Of Testosterone Test (ELISA)

Testosterone concentration was determined using the the Testosterone ELISA kit (DRG Instruments GmBH, Marburg, Germany), for quantitative *in vitro* diagnostic measurements of testosterone in serum or plasma. The assay was done according to the manufacturer's instructions. In brief, 25 µL of each standard (0, 0.2, 0.5, 1.0, 2.0, 6.0, 16 ng/ml) and sample was added into each well containing 200 µL enzyme conjugate and incubated for 60 minutes at room temperature without covering the plate. Following incubation, the wells were washed 3 times with washing buffer (prepared by adding 30 ml of buffer to 1170 ml distilled water). Residual droplets were removed by striking the wells sharply on absorbent paper, 200 µL of substrate solution then added to

each well and incubated for a further 15 minutes at room temperature. The reaction was stopped by adding 100 µL stop solution to each well. This was read at 450 nm using a Microplate Reader Model:LT- 4000 (Labtech, East Sussex, UK). Using the standard curve (an example is illustrated in Figure 21), the concentration of testosterone in the experimental samples was calculated for data analysis. The obtained results are expressed as ng/ml.

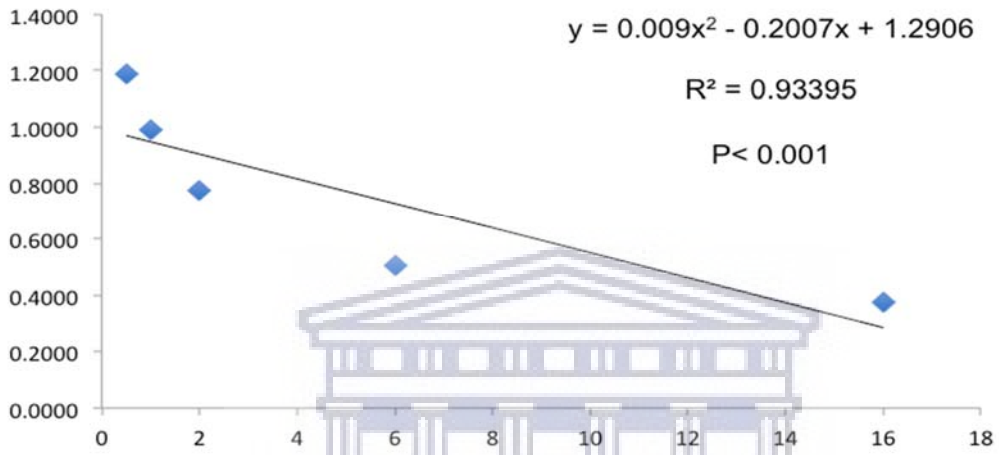

Figure 21: Standard curve representing serum testosterone concentration [ng/ ml].

2.8. Statistical Analysis

Statistical analysis was done by using Medcalc statistical software (Version12.1.3.0, Mariakerke, Belgium). To test for normal distribution, Kolmogorov-Smirnoff test was done, followed by an independent sample t-test if data was normally distributed. However, if data was not normally distributed, the Mann-Whitney test would be performed instead. Data was expressed as mean ± standard deviation, and a P-value of < 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1. Total Body Weight and Organ Weights

3.1.1. Total Body Weight

The summary data of the total body weight (TBW) immediately prior to sacrifice of the experimental animals is shown in Table 7, and the comparative analysis is illustrated in Figure 22. The results show that TBW was significantly higher in the saline group when compared to the Metformin (P = 0.0345), NS200 (P = 0.0057) and NS400 (P = 0.0048) groups. The HSD group displayed a significant increase (P< 0.0001) in mean TBW compared to the NC and all other experimental groups expect the saline group.

Table 7: Data summary for the total body weight (g) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Total Body Weight (g)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	362.8 ± 34.6	347.00	327.00 - 436.00	Normal
Metformin	n=8	323.9 ± 34.3	311.00	287.00 - 385.00	Normal
NS200	n=8	319.5 ± 21.4	320.00	284.00 - 350.00	Normal
NS400	n=8	316.7 ± 24.4	313.00	288.00 - 348.00	Normal
NC	n=8	318.1 ± 35.5	308.00	279.00 - 380.00	Normal
HSD	n=8	388.2 ± 22.8	388.00	355.00 - 425.00	Normal

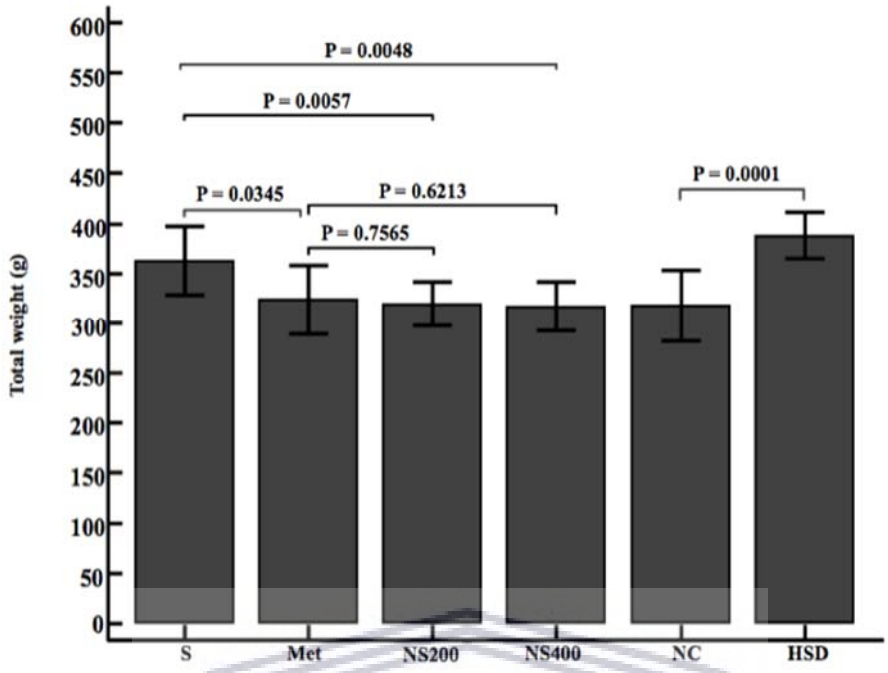

Figure 22: Comparative analysis of the groups for total body weight using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.1.2. Omentum Weight

The summary data of the omentum weight immediately prior to sacrifice of the experimental animals is shown in Table 8, and the comparative analysis is illustrated in Figure 23. The results show that the mean omentum weight was significantly higher in the saline group compared to the NS200 group only (P = 0.0499), borderline significance for the NS400 (P = 0.0630) groups, and not for the Metformin (P = 0.6030) group. The HSD group mean omentum weight was significantly higher when compared for the NC group (P < 0.0001).

Table 8: Data summary for the omentum weight (g) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Omentum Weight (g)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	0.96 ± 0.39	0.917	0.46 - 1.74	Normal
Metformin	n=8	0.86 ± 0.35	0.803	0.41 - 1.53	Normal
NS200	n=8	0.64 ± 0.20	0.619	0.40 - 0.99	Normal
NS400	n=8	0.66 ± 0.18	0.690	0.35 - 1.00	Normal
NC	n=8	0.52 ± 0.14	0.527	0.24 - 0.72	Normal
HSD	n=8	1.07 ± 0.18	1.028	0.89 - 1.47	Normal

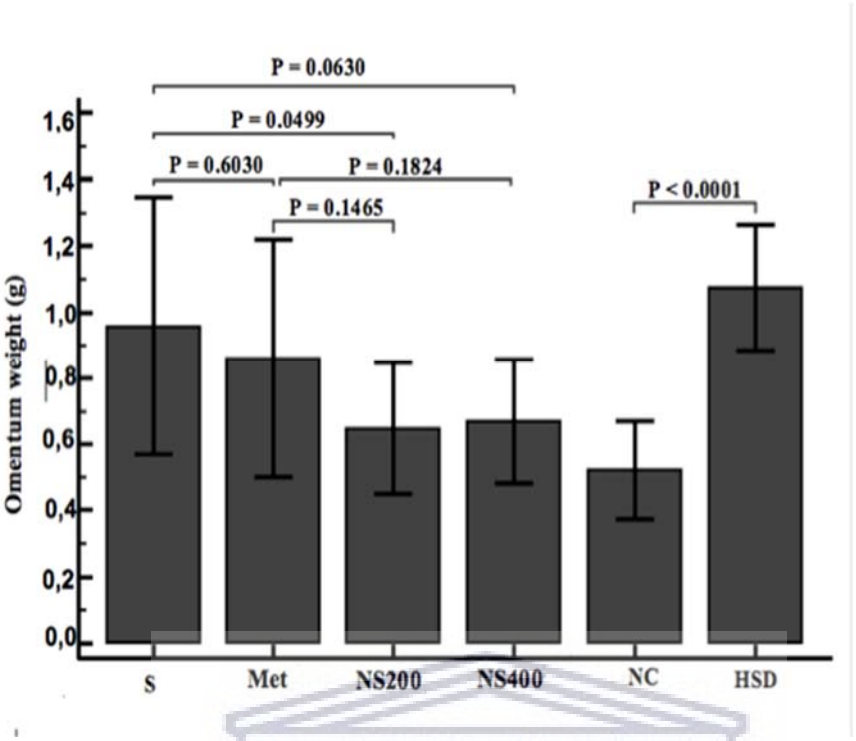

Figure 23: Comparative analysis of the groups for omentum weight using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.1.3. Prostate Weight

The summary data of the prostate weight immediately prior to sacrifice of the experimental animals is shown in Table 9, and the comparative analysis is illustrated in Figure 24. The saline group mean prostate weight was significantly higher compared to the Metformin (P = 0.0088) and NS400 (P = 0.0150) groups. Although there was a decrease in mean prostate weight in the NS200 group, it was however not significant (P = 0.1386). The HSD group mean prostate weight was significantly heavier (P < 0.0001) compared to the NC and all experimental groups except the saline group.

Table 9: Data summary for the prostate weight (g) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Prostate Weight (g)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n = 8	0.95 ± 0.34	0.912	0.44 -1.50	Normal
Metformin	n = 8	0.53 ± 0.18	0.580	0.23 - 0.75	Normal
NS200	n = 8	0.75 ± 0.14	0.765	0.55 - 0.95	Normal
NS400	n = 8	0,60 ± 0.09	0.592	0.47 - 0.74	Normal
NC	n = 8	0.52 ± 0.12	0.509	0.31 - 0.76	Normal
HSD	n = 8	0.99 ± 0.15	0.973	0.81 - 1.32	Normal

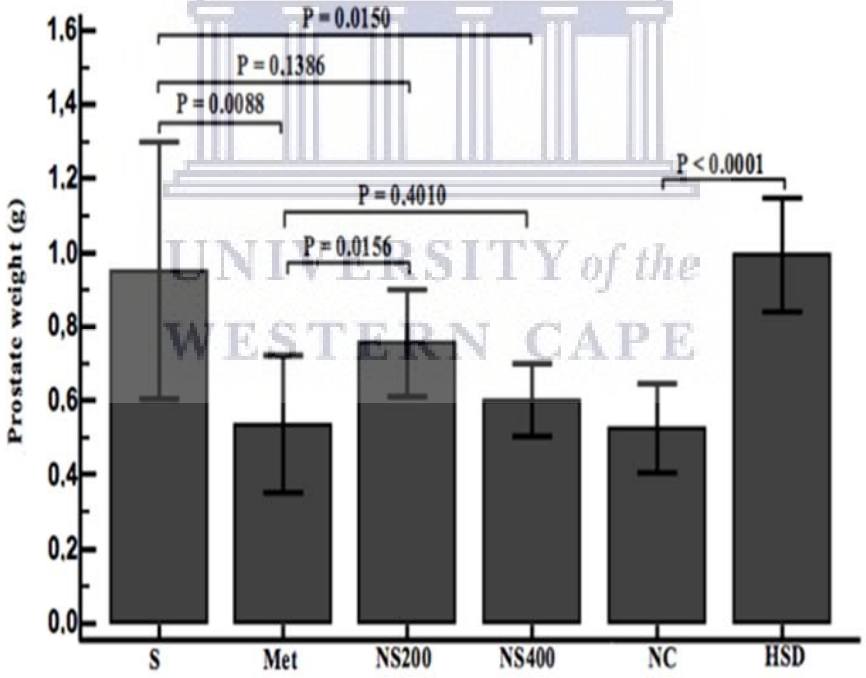

Figure 24: Comparative analysis of the groups for prostate weight using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.1.4. Testis Weight

The summary data of the testicular weight immediately prior to sacrifice of the experimental animals is shown in Table 10, and the comparative analysis is illustrated in Figure 25. Saline group mean testes weight was significantly higher compared to the Metformin group (P = 0.0432) only. Although there was a downward trend in the mean testicular weight for the NS200 and NS400 groups compared to saline group, it was however not statistically significant (P = 0.0816 and P = 0.0570 respectively). The HSD group mean testicular weight was higher compared to the NC group, but this was however statistically significant (P = 0.0548).

Table 10: Data summary for the testis weight (g) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Testes Weight (g)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	1.53 ± 0.16	1.56	1.25 - 1.68	Normal
Metformin	n=8	1.36 ± 0.14	1.35	1.17 - 1.62	Normal
NS200	n=8	1.39 ± 0.12	1.38	1.22 - 1.57	Normal
NS400	n=8	1.32 ± 0. 21	1.37	1.20 -1.63	Normal
NC	n=8	1.46 ± 0.09	1.46	1.35 - 1.59	Normal
HSD	n=8	1.54 ± 0.08	1.55	1.40 - 1.64	Normal

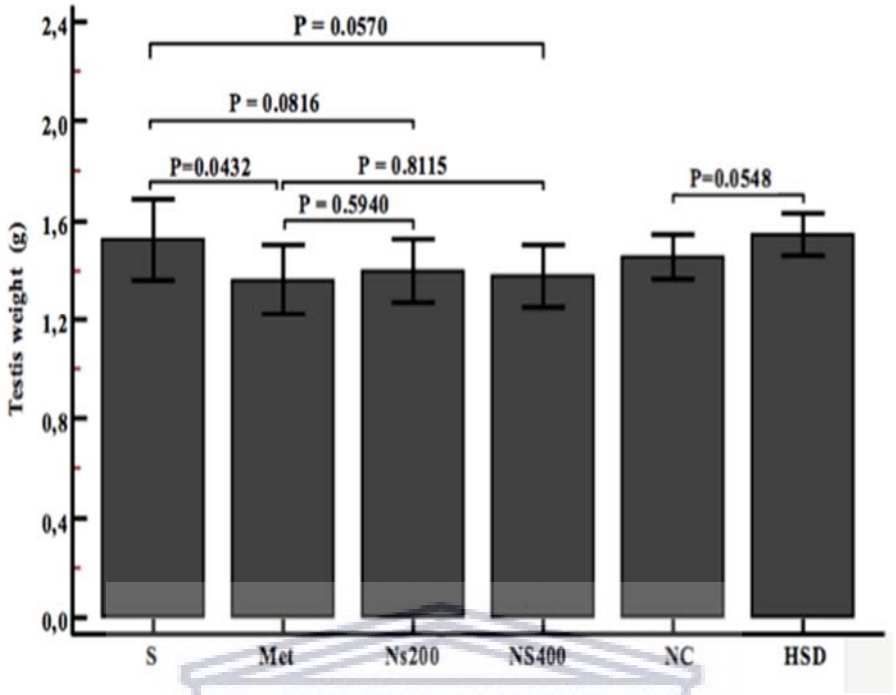

Figure 25: Comparative analysis of the groups for testis weight using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.1.5. Epididymis Weight

The summary data of the epididymis weight immediately prior to sacrifice of the experimental animals is shown in Table 11, and the comparative analysis is illustrated in Figure 26. The saline group mean epididymis weight was significantly higher compared to the Metformin (P = 0.0057), NS200 (P = 0.0053) and NS400 (P = 0.0017) treated groups. The HSD group showed a significant increase in epididymis weight (P < 0.0001) compared to the NC group.

Table 11: Data summary for the epididymis weight (g) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Epididymis Weight (g)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	4.80 ± 1.31	4.90	2.73 - 6.82	Normal
Metformin	n=8	3.16 ± 0.60	2.95	2.36 - 4.03	Normal
NS200	n=8	3.25 ± 0.59	3.37	2.29 - 4.05	Normal
NS400	n=8	2.91 ± 0.47	2.72	2.46 - 3.90	Normal
NC	n=8	2.70 ± 0.43	2.61	2.13 - 3.28	Normal
HSD	n=8	5.61 ± 1.48	4.88	4.17 - 7.85	Normal

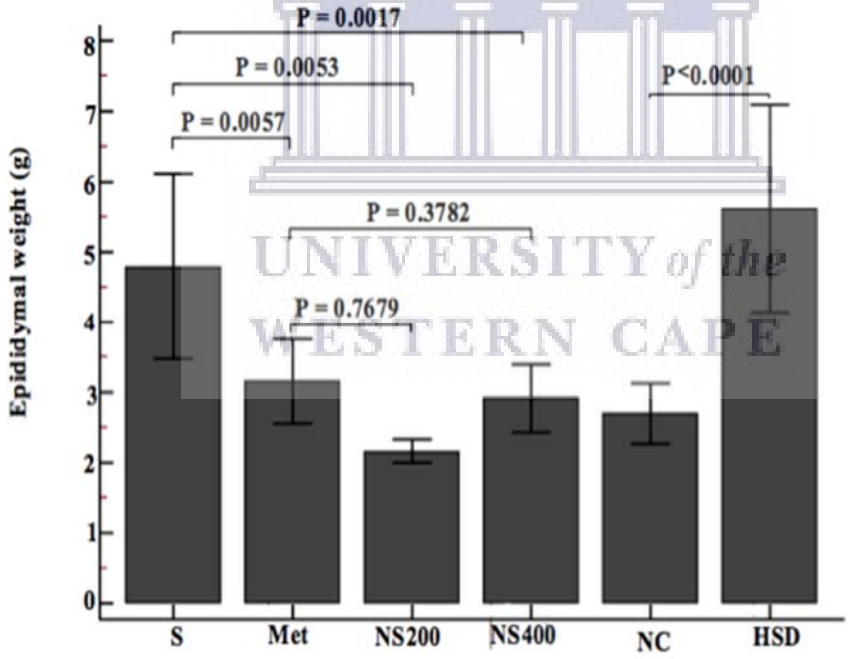

Figure 26: Comparative analysis of the groups for **epididymis** weight using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.1.6. Gastrocnemius Muscle Weight

The summary data of the gastrocnemius muscle weight immediately prior to sacrifice of the experimental animals is shown in Table 12, and the comparative analysis is illustrated in Figure 27. The saline group mean gastrocnemius muscle weight was significantly higher when compared to the NS400 (P= 0.0123) group. Although there was a downward trend in the mean results for the NS200 and Metformin groups compared to saline, this however did not show any statistical significance (P = 0.0726 and P = 0.1226, respectively). However, there was a significant increase in the HSD group mean gastrocnemius muscle weight compared to the NC group (P = 0.0029).

Table 12: Data summary for the gastrocnemius muscle weight (g) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Gastrocnemius Muscle Weight (g)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	2.33 ± 0.21	2.33	2.06 - 2.74	Normal
Metformin	n=8	2.15 ± 0.25	2.07	1.85 - 2.54	Normal
NS200	n=8	2.16 ± 0.16	2.18	1.96 - 2.45	Normal
NS400	n=8	2.06 ± 0.20	1.98	1.83 - 2.34	Normal
NC	n=8	2.12 ± 0.26	2.25	1.83 - 2.50	Normal
HSD	n=8	2.45 ± 0.12	2.50	2.27 - 2.58	Normal

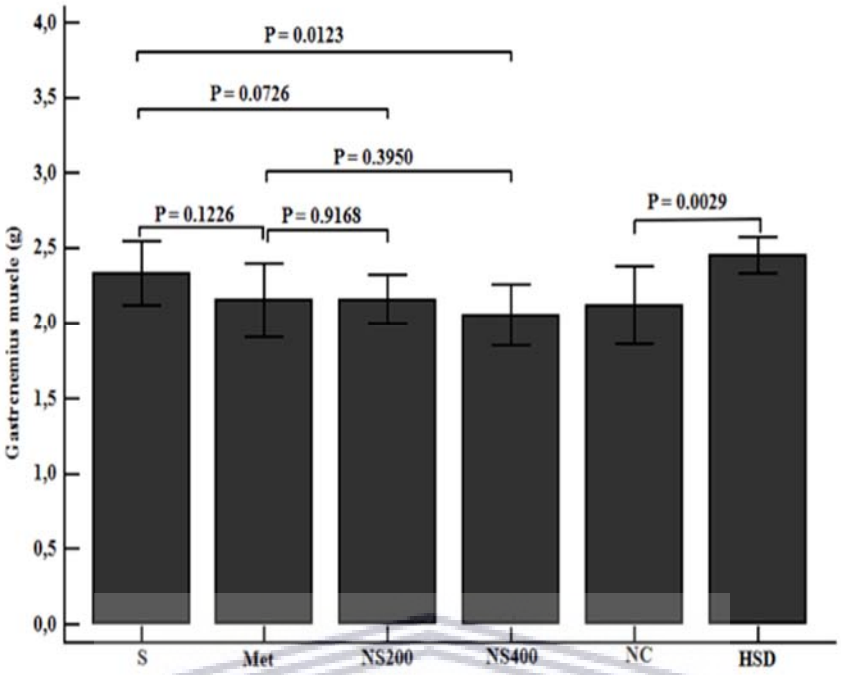

Figure 27: Comparative analysis of the groups for gastrocnemius muscle weight using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.2. Testosterone Concentration

The summary data of the testosterone concentration immediately prior to sacrifice of the experimental animals is shown in Table 13, and the comparative analysis is illustrated in Figure 28. The saline group mean testosterone concentration was significantly lower compared to the Metformin, NS200 and NS400 groups (P = 0.0001, P = 0.0002 and P < 0.0001). However, the testosterone concentration in the HSD group was not significantly different when compared to the NC group (P = 0.4599).

Table 13: Data summary for the testosterone (ng/mL) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Testosterone (ng/mL)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	1.064 ± 0.104	1.01	0.970 - 1.197	Normal
Metformin	n=8	1.264 ± 0.005	1.26	1.256 - 1.271	Normal
NS200	n=8	1.264 ± 0.006	1.26	1.256 - 1.271	Normal
NS400	n=8	1.265 ± 0.004	1.27	1.260 - 1.271	Normal
NC	n=8	1.073 ± 0.106	1.11	0.908 - 1.187	Normal
HSD	n=8	1.101 ± 0.051	1.10	1.045 - 1.202	Normal

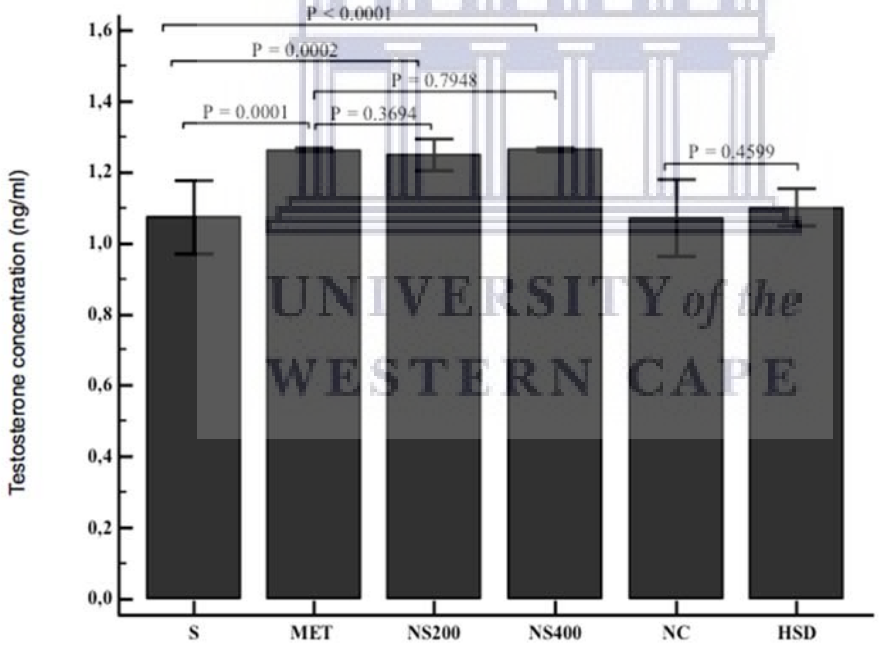

Figure 28: Comparative analysis of the groups for testosterone concentration using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.3. Sperm Parameters

3.3.1. Sperm Concentration

The summary data of the sperm concentration immediately prior to sacrifice of the experimental animals is shown in Table 14, and the comparative analysis is illustrated in Figure 29. The saline group mean sperm concentration was significantly lower compared to Metformin (P = 0.0003) and NS400 (P= 0.0493) groups, but not the NS200 treated group (P = 0.1043). The NC group mean sperm concentration was significantly higher (P = 0.0128) when compared to the HSD group.

Table 14: Data summary for the sperm concentration (M/ml) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Sperm Concentration (M/ml)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	1.51 ± 0.76	1.40	0.60 - 2.80	Normal
Metformin	n=8	3.90 ± 1.31	3.85	1.80 - 6.20	Normal
NS200	n=8	2.30 ±1.15	2.30	0.40 - 3.90	Normal
NS400	n=8	2.48 ± 1.13	2.10	1.00 - 4.30	Normal
NC	n=8	2.52 ± 1.75	2.00	0.60 - 5.90	Normal
HSD	n=8	0.75 ± 0.28	0.85	0.30 - 1.00	Normal

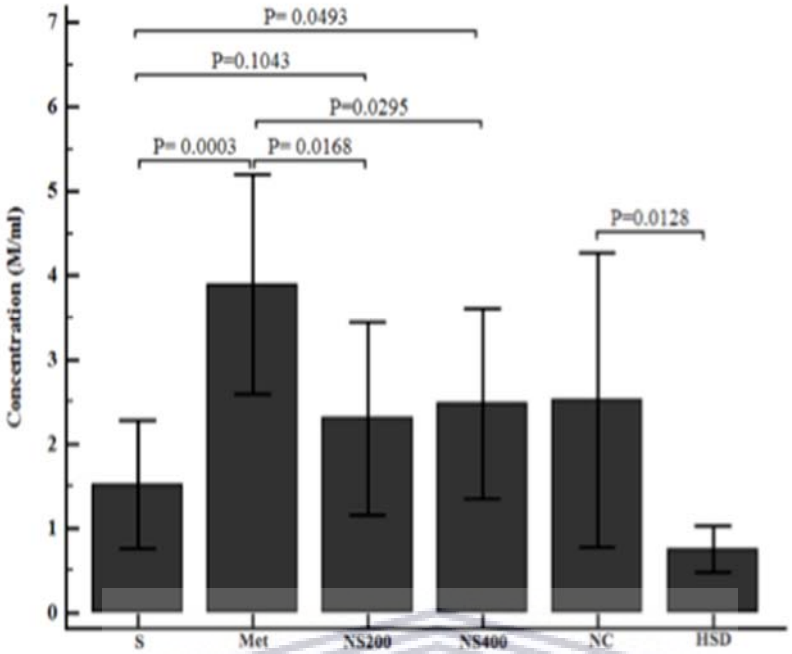

Figure 29: Comparative analysis of the groups for sperm concentration (M/ml) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.3.2. Progressive Motility

The summary data of the progressive motility immediately prior to sacrifice of the experimental animals is shown in Table 15, and the comparative analysis is illustrated in Figure 30. There was a non-significant decrease in the saline group compared to the Metformin group (P = 0.3865) and a non-significant increase in the NS200 and NS400 groups (P = 0.2332 and P= 0.8253, respectively). The HSD group mean progressive motility was significantly lower compared to the NC groups (P = 0.0092).

Table 15: Data summary for the progressive motility (%) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Progressive Motility (%)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	42.65 ± 11.76	44.00	24.10 - 63.40	Normal
Metformin	n=8	44.72 ± 10.11	47.45	22.20 - 56.20	Reject normality
NS200	n=8	32.28 ± 19.57	40.00	1.20 - 58.00	Normal
NS400	n=8	40.77 ± 17.35	43.50	11.40 - 67.00	Normal
NC	n=8	56.66 ± 12.13	56.80	38.00 - 71.00	Normal
HSD	n=8	37.00 ± 14.50	36.80	10.30 - 63.00	Normal

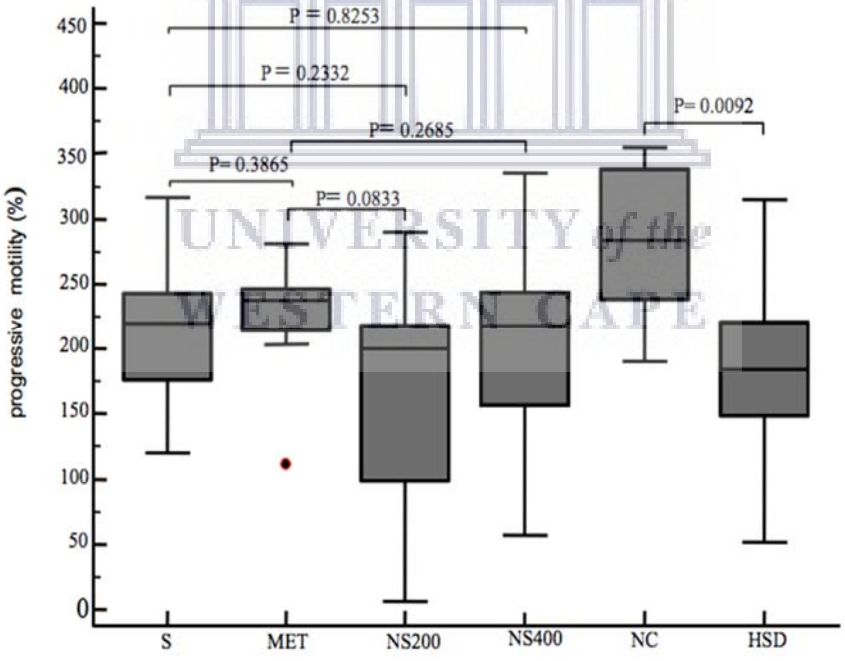

Figure 30: Comparative analysis of the groups for progressive motility (%) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal chow group; HSD = High Sugar Diet group.

3.3.3. Non-Progressive Motility

The summary data of the percentage non-progressive motility immediately prior to sacrifice of the experimental animals is shown in Table 16, and the comparative analysis is illustrated in Figure 31.There was a non-significant decrease in the Saline group compared to the Metformin group (P = 0.5414),as well as a non-significant increase in the NS200 and NS400 groups (P =0.1198 and P = 0.5895,respectively). Also, no statistical difference (P=0.9655) was observed between the NC and HSD treated animals.

Table 16: Data summary for the non-progressive motility (%) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Non-Progressive Motility (%)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	39.70 ± 11.17	40.50	21.20 - 54.40	Normal
Metformin	n=8	42.81 ± 9.08	42.05	29.40 - 54.40	Normal
NS200	n=8	30.89 ± 11.57	35.70	8.90 - 39.50	Normal
NS400	n=8	36.59 ±12.75	33.00	14.00 - 57.40	Normal
NC	n=8	33.32 ± 16.18	31.10	9.50 - 58.70	Normal
HSD	n=8	33.64 ± 14.91	27.70	11.30 - 57.70	Normal

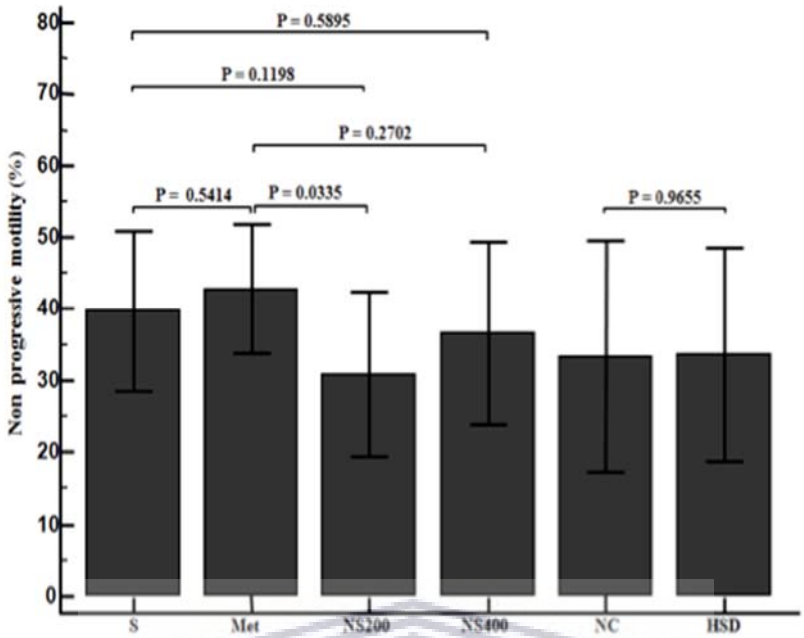

Figure 31: Comparative analysis of the groups for non-progressive motility using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.3.4. Static Motility

The summary data of the percentage static motility immediately prior to sacrifice of the experimental animals is shown in table 17, and the comparative analysis is illustrated in Figure 32. there was no statistical difference in the mean static motility when the saline group was compared to the Metformin, NS200 and NS400 treated animals. (P = 0.3865, P = 0.0851 and P = 0.5660, respectively). However, a significant increase of the mean static motility was observed in he HSD group when compared to the NC group (P = 0.0015).

Table 17: Data summary for the static motility (%) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Static Motility (%)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	17.62 ± 14.66	15.50	0.60 - 45.50	Normal
Metformin	n=8	12.45 ± 16.26	6.300	1.00 - 48.40	Reject normality
NS200	n=8	36.85 ± 30.17	24.80	6.30 - 89.90	Normal
NS400	n=8	22.68 ± 19.17	18.90	4.60 - 65.60	Reject normality
NC	n=8	7.05 ± 3.79	5.400	3.10 - 12.80	Normal
HSD	n=8	29.42 ± 13.31	28.80	10.90 - 45.10	Normal

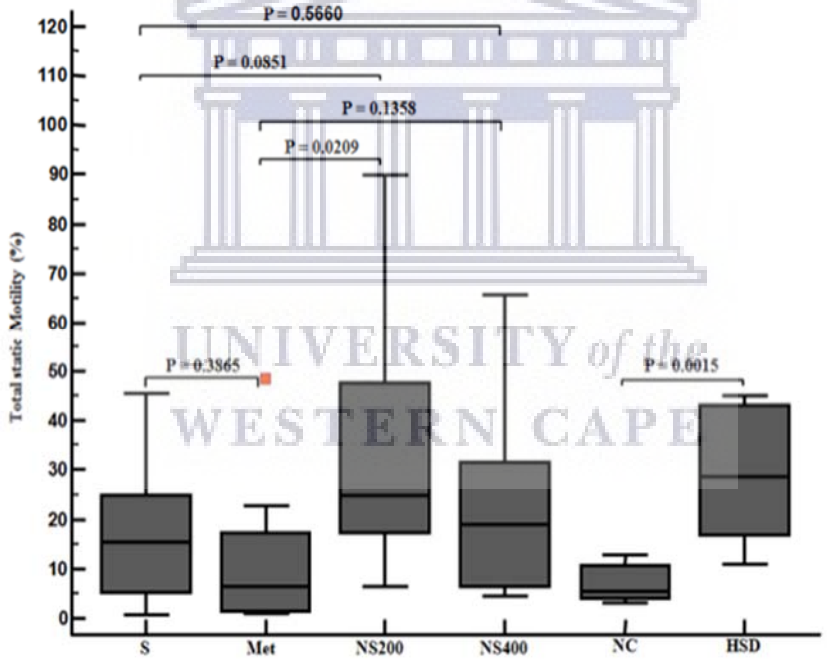

Figure 32: Comparative analysis of the groups for static motility (%) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.3.5. Sperm Vitality

The summary data of the percentage sperm vitality immediately prior to sacrifice of the experimental animals is shown in Table 18, and the comparative analysis is illustrated in Figure 33. The saline group mean sperm vitality was significantly higher when compared to the NS400 treated groups (P = 0.0469). Although there was a downward trend in the mean results for the Metformin and NS200 groups compared to saline group, this was however not significant (P = 0.5966 and P = 0.0637 respectively). There was also no significant difference between the NC groups and the HSD groups (P = 0.9648).

Table 18: Data summary for the sperm vitality (% viable) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal chow group; HSD = High Sugar Diet group.

Sperm Vitality (% viable)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	37.11 ± 9.72	35.00	24.00 - 55.00	Normal
Metformin	n=8	32.50 ± 10.15	35.50	9.00 - 42.00	Reject normality
NS200	n=8	27.00 ± 9.30	30.00	12.00 - 39.00	Normal
NS400	n=8	24.78 ± 11.05	29.00	11.00 - 41.00	Normal
NC	n=8	32.22 ± 20.13	24.00	13.00 - 61.00	Normal
HSD	n=8	27.11 ± 8.50	25.00	13.00 - 39.00	Normal

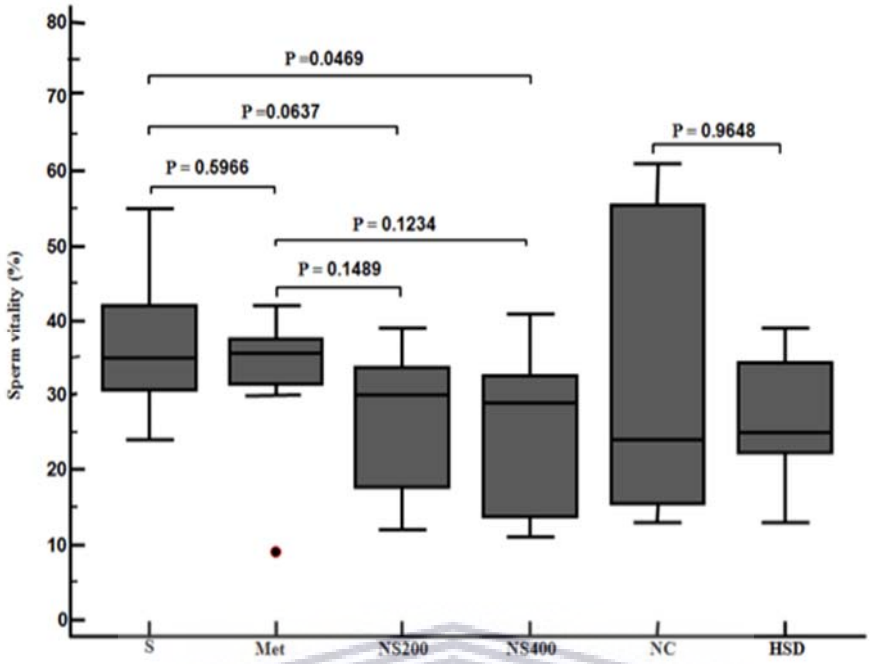

Figure 33: Comparative analysis of the groups for sperm vitality (% viable) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.3.6. Mitochondrial Membrane Potential (MMP)

The summary data of the percentage MMP (% intact) immediately prior to sacrifice of the experimental animals is shown in Table 19, and the comparative analysis is illustrated in Figure 34. The Saline group MMP was significantly lower compared to the Metformin (P = 0.0003) and NS400 (P < 0.0001) groups, and no statistical difference was observed when compared to the NS200 group (P = 0.3067). The HSD group was not significantly different compared to NC group (P =0.2808).

Table 19: Data summary for the MMP (% Intact) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

MMP (Intact %)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	54.78 ± 17.29	57.00	26.00 - 83.00	Normal
Metformin	n=8	90.37 ± 12.92	96.50	65.00 - 99.00	Normal
NS200	n=8	62.22 ± 12.18	61.00	49.00 - 85.00	Normal
NS400	n=8	91.00 ± 4.84	92.00	83.00 - 98.00	Normal
NC	n=8	70.11 ± 21.48	66.00	36.00 - 99.00	Normal
HSD	n=8	61.44 ± 9.01	63.00	48.00 - 74.00	N0rmal

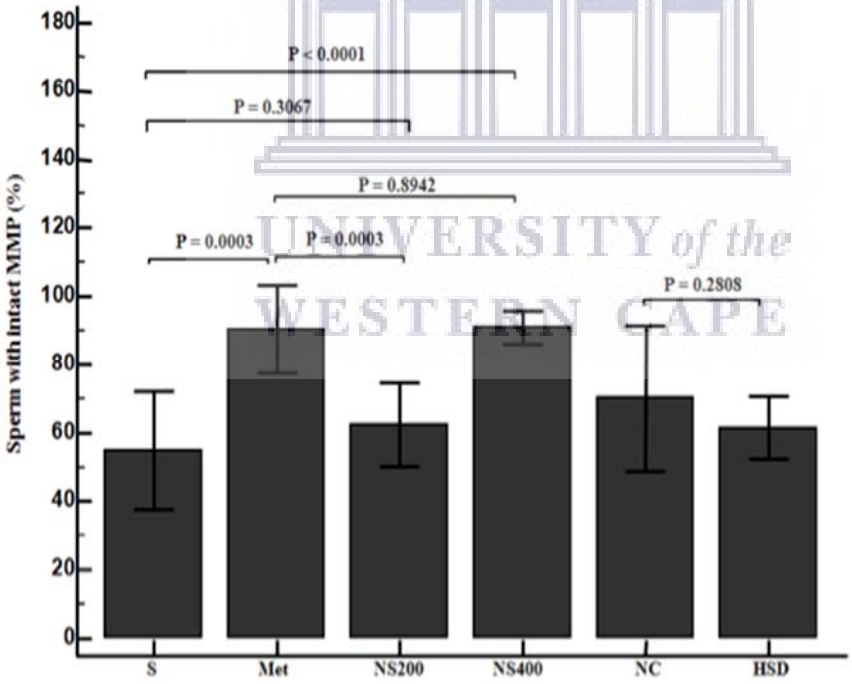

Figure 34: Comparative analysis of the groups for MMP (% intact) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.4. Histological Examination of Reproductive Organs

3.4.1. Seminiferous Tubules

The summary data of the seminiferous tubules largest expansion (µm) immediately prior to sacrifice of the experimental animals is shown in Table 20, and the comparative analysis is illustrated in Figure 35. The mean largest expansion of seminiferous tubules in the saline group was significantly smaller compared to the Metformin ($P < 0.0001$), NS200 ($P < 0.0001$) and NS400 ($P = 0.0001$) groups. More so the HSD group mean largest expansion of the seminiferous tubules was significantly smaller ($P = 0.0001$) as compared to the NC group.

Table 20: Data summary for the largest expansion (µm) of seminiferous tubules. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sat*iva 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Seminiferous Tubules largest expansion (µm)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	74.71 ± 22.66	73.108	43.3 - 105.9	Normal
Metformin	n=8	437.92 ± 144.82	442.97	265.1 - 682.6	Normal
NS200	n=8	458.50 ± 143.60	380.82	334.3 - 734.8	Normal
NS400	n=8	403.19 ± 173.07	310.23	243.3 - 687.4	Normal
NC	n=8	426.07 ± 159.30	399.62	211.2 - 746.7	Normal
HSD	n=8	101.45 ± 31.30	89.333	71.3 - 151.2	Normal

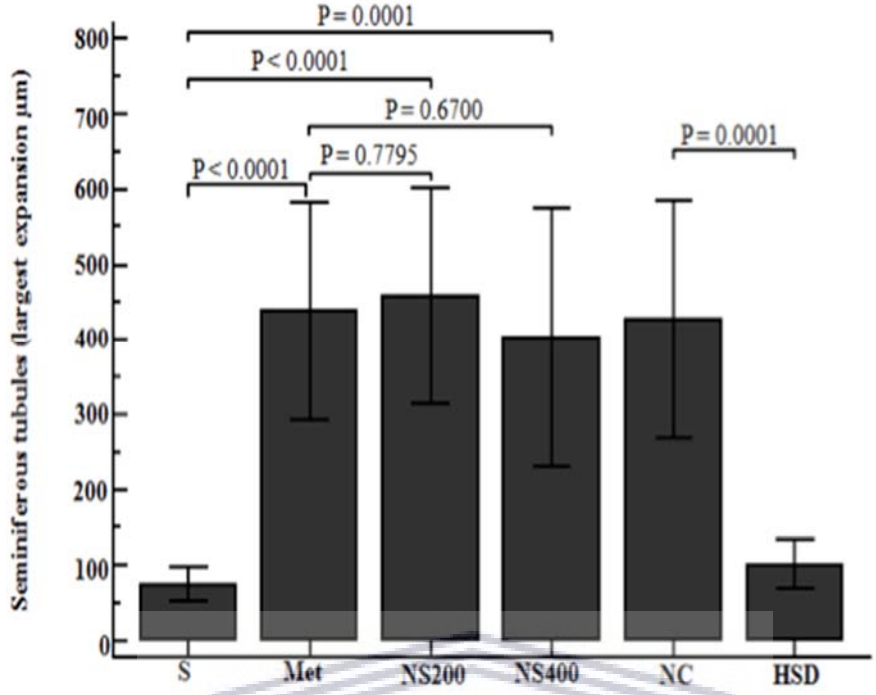

Figure 35: Comparative analysis of the groups for seminiferous tubules largest expansion (µm) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

The summary data of the seminiferous tubules width expansion (µm) immediately prior to sacrifice of the experimental animals is shown in Table 21, and the comparative analysis is illustrated in Figure 36. The saline group mean width expansion of seminiferous tubules was significantly lower compared to those values in the Metformin (P< 0.0001), NS200 (P = 0.0001) and NS400 (P = 0.0001) groups. In animals from the HSD group, the mean width expansion of seminiferous tubules was significantly lower as compared to the NC group (P = 0.0002).

Table 21: Data summary for the seminiferous tubules width expansion (µm) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Seminiferous Tubules Width Expansion (µm)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=9	85.1 ± 36.1	73.48	51.70 - 143.5	Normal
Metformin	n=8	264.0 ± 44.9	264.23	198.4 - 325.6	Normal
NS200	n=8	363.8 ± 137.9	339.58	233.5 - 549.8	Normal
NS400	n=8	300.4 ± 109.8	273.52	202.3 -528.5	Normal
NC	n=8	315.4 ± 106.3	262.97	223.5 - 476.3	Normal
HSD	n=8	99.8 ± 54.9	91.15	35.66 - 167.4	Normal

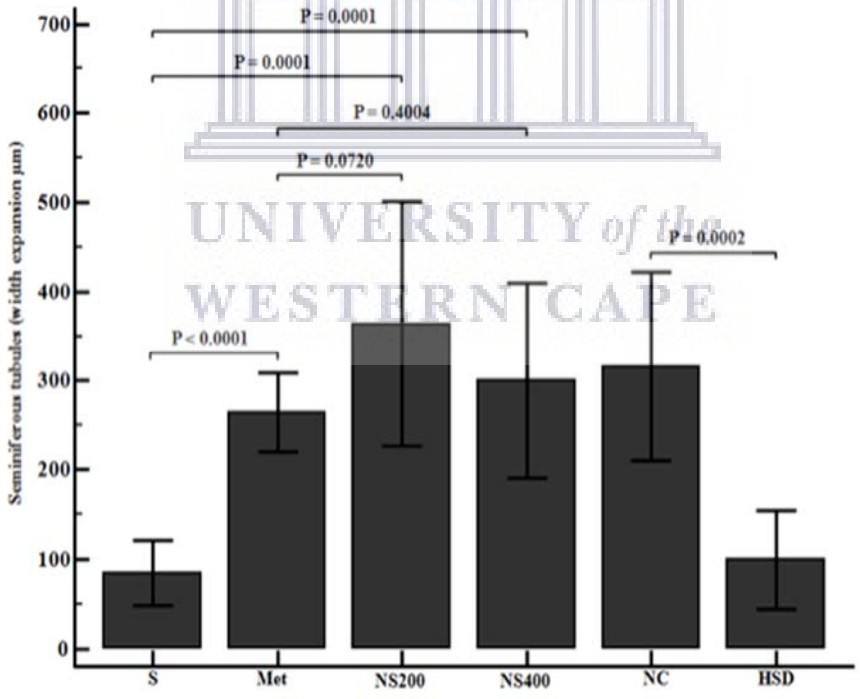

Figure 36: Comparative analysis of the groups for seminiferous tubules width expansion (µm) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

The summary data of the seminiferous tubules epithelial cell height (μm) immediately prior to sacrifice of the experimental animals is shown in Table 22, and the comparative analysis is illustrated in Figure 37. The saline group epithelial cell height of seminiferous tubules was significantly lower when compared to the Metformin and NS400 (P= 0.0398 and P= 0.0500 respectively). Although there was a downward trend for NS200, it was borderline significant (P = 0.0505). In animals from the HSD group, the mean epithelial cell height showed a downward trend compared to the NC group, but this was not statistically significant (P = 0.0901).

Table 22: Data summary for the seminiferous tubules epithelial cell height (μm) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Seminiferous Tubules Epithelial Cell Height (μm)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	77.1 ± 46.1	84.81	14.21- 148.9	Normal
Metformin	n=8	254.8 ± 216.8	200.10	55.23 - 607.6	Normal
NS200	n=8	219.5 ±182.5	136.09	54.53 - 514.9	Normal
NS400	n=8	186.4 ±136.5	165.82	58.04 - 358.9	Normal
NC	n=8	192.5 ±148.1	156.20	51.06 - 461.5	Normal
HSD	n=8	93.1 ± 43.51	99.415	12.82 - 141.3	Normal

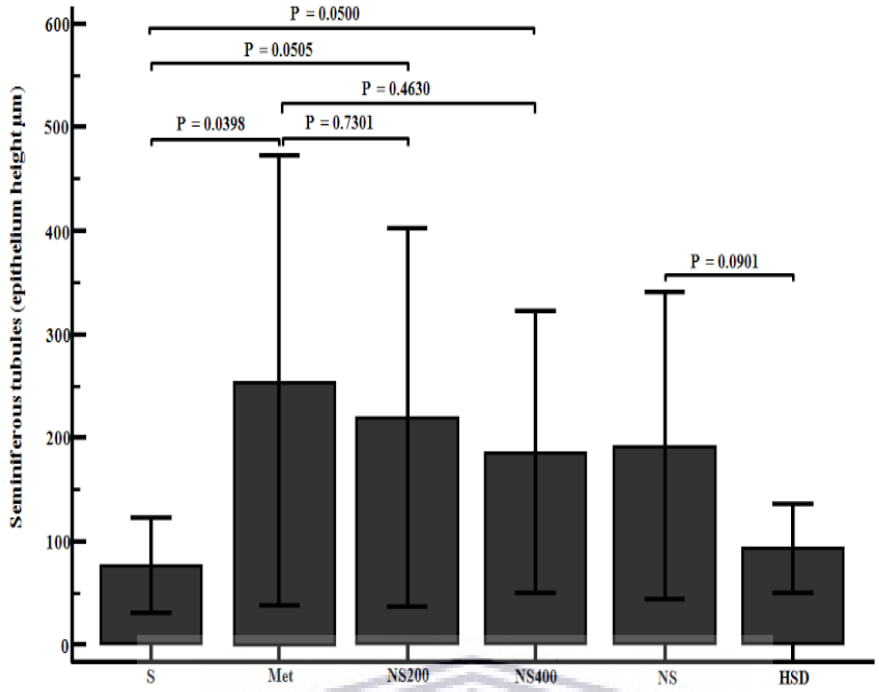

Figure 37: Comparative analysis of the groups for seminiferous tubules epithelial cell height (µm) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.4.2. Cauda Epididymidis

The summary data of the largest expansion of cauda epididymidis (µm) immediately prior to sacrifice of the experimental animals is shown in Table 23, and the comparative analysis is illustrated in Figure 38. The Saline group mean largest expansion was significantly lower when compared to those values in the Metformin ($P < 0.0001$), NS200 ($P < 0.0001$) and NS400 ($P < 0.0001$) treated groups. As expected, animals administered the HSD showed the greatest expansion of cauda epididymidis ($P < 0.0001$) when compared to the NC group.

Table 23: Data summary for the cauda epididymidis largest expansion (µm) of the rats in each group. NS200 = *Nigella* sativa 200 mg/Kg/day group; NS400 = *Nigella* sativa 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Cauda Epididymidis largest expansion (µm)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	75.5 ± 14.4	74.4	55.8 - 97.2	Normal
Metformin	n=8	409.4 ± 148.1	380.3	224.4 - 617.0	Normal
NS200	n=8	531.1 ± 196.0	515.8	286.5 - 846.7	Normal
NS400	n=8	493.2 ± 149.6	494.5	209.8 - 660.4	Normal
NC	n=8	544.2 ± 155.1	543.4	333.2 - 744.9	Normal
HSD	n=8	99.1 ± 19.8	92.4	80.8 - 133.2	Normal

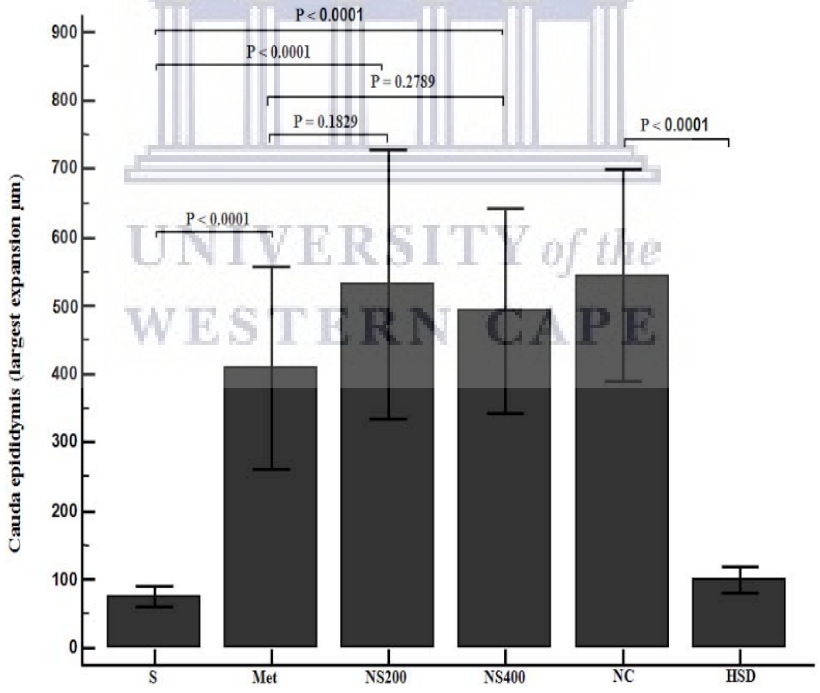

Figure 38: Comparative analysis of the groups for cauda epididymidis largest expansion (µm) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

The summary data of the width expansion of cauda epididymidis (µm) immediately prior to sacrifice of the experimental animals is shown in Table 24, and the comparative analysis is illustrated in Figure 39. The saline group was significantly lower when compared to those of the Metformin ($P < 0.0001$), NS200 ($P < 0.0001$) and NS400 ($P = 0.0005$) treated groups. The mean values of the width expansion of cauda epididymidis from the HSD group was significantly lower ($P< 0.0001$) compared to the NC group.

Table 24: Data summary for the cauda epididymidis width expansion (µm) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Cauda Epididymidis Width Expansion (µm)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	66.2 ± 29.4	58.5	25.8 - 116.6	Normal
Metformin	n=8	359.6 ± 129.1	287.1	258.3 - 571.2	Normal
NS200	n=8	307.7 ± 63.0	292.21	226.8 - 415.9	Normal
NS400	n=8	315.5 ± 155.3	289.8	66.2 - 524.6	Normal
NC	n=8	386.6 ± 145.3	351.5	238.3 - 573.5	Normal
HSD	n=8	81.2 ± 34.6	75.3	41.5 - 147.3	Normal

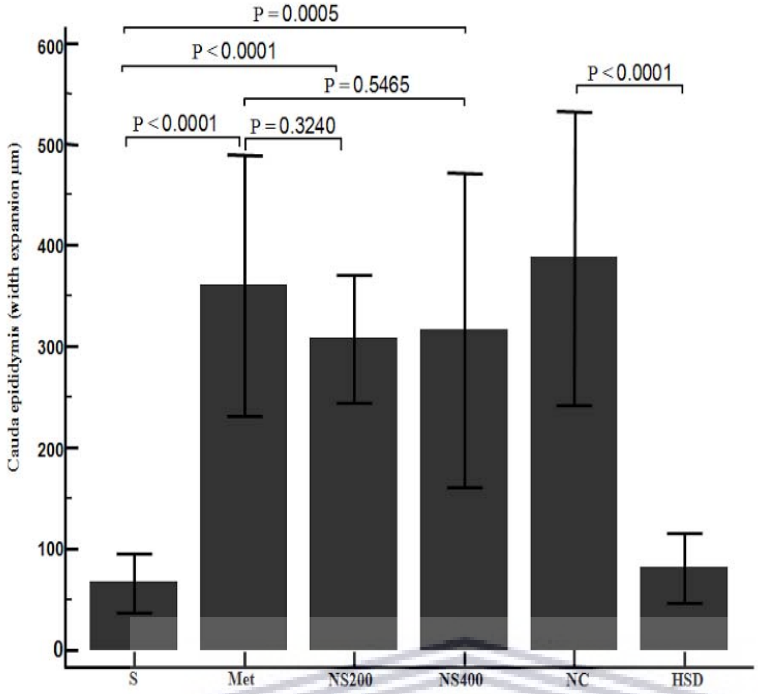

Figure 39: Comparative analysis of the groups for cauda epididymidis width expansion (µm) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

The summary data of the epithelial cell height of cauda epididymidis (µm) immediately prior to sacrifice of the experimental animals is shown in Table 25, and the comparative analysis is illustrated in Figure 40. The saline group mean epithelial cell height of cauda epididymis was significantly smaller compared to those values in the Metformin ($P < 0.0001$), NS200 ($P < 0.0001$) and NS400 ($P < 0.0001$) groups. In animals from HSD group the mean epithelial cell height of the cauda epididymis was significantly higher ($P< 0.0001$) as compared to the HSD group.

Table 25: Data summary for the epithelial cell height of the Cauda Epididymidis (µm) of the rats in each group. NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

Cauda Epididymis Epithelial Height (µm)					
	Sample Size	**Mean ± SD**	**Median**	**Range**	**Distribution**
Saline	n=8	3.95 ± 0.74	4.00	2.84 - 5.30	Normal
Metformin	n=8	15.49 ± 2.61	16.30	9.88 - 17.98	Normal
NS200	n=8	20.11 ± 5.83	20.14	13.34 - 31.49	Normal
NS400	n=8	18.06 ± 3.89	17.82	12.58 - 22.92	Normal
NC	n=8	19.44 ± 5.53	19.46	12.67 - 29.46	Normal
HSD	n=8	5.340 ± 0.77	16.96	13.58 - 26.06	Normal

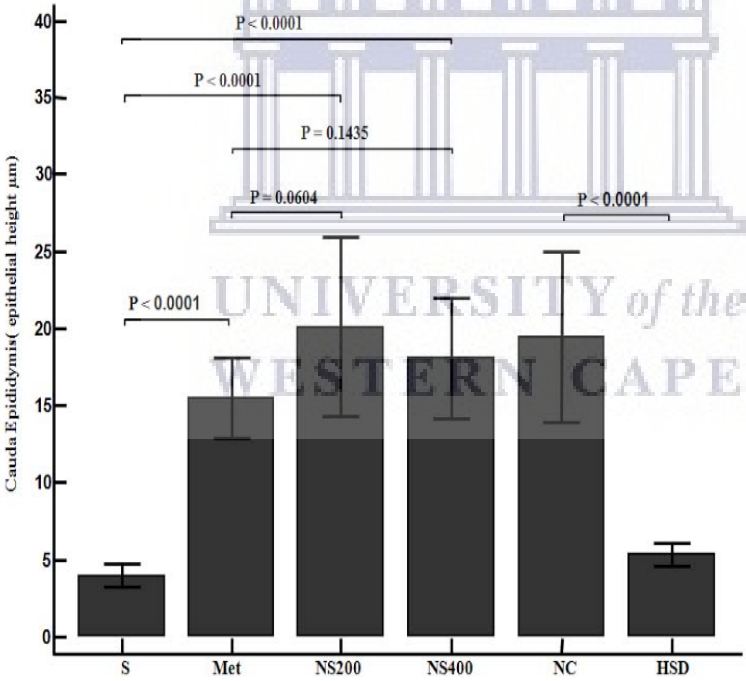

Figure 40: Comparative analysis of the groups for cauda epididymidis epithelial cell height (µm) using bar graphs expressed as mean ± SD. S = Saline group; Met = Metformin group; NS200 = *Nigella sativa* 200 mg/Kg/day group; NS400 = *Nigella sativa* 400 mg/Kg/day group; NC = Normal Chow group; HSD = High Sugar Diet group.

3.5. Testicular Histology

In the saline group, the testicular architecture shows several layers of epithelial cells and cuboidal spermatogonia with clear cytoplasm and rounded nuclei (Figure 41-A). Upon treatment with Metformin, NS200 and NS400, there was a reversal of the testicular histology towards normal and the micrographs shows regeneration of spermatocytes (Figure 41-B; C; D). Histological evaluation of the testis in the NC group shows normal histo-architecture with three layers of the seminiferous tubules (Figure 41-E). In contrast, the HSD group shows Sertoli cells and large epithelial cells with distortion in the germinal layers of the seminiferous tubules as well as abnormal spermatocytes (Figure 41-F).

Figure 41: Morphology of seminiferous tubules sections in the experimental rats; (A) Saline group; (B) Metformin group; (C) *Nigella sativa* 200 mg/Kg/day group; (D) *Nigella sativa* 400 mg/Kg/day group; (E) Normal Chow group; (F) High Sugar Diet group. L = Lumen, RRFS= Remarkable Regenerative Features of Spermatocytes, ST = Seminiferous Tubules, LC= Leydig cell, DS= Damaged Spermatocytes

3.6. Epididymitis Histology

Histological evaluation showed unremarkable architecture of the epididymidis and lumen containing lots of spermatozoa in the saline groups (Figure 42-A). In the Metformin group (Figure 42-B), the epididymidis showed a clear normal structure and there was a clumping of the nucleus of epithelial cells and cytoplasmic vacuoles with sperm leakage. Furthermore, histology of epididymidis of animals from the NS200 and NS400 treated rats showed congestion indicating marked distribution of sperm cells and appearing as dense fibers within the lumen (Figure 42-C; D). Also, the NC group showed that epididymis with normal histo-architecture and well distributed sperm cells (Figure 42-E). Contrary to what was observed with the others groups, the rats in the HSD group showed damaged in epididymis with irregular shape, showing a loss of histo-architecture and a reduced the number of sperm cells by the diminutive of the congestion in the lumen (Figure 42- F).

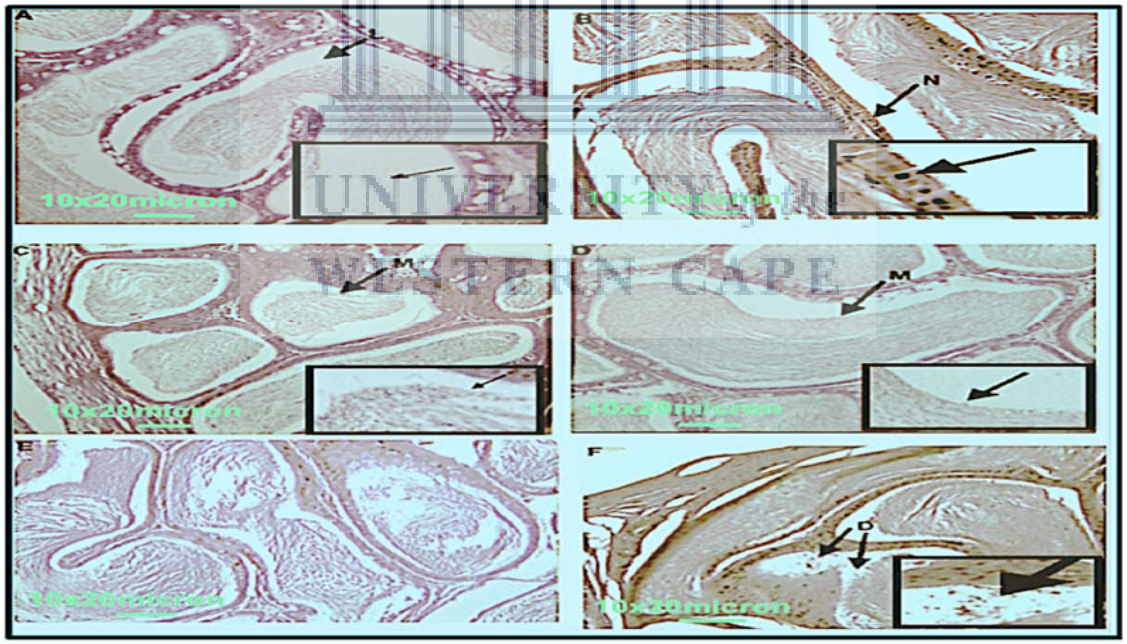

Figure 42: Morphology of Epididymis sections in the experimental rats; (A) Saline group; (B) Metformin group; (C)*Nigella sativa* 200 mg/Kg/day group; (D)*Nigella sativa* 400 mg/Kg/day group; (E) Normal Chow group; (F) High Sugar Diet group. D = Damage of Spermatozoa, L = Lumen, M = Marked distribution of sperms, N= Nucle

3.7. Prostate Histology

Evaluation of prostatic tissue showed a prostate architecture with congestions and few mucosa infoldings in the saline group (Figure 43-A), which were reduced in the Met, NS200 and NS400 groups (Figure 43- B; C; D). As expected, the NC group showed an unremarkable prostate architecture without any histological defect (Figure 43-E). However, the HSD treated rats showed disruption in the prostate architecture, a greater severity of mucosal infoldings and congestion (Figure 43- F).

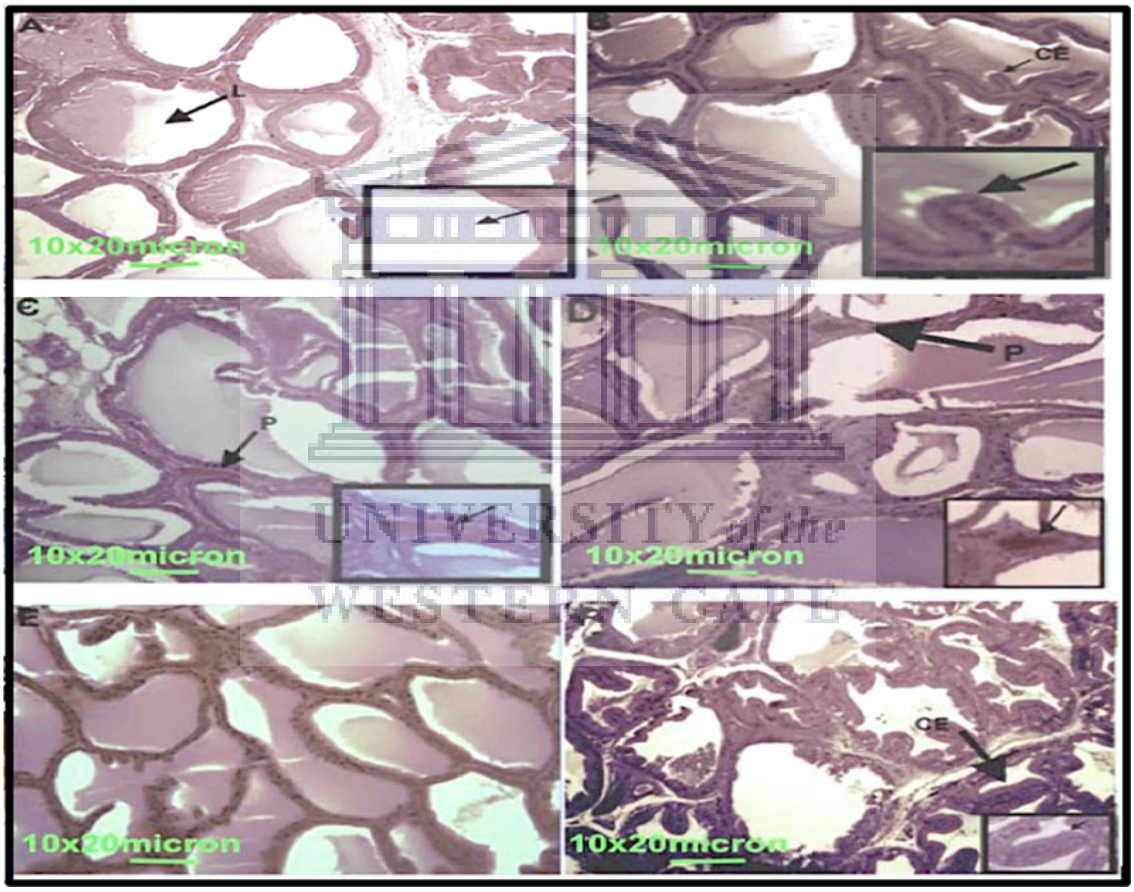

Figure 43: Morphology of the prostate sections in the experimental rats (A); Saline group; (B) Metformin group; (C) *Nigella sativa* 200 mg/Kg/day group; (D) *Nigella sativa* 400 mg/Kg/day group; (E) Normal Chow group; (F) High Sugar Diet group. L= Lumen, P= prostatic concretion, M= Mucosa infoldings.

CHAPTER 4

DISCUSSION

4.1. Introduction

Obesity is a growing public health concern in developed countries as a result of changes in lifestyle and behaviour such as eating fast foods, sugar rich diets, unhealthy snacks, increased red meat, lack of fruits, vegetables and fibre, fewer wholegrain cereals and a lack of exercise. Weight gain is considered clinically problematic if it results in a BMI ≥ 30 kg/m^2 (Kuczmarski *et al.* 2007). In this study, adult male Wistar rats (n = 53), weighing 180 – 220 g, were included and kept in an animal husbandry at controlled temperature between 21-23°C and a fixed artificial 12-hour light/dark cycle with water provided ad libitum. The experimental design was based on an obesogenic high sugar diet model (Naderali *et al*., 2001; Panchal *et al*., 2011; Huisamen *et al*., 2012; Solomon & Henkel. 2015) exposed to all groups except the Normal chow (NC) group. The experimental groups were treated with *Nigella sativa* (Ns) 200 mg/Kg/day (NS200), Ns 400 mg/Kg/day (NS400) and Metformin 75 mg/Kg/day (Met), and compared to a saline (100 µl/day) control group. A high sugar diet (HSD) and NC group without intervention were used as a control group for the obesogenic diet model.

Following dietary exposure for 14 weeks, and experimental or control feeding for the last 8 weeks, the animals were sacrificed via cervical dislocation. Outcomes analyzed included body and organ weights, sperm analysis and serum testosterone. Further outcomes included histology on reproductive organs.

4.2. Impact Of *Nigella Sativa* On Total Body And Organ Weight

4.2.1. Total Body Weight

The results show the mean total body weight (TBW) was significantly heavier in the HSD group (P=0.0001) compared to the NC group. As a control for the HSD animal model, this result indicates the diet has an obesogenic effect. This is consistent with similar high sugar diet models. Naderali *et al*. (2001), in an experimental study with male Wistar rats, used a normal rat chow diet with supplemented condensed milk, saccharine and water for 15 weeks that induced obesity in rats. Huisamen *et al*. (2012) used a diet supplemented with sucrose and condense milk to induce obesity in Wistar rats, and in another study used fructose and cooking fat to induce a pre-diabetes obese animal model (Huisamen *et al*., 2013). Solomon & Henkel (2015) induced obesity in male Sprasgue-Dawley rats using supplementation of normal chow with 33% condensed milk, 7% sucrose and 27% water for 20 weeks. Lecomte *et al*. (2015) induced obesity in young male rates with supplementation using high carbohydrate and condensed milk, increasing body weight, and adipose tissue mass. Panchal *et al*. (2011) induced obesity and increased omental adiposity on male Wistar rats (8 - 9 weeks old) using a high-carbohydrate diet including condensed milk, beef tallow and fructose for 16 weeks. This is further consistent with studies indicating that high energy sugar intake induces obesity in humans compared to those eating low sugar diets, where the effect of increased sugar intake is closely correlated with increased total body weight and abdominal adiposity in humans (Vermunt *et al*., 2003).

This study showed a significant decrease in weight in the metformin group compared to saline group (P=0.0345). This is consistent with studies indicating that metformin causes a decrease in the body weight in obese and diabetic animals and humans (Mathur *et al*., 2011; Nayak *et al*., 2017). Previous studies indicate that there is a significant decrease in weight and changes in skinfold density, body surroundings, visceral adipose tissue when using metformin therapy (Mathur *et al*., 2011; Yanovski *et al*., 2011; Yan *et al*., 2015).

This study showed that the low (NS200) and high (NS400) dose of Ns oil significantly reduced the TBW of the experimental rats compared to saline (P = 0.0048 and P = 0.0057 respectively). This is consistent with studies that reported a reduction of body weight with the use of Ns in obesity. It has been found that oral administration of Ns oil (500 mg/Kg/day) showed a significant decreased in body weight in Wistar albino rats, fed a high fructose diet for induction of insulin resistance. This further was associated with an improvement in serum lipid profile, and insulin receptor sensitivity (Elseweidy *et al*., 2018). Ns at 2 g/Kg/day in male rats lead to diminishing weight without any toxic effect, equivalent to that of metformin compared to control groups (Meddah *et al*., 2009). Ns has further been shown reduce the rate of body weight, BMI and WC in normal rats fed a high fat diet compared to placebo groups (Namazi *et al*., 2018). Weight loss in Ns oil may be mediated by constituents including thymoquinone (TQ), pcymene, carvacrol, α-pinene, 4-terpineol, longifoline, carvone, and t-anethole (Abdellatif *et al*. 2013). Furthermore, Abduallah *et al* (2017) reported that TQ compound of Ns oil decreases body weight at deferent dosages with Streptozotocin (STZ) induced diabetes in female rats weighing 200 – 250 mg/kg receiving Low dose (5 mg/Kg) and high dose TQ (10 mg/Kg). Also in female ovariectomized rats for a metabolic obese model in menopause, Ns reduced weight at dosages of 300, 600 and 1200 mg/kg/day over 3 weeks (Parhizkar *et al*., 2011). In human studies, 3 g daily dose of Ns reduced body weight in men (Ermumcu & Şanlıer, 2017). The decrease of weight appears to be dose dependent (Abdellatif, 2013). This is consistent with clinical trials that recommend a larger dose and longer term of Ns consumption will give better results with significant reduction of body weight, waist circumference, and serum free testosterone (Datau *et al*., 2010). Therefore, NS clearly shows potential to reduce adiposity in obesity and associated metabolic complications which is consistent with the results of this study.

The toxic dose of Ns has been investigated. Abukhader *et al*. (2012) reported that male and female rats which received intraperitoneal (IP) injection of TQ have different signs of toxicity than those that received oral ingestion, which

was linked to acute pancreatitis. Maximum dose for intraperitoneal injection was 22.5 mg/kg in male rats and 15 mg/kg in females, associated with toxicity, acute pancreatitis and weight loss (Abukhader *et al*., 2012).

Furthermore, this study showed that there was no significant differences in the reduced TBW between Metformin and the NS200 (P = 0.7565) and NS400 (P = 0.6213) groups. This suggests that NS was as effective as metformin in reduced weight in an obesity context.

4.2.2. Omentum Weight

The study results show that mean omentum weight was significantly heavier in the HSD group (P< 0.0001) compared to NC group. This is consistent with studies suggesting the supplementation with high-energy sugar in animal models induced visceral adiposity (Naderali *et al*., 2001; Vermunt *et al*., 2003; Panchal *et al*., 2011; Huisamen *et al*., 2012; Solomon & Henkel 2015).

This study showed a non-significant decrease in mean omentum weight in the metformin group compared to saline group (P=0.6030). Male Wistar rats weighing about 180 – 200 g fed with normal diet and treated with 15 mg/kg metformin twice daily did not change the body weight, food intake or visceral fat (Apaijai *et al*., 2012). Wang *et al*. (2017) reported that metformin did not alter visceral adipose tissues in diabetic obese rats at 500 mg/Kg/day by oral gavage for 12 weeks. Studies of diabetic male and female humans between the ages of 30 and 70 years showed that metformin therapy at (2,550 mg/day) reduced adipocyte size but not total body weight (Ciaraldi *et al*., 2002).

This study indicates that mean omentum weight of NS200 and NS400 was decreased, reaching significance in the NS200 group (P = 0.0499), but only borderline significance in the NS400 group (P = 0.0630) compared to saline. This was more effective than metformin, although this did not reach statistical significance. This is aligned to the reported benefit on weight loss in obese and diabetic models reported above.

Furthermore, Didi *et al*. (2014) showed that Ns causes a significant decrease in abdominal adipose tissue weights, alongside body weight, using one-month-old male Wistar rats receiving 4 % Ns oil. White male albino rats weighing 80-90 g fed NS derived polyphenols for 14-weeks showed significant antioxidant properties which can inhibit lipid peroxidation to protect tissue from damage (Amin & Nagy, 2009). TQ (2.5, 5 and 10 mg/kg) and Ns (0.048, 0.192 and 0.384 mg/kg) can lead to a reduction in lipid peroxidation in the body (Hosseinzadeh *et al.,* 2007)

4.2.3. Prostate Weight

The results show the mean prostate weight was significantly heavier in the HSD group ($P < 0.0001$) compared to the NC. Obese hyperinsulinaemic rats on a high calorie diet are experimentally associated with increased prostate weight and volume via hyperplasia, where testosterone treatment leads to augmented prostate growth (Vikram *et al*., 2010). In addition, mean gain of the ventral prostate was higher in obese rats fed high-fat diet compared to control treated for 2 weeks (Ribeiro *et al.,* (2012). However, Adekunbi *et al*. (2016) reported no significant change in the weight of the epididymis and prostate gland in rats fed sucrose, with reduced motility, concentration and viability found. Obesity can lead to increased prostrate size and weight in men (Freedland *et al*., 2006), and elderly obese men have a higher risk of BPH (Parikesit *et al*., 2016). These results are consistent with obesity in men being associated with increased prostate volumes, weight and lower serum levels of testosterone (Muller *et al*., 2013).

The results show that mean prostate weight was significantly decreased in the metformin group ($P = 0.0088$) compared to the saline group. Mosli *et al*. (2015) showed that treatment with 500 and 1000 mg/kg metformin orally for 14 days initiated a testosterone mediated improvement in prostate weight and prostate index (prostate weight (mg): total body weight (g)) in experimental models of benign prostatic hyperplasia in rats. This was further associated with increased oestrogen-beta receptor expression compared to estrogen-

alpha receptors, reduced IGF-1 and increased P21 and Bax/Bcl-2 gene expressions (Mosli *et al*., 2015). Xu *et al*. (2018) reported a decrease in the level of prostatic hyperplasia and the hormonal mediator insulin-like growth factor-1 (IGF-1) in sprague-Dawley rats fed a high calorie diet with metformin treatment.

The results of this study showed that the mean prostate weight was significantly decreased in the NS400 group (P=0.0150) compared to saline, with a non-significant decrease in mean prostate weight in the NS200 group (P = 0.1386) compared to the saline group. NS seed extract (100 mg/day) has been found to significantly reduce the weight of seminal vesicles and prostate (Singh, 2015). NS oil shows a non-significant increase on organ weights of rats, similar to the results especially when the rats treated with low dose NS200 (Mansour *et al*., 2013). However, Al-Taee *et al*., (2008) showed no change in weight of testes, seminal vesicle and prostate after treatment of 100 and 200 mg/Kg Ns. In BPH rat models, NS extracts significant increase the prostate weight at 50 mg/Kg (Al-Trad *et al*., 2017).

Furthermore, the NS400 group was not statistically different from the metformin group, suggesting that at the higher dose, NS reduced prostate weight in obesity as effectively as metformin. Although NS200 decreased prostate weight compared to the control group, this was not as effective as metformin.

4.2.4. Testis Weight

The results show that mean testis weight was heavier in the HSD group compared to the NC group, but this did not reach statistical significance (P = 0.0548). Adekunbi *et al*. (2016) reported a non-significant decrease in testicular weight in rats fed a sucrose enriched diet over 6 weeks. Elobeid *et al*. (2015) showed no significant changes in testis weight with a high fat diet induced obesity model in rats. Oyelowo *et al*. (2014) reported an increase in testes and seminal vesicle weight with supplementation of high sucrose solution and honey (HSS+ H). However, Vendramini *et al*. (2014) reported a

decrease in testicular and epididymis weights in obese sexually mature rats. Shibata & Fukuwatari. (2013) also reported that the testicular weight was also lower in rats fed with fructose enriched chow.

This study shows that the mean testes weight was significantly decreased in the metformin group (P = 0.0432) compared to the saline group. The HSD therefore caused an increase in testicular weight, which was rescued by metformin treatment. Mahmoud *et al*. (2014) reported that metformin at a dose of 350 mg/kg daily in diabetes mellitus animal model showed there was no significant difference in testis weight in the group treated with metformin. Similarly, metformin at a dose of 30 mg/kg daily for twenty-one consecutive days in male albino rat showed no significant difference in testis weight (Adaramoye *et al*., 2012). However, Kianifard *et al*. (2012) reported a decrease in testicular weight in streptozotocin induced diabetic rats treated with metformin compared to control.

This study indicates that there was a downward trend in the mean testis weight for the NS200 and NS400 groups compared to saline, although this did not reach statistical significant (P = 0.0816 and P = 0.0570, respectively). Furthermore, these results were also not statistically different to the metformin group. Parandin *et al*. (2012) reported an increase in testicular size in rats receiving 200 and 400 mg/kg alcoholic extract of NS for 60 days orally. This was not statistically significant at 200 mg/Kg, but was significant at 400 mg/Kg dosage. Atta *et al*. (2017) reported that TQ treatment in diabetic rats increased testicular weight compared to control, where induction of diabetes decreased testicular weight in Wistar rats. Al-Sa'aidi *et al*. (2009) showed an alcoholic extract of NS increased testis weight and fertility parameters in white male rats treated with a different dosage of the alcoholic extract (0.5 and 1.5 g/Kg) for 53 days. In addition, further studies indicate that dose of NS 300 mg/kg body weight for 60 days leads to an increase in the number of spermatocytes and spermatids alongside a significant increase in the weight of reproductive organs in adult male albino rats (Mohammad *et al*., 2009). Tüfek *et al*. (2015) further demonstrated an increase in testicular weight after TQ treatment of obese rats fed with high-energy foods.

4.2.5. Epididymis Weight

The results show that the mean epididymis weight of the HSD group was significantly heavier (P<0.0001) compared to the NC group. This is consistent with Burgeiro *et al*. (2017) reporting that a high sucrose diet resulted in an increased epididymal fat pad weight compared to body weight ratio in male Wistar rats. According to a study by Archer *et al.* (2003), the epididymis was significantly heavier in rats fed with high energy (HE) diet containing corn oil and sweetened condensed milk for 14 weeks. In addition, Holmes *et al*., (2015) reported that the weight of the left epididymis was significantly higher in diet-induced obese using male Sprague-Dawley rats fed with high calories for 12 weeks .

This study shows that the metformin group mean epididymis weight was significantly decreased compared to the saline group (P = 0.0057). Metformin treatment of 150 mg/kg daily by oral gavage leads to decreased epididymal adiposity after 2 weeks in male Sprague-Dawley rats fed high energy diets (Thomas *et al*., 2016). Similarly, Song *et al*. (2001) reported metformin (120 mg/kg) decreased epididymal fat weight in high calories diet animal model.

This study shows that there was a significant decrease in the mean epididymis weight for the NS200 and NS400 groups compared to saline group (P = 0.0053 and P = 0.0017, respectively). TQ from NS, at a dose of 50 mg/kg daily in diabetic rats, lead to a relative decrease in the weight of epididymis, alongside the testis, prostate and seminal vesicles, compared to the control group. Moreover, the decrease of epididymis weight in diabetic rats depends on highest testosterone concentration, that leads us to say TQ may increase the levels of testosterone significantly in diabetic rats, which stimulates the growth and action of reproductive organs (Azzouni ,2012; Atta *et al*., 2017). However, Mahmoud *et al*. (2014) and Singh (2015) reported that no signicant difference in epididymis weight of 500 – 1000 mg/Kg per day dosage in animal studies. Furthermore, Bashandy (2007) and Parandin *et al*. (2012) did

not report any significant changes in epididymis weight using NS extracts at 200 and 500 mg/Kg respectively

This study shows that there was no statistical significance in mean epididymis weight in the NS200 group (P = 0.7679) and NS400 group (P=0.3782) compared to the metformin group. This suggests that NS was effective as metformin in reduced of epididymis weight.

4.2.6. Gastrocnemius Muscle Weight

The results shows that mean gastrocnemius muscle weight was significantly increased in the HSD group compared to the NC group (P= 0.0029). According to Swithers *et al*. (2011), rats exposure to high-calorie food had a greater effect on lean body mass compared to non-fat food. LIzárraga *et al*. (2015) reported that the lean body mass was increased at 9 months when feeding with high-calorie diet, using female Wistar rats. Rats on a sucrose-rich diet showed an increase in weight, with an increase in gastrocnemius muscle weight included (Chicco *et al*., 2003). Male Wistar rats fed with high sucrose diet and a high fructose diet for two months reported an increase in body weight where more than half was fat mass, and a quarter of the body was lean mass (Rosas-Villegas *et al*., 2017). Gómez-Pérez *et al*. (2012) reported that gastrocnemius and soleus muscle weight increased with high energy composition in Wistar rats. However, Paulino *et al.* (2010) reported that visceral fat mass weight levels were higher in rats on a high-fat and sucrose diet compared to lean rats in old male Wistar rats, with no clear change in weight of skeletal muscle. Fatan *et al.* (2012) further suggested that there were no significant differences in gastrocnemius muscle mass in adult male Wistar rats fed HSD for 15 weeks.

This study shows that there was no statistical significance in the mean gastrocnemius muscle weight for the metformin group compared to saline group (P = 0.1226). Oliveira & Gomes-Marcondes (2014) reported no significant differences in gastrocnemius muscle mass metformin treatment (33

mg/Kg/day) in young Wistar rats. Diniz Vilela *et al*. (2016) indicated that metformin treatment can minimize damage in the gastrocnemius muscle in diabetic rats induced by obesity, by decreasing muscle weight in male Wistar rats. Furthermore, Wessels *et al*. (2014) indicates that Zucker diabetic obese rats treated by oral gavage with metformin (30, 100 or 300 mg/Kg daily) showed impaired and decreased *in vivo* muscle capacity weight. Diabetic obese rats treated with metformin (200 mg/Kg) and cellulose (10 mg/Kg) leads to a decrease of lead body mass, opposite to metformin alone which increases lean body mass in experimental models (Reimer *et al*., 2014)

This study shows that there was a downward trend in mean gastrocnemius muscle weight in the NS200 group, although this did not reach statistical significance (P = 0.0726) compared to saline group. However, the NS400 group showed a significant decrease in the gastrocnemius muscle weight (P= 0.0123) compared to saline group. There are limited studies investigating the effect of Ns on lean body mass and skeletal muscle by. TQ compound was shown to protect the muscle by lower limb in nativel muscle tissues in Dawley male rats with dose 25 mg/kg of TQ (Erkut *et al*., 2016).

This study shows that there was no statistical difference in mean gastrocnemius muscles in the NS200 group (P = 0.9168) and NS400 group (P=0.3950) compared to the metformin group. This suggests that Ns was as effective as metformin in reduced weight of a gastrocnemius muscles in this study.

4.3. Blood Biochemistry

4.3.1. Testosterone Concentration

The results show that the mean testosterone concentration was non-significantly different in the HSD group (P = 0.4599) compared to the NC group. Adekunbi *et al*.(2016) reported that in the Male Sprague–Dawley rats fed with sucrose, reported a significant decrease in testosterone level. Sakamur *et al*. (2016) reported that male Sprague Dawley rats fed with

fructose also decreased testosterone levels. Furthermore, Macrini *et al*. (2016) reported that Male Wistar rats fed with a hypercaloric diet shows a significant decrease in testosterone levels compared with the control group. (Li *et al*., 2017).

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This study shows that mean testosterone concentration was significantly increased in the metformin group (P = 0.0001) compared to the saline group. This is consistent with Nasrolahi *et al*. (2013), reporting that Metformin at 100 mg/Kg with diet induced diabetic male rats could increase testosterone concentrations. Metformin (500 mg/Kg) further increased testosterone in male Sprague-Dawley diabetic rats (El Shaheed *et al*., 2012). Faure *et al*. (2018) reported that metformin (850 mg/day) leads to increased testosterone production in infertility human male patients.

This study shows that the mean testosterone concentration was significantly higher in the NS200 and NS400 group (P = 0.0002 and P < 0.0001) compared to saline. Bashandy (2007) reported an improvement of fertility index as manifested by an increase in seminal vesicle weight and testosterone concentration by using oral administration of Ns oil (0.5 mL/rat daily) for two months. Furthermore, Ns in dosage of 0.4 ml/100 gm of body weight resulted in increased testosterone levels, weight, and improved function of the testes, epididymis and prostate gland (Al-Taee, 2008). An alcoholic extract of Ns (200 and 400 mg/Kg for 60 days) increased fertility potential and testosterone concentration in male rats (Parandin *et al*., 2012). In addition, treatment of Ns and TQ extract in obesity induced diabetic rats increased testosterone levels by using dosage of 300 mg/Kg (Ns) and 4 mg/Kg (TQ) (Aithal *et al*., 2016). Mahmoud *et al*. (2014) reported an improvement in testosterone levels in diabetic rats treated with Ns 1 mL/Kg. In addition, Ns oil and TQ (3 g/day) caused a very significant reduction in body weight, and could prevent the decrease of the serum free testosterone level (Ibraheim, 2002; Tavakkoli *et al.,* 2017).

Furthermore, there was a no significant difference for testosterone concentration between metformin group and the NS200 (P = 0.3694) and NS400 (P = 0.7948) groups. Ns therefore improved testosterone as effectively as metformin in this study.

4.4. Sperm Parameters

4.4.1. Sperm Concentration

The results show the mean sperm concentration was significantly lower in the HSD group (P = 0.0128) compared to the NC group. This is consistent with the negative impact of obesity on male reproductive parameters (Leisegang *et al*., 2014; McPherson & Lane, 2015; Leisegang *et al*., 2016). In humans, it is well established that obesity and metabolic syndrome reduce fertility parameters, including concentration, motility, vitality, normal morphology and functional parameters such as DNA integrity, and is closely related with male factor infertility (Palmer *et al*., 2012; Leisegang *et al*., 2014; McPherson & Lane, 2015; Leisegang *et al*., 2016). Adekunbi *et al*. (2016) reported that rats exposed to sucrose to induce obesity reduced sperm concentration, motility and viability.

This study shows that the metformin group mean sperm concentration was significantly higher (P = 0.0003) compared to the saline group. Yan *et al*. (2015) indicates the protective effects of metformin to improve semen parameters including concentration, motility, viability and normal morphology in obese male rats at dosage 100 mg/kg daily. Furthermore, metformin at a dosage of 30 mg/kg/day for 6 weeks in diabetic rats leads to increasing epididymal sperm count, motility, and normal morphology (Adaramoye *et al*., 2012; Banihani, 2016).

The results of this study showed that the mean sperm concentration was increased in both groups of Ns, not significant for NS200 (P = 0.1043), but statistically significant in the NS400 group (P = 0.0493) compared to the saline group. Bashandy (2007) reported that male rats on an hyperlipidemic

and obesogenic HFD for 2 months and treated with Ns oil (0.5 ml/rat) resulted in improved sperm concentration, motility, and a decrease in sperm abnormalities (alongside increasing seminal vesicles weight and testosterone concentration). Parandin *et al*. (2012) reported that an alcoholic extract of Ns could increase fertility potential and sperm parameters, including increasing motility, concentration and viability in adult Wistar rats. Similar to this study, this was more pronounced at a higher dose of 400 mg/Kg than 200 mg/Kg body weight over 60 days. This also appears to be consistent in men, where 2.5 ml Ns oil for 2 mouths leads to improved semen volume, sperm count, motility and morphology in Iranian infertile men with abnormal semen quality (Kolahdooz *et al*., 2014).

Furthermore, sperm concentration in the NS200 (P = 0.0168) and NS400 (P = 0.0295) groups were significantly reduced compared to metformin. Although NS200 and NS400 increased sperm concentration compared to the control group, metformin was reported to be superior to Ns on this parameter.

4.4.2. Motility

The results show the mean progressive (P = 0.0092) and static (P = 0.015) motility of sperm was significantly lower and higher respectively in the HSD group compared to the NC group. However, there was no significant difference between the groups for non-progressive motility. Fernandez *et al*. (2011) reported a decreased percentage of progressively motile spermatozoa in Wistar rats fed a high obesogenic diet for 15 weeks. Diet-induced obesity (DIO) using sucrose and condensed milk for a period 16 weeks leads to decreased motility, motion parameters and viability in male Wistar rats (Oyeyipo *et al*., 2015). Young male rats exposed to high calories diet also decreased motility over 8 weeks (Yen *et al*., 2015). It is further well defined that obesity is associated with reduced motility in humans (Leisegang *et al*., 2014; McPherson & Lane, 2015; Leisegang *et al*., 2016). In healthy young men, sugar-sweetened beverages result in lower sperm motility (total and progressive) (Chiu *et al*., 2014). Furthermore, Rufus *et al*. (2018) reported that reducing body mass index (BMI) among male partners of infertile couples

improves compromised semen parameters, including sperm concentration and progressive motility.

The results show the mean progressive (P=0.3865), non-progressive (P = 0.5414) and static (P = 0.3565) motility of sperm was not significantly different in the metformin group compared to the saline group. Metformin by oral treatment in diabetic male rats decreases sperm motility when treated with metformin of 5 and 30 mg/kg for 21 days (Adaramoye *et al*., 2012; Banihani, 2016). In addition, metformin at dose 500 mg/kg of body weight in diabetic rats for 56 days showed that the sperm motility was decreased significantly in diabetic rats treated with metformin (Kianifard *et al*., 2012). However, Yan *et al*. (2015) indicates the protective effects of metformin to improve semen parameters including motility in obese male rats.

The results of this study showed that for the NS200 and NS400 group, there was a no significant change in mean sperm progressive motility (P = 0.2332 and P = 0.8253 respectively), non-progressive motility (P = 0.5895 and P = 0.1198, respectively) and static motility (P = 0.0851 and P = 0.5660, respectively) compared to the saline group. According to a study by Iranpour *et al*. (2017), TQ compound of Ns above 20 µg/ml caused a decrease of total motility percentages using *in vitro* models from human semen. Sadeghnejad *et al*. (2016) found that 5 and 10 mg/ml Ns *in vitro* incubation with semen also resulted in a reduction the percentage of motile spermatozoa (progressive and non-progressive sperms). Using an *in vivo* model, Cho Ping *et al*. (2014) reported that Ns oil at 2 mL/100g body weight for 60 days did not show any significant results on sperm parameters including sperm motility, using male Sprague-Dawley rats. Parandin *et al*. (2012) reported that an alcoholic extract of Ns could increase fertility potential and sperm parameter, including increasing motility and viability in adult Wistar rats. This was more pronounced at a higher dose of 400 mg/Kg than 200 mg/Kg body weight for 60 days. In addition, *in vivo* administration of TQ improved spermatogenesis and motility of spermatozoa, with the percentage of non-progressive and immotile sperms reduced (Fazelian, 2014).

4.4.3. Sperm Vitality

The results show the mean sperm vitality was not significant lower in the HSD group (P = 0.9648) compared to the NC group. However, numerous studies reflect a negative impact of obesity on vitality (Palmer *et al*., 2012; Leisegang *et al*., 2014; McPherson & Lane, 2015; Leisegang *et al*., 2016) not observed in this study results. Defo *et al*. (2017) suggested that a high-energy diet (including palm oil diet, fats, proteins, vitamins, and minerals) decreased sperm parameters including vitality in adult Wistar rats, alongside reduced normal spermatozoa morphology in the head and tail. In addition, diet-induced obesity using sucrose and condensed milk for a period of 16 weeks lead to reduced vitality of rat spermatozoa (Oyeyipo *et al*., 2015).

This study shows that the metformin group mean sperm vitality was non-significantly decreased (P = 0.5966) compared to the saline group. Yan *et al*. (2015) showed that metformin improved vitality in obese male rats at dosage 100 mg/kg daily. Furthermore, metformin (5 and 30 mg/kg for 21 days) in rats with the type 2 diabetes showed insignificant effect on the ratio of live sperm cells (Adaramoye *et al*., 2012).

The results of this study showed that the mean sperm vitality was significantly lower in the NS400 group (P = 0.0469), and there was no significant decrease in mean sperm vitality in the NS200 group (P = 0.0637) compared to the saline group. Cho Ping *et al*. (2014) reported that Ns at a dose 6.0 ml/100g body weight for 100 days treatment resulted in lower the percentage of dead sperm. According to a study by Parandin *et al*. (2012) using an alcoholic extract of Ns at dose of 200 and 400-mg/kg body weight daily for 60 days in male rats, there was a positive effect on viability of the rats. Mahmoud *et al*. (2014)reported that diabetic rats treated with 350 mg/Kg Ns daily also resulted in improvement in sperm viability.

Furthermore, sperm vitality in the NS200 (P = 0.1489) and NS400 (P = 0.1234) groups were not significantly lower compared to metformin. Although NS200 and NS400 improve sperm vitality depends in the high dosage of Ns,

compared to the control group, NS400 was reported to be superior to metformin on this parameter.

Mitochondrial Membrane Potential (MMP)

The results show the mean sperm mitochondrial membrane potential (MMP), expressed as the percentage of spermatozoa with intact MMP was decreased in the HSD group compared to the NC group, although this did not reach statistical significance (P = 0.2808). Although inconsistent, obesity is associated with reduced MMP (Leisegang *et al*., 2014; McPherson & Lane, 2015; Leisegang *et al*., 2016; Raad *et al*., 2017). Sperm motility is dependent on the functional solidity of the mitochondria (Paoli *et al*., 2011). Furthermore, there was a study by Fariello *et al*. (2012) that indicates the increase of BMI can lead to decreased mitochondrial activity and progressive motility in overweight male patients.

This study shows that metformin group MMP was significantly increased (P = 0.0003) compared to the saline group. Calle-Guisado *et al*. (2019) reported that metformin did not affect viability and mitochondrial membrane potential (MMP) without affecting parameters including viability, MMP and mitochondrial superoxide anion generation using *in vitro* incubation of 10 mM metformin for 8 and 20 hours. This present study appears to be the first i*n vivo* report suggesting a positive effect of metformin on MMP in an obesogenic environment.

The results of this study showed that the mean sperm MMP was significantly higher in the NS400 group (P < 0.0001), and a non-significant increase in the NS200 group (P= 0.3067) compared to the saline group. However, there has not been significant previous studies of Ns on this parameter. An *in vitro* study showed that Ns was able to deactivate sperm cells in a dose-dependent manner, where 20 µg/ml of TQ inactivated all spermatozoa including decreased mitochondrial membrane potential (MMP) (Iranpour *et al*., 2017). Moreover, both the Ns extraction and TQ compound has been found to inhibit membrane lipid peroxidation and to promote the antioxidant defense systems

which cause damage to the various lipid and protein components of the spermatozoa (Verma *et al*., 2007; Saulsbury *et al*., 2009; Kalender *et al*., 2012; Mosbah *et al*., 2016).

Interestingly, there was a no significant difference for sperm MMP in the NS400 (P = 0.8942) compared to metformin, suggesting the high dose of Ns was as effective as metformin in improving MMP. NS200 was not as effective as metformin nor was it statistically different to the saline control group.

4.5. Histological Examination Of Reproductive Organs

4.5.1. Seminiferous Tubules

The results show the mean largest expansion (P = 0.0001) and the mean width expansion of the seminiferous tubules (P = 0.0002) in the HSD group was significantly smaller compared to the NC group. This reflects a decreased lumen size. Additionally, the mean epithelial cell height of HSD group were non-significantly lower as compared to the NC group (P = 0.0901). Ibáñez *et al*. (2017) reported that DIO in male Wistar rats caused an abnormal seminiferous tubule with a reduction in seminiferous epithelium height and seminiferous tubular diameter. This is consistent with Yang *et al*. (2018), where DIO in Sprague-Dawley male rats showed atrophic and reduced seminiferous tubule diameters, with reduced cells through increased apoptosis.Campos-Silva *et al*.(2015) reported that male Wistar rats undergoing DIO showed a decrease in the seminiferous tubule diameter in the high caloric intake groups. De Oliveira *et al*. (2018) reported that Wistar rats receiving cafeteria diet (rat chow mixed with condensed milk and hydrogenated vegetable fat) showed a reduction in the histological structure and epithelium height of seminiferous tubules in cafeteria diet. HSD therefore induced expected changes in the seminiferous tubules in obesity in this study consistent with the literature.

This study shows that the metformin group mean largest expansion and mean width expansion of seminiferous tubules was significantly increased compared

to the saline group (P< 0.0001). This reflects an improved lumen size with metformin. Furthermore, the epithelial cell height of the seminiferous tubules was significantly higher (P = 0.0398) compared to saline group. Ayuob *et al*. (2015) demonstrated a beneficial effect of metformin (100 mg/Kg/day) on seminiferous tubule diameter in chemically induced diabetic rats. Nasrolahi *et al*. (2013) reported that metformin (100 mg/Kg/day) by oral gavage increased germinal epithelium height and seminiferous tubules diameter. A combination of Zinc (10 mg/Kg/day) and metformin (250 mg/Kg/day) improved histopathological structure of the testis, increasing the seminiferous tubule diameter (Aziz *et al*., 2018). Tag *et al*. (2015) reported that diabetic male albino rats treated with metformin (350 mg/Kg/day), leads to increased epithelial height. Kianifard *et al*. (2012) showed that diabetic Sprague-Dawley rats treated with metformin (100 mg/Kg/day) increased epithelium height of seminiferous tubule.

The results of this study showed that the NS200 and NS400 group mean largest expansion and the mean width expansion of seminiferous tubules was significantly increased(P<0.0001) compared to the saline group. Furthermore, NS200 and NS400 improved epithelial cell height of the seminiferous tubules (P = 0.0505 and P=0.0500, respectively) compared to saline group. Ns therefore has a positive impact on reduced lumen size in DIO rats. According to Atta *et al*. (2017), diabetic male Wistar rats induced by intraperitoneal injection of streptozotocin and treated with TQ (50 mg/kg) showed an improved seminiferous tubule diameter compared to diabetic group only. This leads to extensive structural repairs of seminiferous tubules, activation of Leydig cells and shedding of the germinal epithelium, through downregulation of inducible nitric oxide synthase (iNOS) and nuclear factor kappa (NF-κB) p65 protein expression levels in testes. Al-Sa'aidi *et al*. (2009) reported that mature rats treated with an alcoholic extract of Ns (0.5 and 1.5 g/Kg) showed a significant increase in the diameter and wall thickness of the seminiferous tubules and corresponding height of epithelial cells. This is supported by reporting of rats treated with Ns extract (0.4 ml/100 gm b.w) resulting in an increase of seminiferous tubule diameter and thickness of the germinal layer (Al-Taee, 2008).

Furthermore, there was a no significant increase in the mean large expansion of seminiferous tubules between NS200 (P = 0.7795) and NS400 (P= 0.6700) groups compared to metformin group. Ns appears to rescue HSD induced seminiferous tubule reduction as effective as metformin. Additionally, there was a no significant increase in the mean epithelial cell height of the seminiferous tubules between NS200 (P = 0.7301) and NS400 (P= 0.4630) groups compared to metformin. Ns was as effective as metformin in improving seminiferous tubule diameter and epithelial cell height.

4.5.2. Cauda Epididymis

The results show the mean largest expansion and the mean width expansion of the cauda epididymis in the HSD group was significantly smaller (P< 0.0001) compared to the NC group. Furthermore, the results show the mean epithelial cell height of the cauda epididymis was significantly smaller (P< 0.0001) as compared to the HSD group. Soudamani *et al.* (2005) reported that obesity induced diabetic Wistar rats showed reduced epididymis diameter and epithelial cell height of caput epididymis. According to the study of Ibáñez *et al*. (2017), Wistar male rats fed with HFD show a decrease in the stromal compartment of epididymal caput. Pushpendra & Jain (2015) reported that high cholesterol diet in rats showed a reduction in epithelial cell height of caput of cauda epididymis.

This study shows that the mean largest expansion and the mean width expansion of the cauda epididymis in the metformin group was significantly increased (P<0.0001) compared to saline group. Furthermore, metformin significantly improved the mean epithelial cell height of cauda epididymis (P<0.0001). Thomas *et al*. (2016) reported that T2DM induced through obesity and treated with metformin (150 mg/Kg) reduced epididymal adiposity and improvement of lumen size. Adaramoye & Lawal (2014) reported a link between metformin (30 mg/kg/day) in T2DM rats and an improvement epididymal morphology and epididymal sperm count.

This study shows that the mean largest expansion and the mean width expansion of the cauda epididymis in the NS200 and NS400 group was significantly higher ($P < 0.0005$) compared to saline group. Furthermore, Ns improved the mean epithelial cell height of cauda epididymis in both groups ($P < 0.0001$). Mohammad *et al*. (2009) showed that Ns extract in adult male albino rats (300 mg/Kg) resulted in an increase in epithelial cell height (cauda and caput) and significant increase in lumen size.

There was a no significant difference between the experimental groups for the mean largest expansion of the cauda epididymis (NS200: $P = 0.1829$ and NS400: $P= 0.2789$) groups compared to metformin group. Furthermore, there was a no significant difference in the mean width expansion of cauda epididymis between NS200 ($P = 0.3240$) and NS400 ($P= 0.5465$) groups compared to metformin.

4.6. Morphological Examination Of Reproductive Organs

4.6.1. Morphology Of Testis

In this study, the HSD group showed an increased height of epithelial cells and spermatocyte damage compared to NC. Vendramini *et al*. (2014) reported that obesity in rats caused damage in testicular tubules. Yang *et al*. (2018) reported that Sprague-Dawley male rats fed with high calories showed pathological changes by disfigured seminiferous tubules in the testicular tissue of the high calories group. Roushandeh *et al*. (2015) reported that male Wistar rats fed with HFD showed that the spermatocyte I and spermatids was significantly lower than in the control group.

In this study, the metformin group showed a remarkable regeneration of spermatocytes. Yang *et al*. (2018) reported that rats fed with high calories and treated with metformin (100 mg/Kg) resulted in reduced testicular cell apoptosis and increased spermatogenic cells in nearly all the seminiferous tubules. Nasrolahi *et al.* (2013) reported that rats fed with honey and treated with metformin showed normal spermatogenesis and improvement in

germinal epithelium and cell thickness. A study by Tag *et al*. (2015) reported a marked repairing of testicular abnormalities and expansion of histological structure in diabetic rats treated with metformin (350 mg/Kg/day).

In this study, the NS200 and NS400 groups showed that the morphological structure of the testes went through remarkable regeneration of spermatocytes, Sertoli cells and large epithelial cells. Samsudin (2015) reported that Male Sprague Dawley rats given Ns (60 µl/Kg) showed that the seminiferous tubules have been densely packed. Hala (2011) reported that Ns (250 mg/Kg) administration resulted in a normal morphological structure and normal spermatogenic chain. Furthermore, additional studies suggest a recovery of histological features of germ cells lining of the seminiferous tubules, with clear cell membranes, normal sperm in the lumen and clear Leydig cells after treatment of Ns at dose of 1000 mg/Kg/day (Gali-Muhtasib *et al* ., 2006; Elshama *et al* ., 2013).

HSD therefore has a detrimental impact on testicular morphology. This can be improved through administration of metformin as well a Ns oil and seed extractions. Metformin appeared to improve testicular morphology similarly to that of Ns.

4.6.2. Morphology Of Epididymis

In this study, the HSD group showed spermatozoa damage in the HSD group. According to Soudamani *et al*. (2005), obesity induces a regression in the epididymidal tubular size and increase in the interstitial stroma and shrinking of epithelium cells, a reduction in the lumen diameter and a thickening of basement membrane. A high calorie diet in rats results in an increase in the diameter of tubule, lumen, and reduced size of epithelium compared to the control group (Amini Mahabadi *et al*., 2013). Ibáñez *et al*. (2017) reported that Wistar male rats fed with a high-fat diet (HFD) for 30 days resulted in a decrease in the stroma and epithelium and increased in lumen diameter.

In this study, the metformin group showed remarkable regenerative feature in spermatocytes. According to a study of Ayuob *et al*. (2015), metformin in male diabetic rats showed remarkable regenerative feature in spermatocytes and showed a significant reduction in the epithelial cell height of both corpus and cauda of the epididymis. Owolabi & Omogbai. (2012), reported that metformin resulted in favourable histological changes in the testis and epididymis, increasing epididymal tubules and height of epithelium.

In this study, the NS200 and NS400 groups showed a congestion of spermatogonia. Al-Sa'aidi *et al* .(2009) reported that male rats were treated with the alcoholic extract of Ns (0.5 and 1.5 g/Kg), increased epitheliam cell height. According to Mohammad *et al*. (2009), Ns (300 mg/Kg) increased epithelial cell height in and increased the number of spermatogonia.

HSD has a detrimental impact on epididymal morphology. This can be improved through administration of metformin as well a Ns oil and seed extractions. Metformin appeared to improve epididymal morphology similarly to that of Ns.

4.6.3. Morphology Of Prostate

In this study, the HSD group showed disruption in the prostate architecture, with a greater severity of mucosal infoldings and congestion. According to a study by Ribeiro *et al*. (2012), rats exposed to high calorie diet for 15 weeks showed an increase in cell proliferation in both prostate epithelium and stroma. Silva *et al*. (2015) indicated that adult male Wistar rats fed a protein, carbohydrate and fat diet showed epithelial hyperplasia was the most prevalent in the prostate of obese rats,. Furthermore, Tikoo *et al*. (2017) reported that Sprague-Dawley rats fed with high calories of carbohydrate, protein and fat showed an upregulation of inflammation by promoting an increase of cellular tumor antigen and phosphoprotein (p53), accelerating prostate epithelial hyperplasia.

In this study, the metformin group showed improvement in the disruption of the prostate architecture compared to saline, reducing the severity of columnar epithelial lining and congestion. Mosli *et al*. (2015) reported that diabetic Sprague–Dawley rats treated with metformin (500 and 1000 mg/Kg) for 14 days leads to a change in the epithelial structure by reduction of epithelial cells thickness compared to the control group. Nasrolahi *et al*., 2013, reported that diabetic rats treated with metformin (100 mg/Kg) showed an improvement in the germinal epithelium thickness. A study by Xu *et al*. (2018) reported that the rats fed high calories and treated with metformin showed a reduction of prostatic hyperplasia and a favourable change in the epithelial structure. Akinyek *et al.* (2013) reported that mice fed with normal chow and treated with metformin (200 mg/Kg) showed a magnification of nuclei and nonuniform, atypical and diminutive growth of epithelial cells.

The NS200 and NS400 groups showed a reduction in the disruption of the prostate architecture compared to saline and decreased severity of columnar epithelial lining changes and congestion compared to the NC group. Lina *et al*. (2014) reported that Sprague-Dawley juvenile male rats treated with Ns (6 µl/100g) oil showed an improvement in histology structure, namely reduction in the epithelial height of the mucosal linings and with the appearance of an acidophilic secretion in the lumen as compared to the control group. Furthermore, Khan *et al*. (2011) reported that TQ improved endothelial cells and increased cell proliferation. Radad *et al*. (2014) and Sayed *et al*. (2014) reported that male Sprague-Dawley rats given a dose of 10 mg/Kg TQ twice weekly showed normal histological appearance with moderate congestion of some blood vessels and crowding of the lining epithelium of prostate in the experimental group .

HSD has a detrimental impact on prostatic morphology by disruption of the prostate architecture, with hyperplasia and increased height in columnar epithelial lining and congestion. This may be improved through administration of metformin as well as Ns oil. Metformin appeared to improve prostatic morphology similarly to that of Ns.

4.7. Conclusions

The results of this study demonstrated that the administration of Ns (200 mg/Kg and 400 mg/Kg) and Metformin (75 mg/Kg) for 6 weeks alongside an obesogenic diet resulted in weight loss, increased serum testosterone and improved histological features of the rat testes, epididymis and prostate gland. Furthermore, Ns and Metformin could increase the sperm motility, vitality, MMP in obese rats. There was no significant decrease in mean sperm vitality in the NS group, and the mean sperm concentration was increased in both groups of Ns. NS200 and NS400 showed remarkable improvements of morphological structure of the testes, epididymis and prostate similarly to metformin. Ns seed oil, alongside metformin, may protect and improve obesity induced reproductive dysfunction. This warrants further investigation into the mechanisms of action and clinical confirmation of these medicines for obesity induced male infertility.

CHAPTER 5

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