

CHAPTER 1

INTRODUCTION

1.1 Global Infertility Scenario

International Committee for Monitoring Assisted Reproductive Technology and the World Health Organization defined infertility as ‘a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse’ (Zegers-Hochschild *et al.*, 2009). American Society for Reproductive Medicine (2006a) defines infertility as a disease. On the other hand, infertility is defined by demographers as absence of live birth in a sexually active and non-contracepting woman (Larsen, 2005). Demographers take live births into consideration instead of conceptions due to the fact that complete data on conception in population-based studies are difficult to collect (Casterline, 1989).

It has been reported that 10–15 % of couples are affected globally (Mosher and Pratt, 1991); approximately 72.4 million couples had fertility problems (Boivin *et al.*, 2007). Male infertility is more prevalent compared to female (Ibrahim *et al.*, 2008). Shefi and Turek (2006) reported that approximately 50 % of infertility cases are related to male factors. Male infertility generally can be associated with sperm defects such as low sperm production (oligozoospermia), low sperm motility (asthenozoospermia) or sperm with abnormal morphology (teratozoospermia). However, oligoasthenoteratozoospermia (OAT), which is a combination of these sperm defects, is the most common factor that leads to male subfertility (Guzick *et al.*, 2001). In 2002, National Survey for Family Growth conducted by Centers for Disease Control and Prevention (CDC) reported that about 7.5 % of all sexually active men (3.3-4.7 million men) would seek clinical help in having a child and 13.7 % of those seeking help have

sperm or semen problem (Anderson *et al.*, 2009). It has been suggested that these infertile couples also seek for solution by turning to traditional medicine made from natural plants and modern medicine as well (Feldman & Laura, 2004).

1.2 Fertility in Malaysia

Demographics of Malaysia are made up of various ethnic groups. Sixty five percent of the populations are Malays and bumiputera, 26 % Chinese, 8 % Indians and other ethnic groups 1 %. Meanwhile, population of Malaysia has an annual growth rate of 2.4 % (Vital Statistics Malaysia, 2012).

From the overall population of Malaysia, 80 % of them resides in Peninsular Malaysia in the year 2000, while 11 % and 9 % of the remaining population reside in Sabah and Sarawak, respectively (Tey *et al.*, 2012). In Peninsular Malaysia, the Malays, Chinese and Indians made up 60 %, 26.4 % and 9.1 %, respectively of the total population (Tey *et al.*, 2012). Hence, for a clearer picture of fertility problems in Malaysia, only the three main ethnic groups in Peninsular Malaysia would be discussed.

Fertility rate could be defined as the average number of children that would be born to a woman over her lifetime if she followed the current average pattern of fertility among a given group of women and survived through her reproductive years. The latter factor is to be used as an indicator of strength of population growth. Demographers often compare fertility rates to replacement level fertility. Replacement level fertility refers to the number of children that a woman must have in order to replace the existing population. Fertility rate of Malaysian reached the replacement level of 2.1 in 2010, increased to 2.2 in 2011 parallel with increased birth rate (Vital Statistics Malaysia, 2012).

Malay and Bumiputera woman aged 15-49 years have fertility rates of 2.7 and 2.3, respectively, which were higher than the replacement levels (Vital Statistics Malaysia, 2011). Contradictorily, fertility rates of Chinese, Indians and others were lower than replacement level with 1.5, 1.6 and 1.0, respectively in 2011 (Vital Statistics Malaysia, 2011). However, the main basis for these values has not been well established. Possible factors include rising costs of living and daily stresses undergone by Malaysian citizens that eventually lower productivity and fertility.

1.3 Etiology of Male Infertility

There are numerous known potential causes of male infertility, however, a large portion of male infertility cases remain idiopathic. This scenario by chance suggests the insufficient and poor understanding of spermatogenesis process and its regulatory mechanisms (Rajender *et al.*, 2010). Over the past 50 years, qualities of sperm are deteriorating gradually (Carlsen *et al.*, 1992) and this could be correlated to the increase in environmental pollution (Osser *et al.*, 1984). The detrimental effects of environmental threats on male reproductive function were discovered some 30 years ago when pesticide manufacturer and agricultural workers exposed to nematocide suffered infertility due to severely impaired spermatogenesis (Whorton *et al.*, 1977; Slutsky *et al.*, 1999). To date, many factors are known to affect fertility. These factors include heavy metals, solvents, agricultural chemicals, phytoestrogens, radiation exposure and heat (Lahdetie, 1995).

One of the major factors that lead to infertility in male is smoking. Smoking is one of the lifestyle factors that lead to chronic diseases in the long run and can also cause fertility problems during the reproductive years (Augood *et al.*, 1998; Nestler *et al.*, 1989). Langgassner (1999) stated that most smokers were young adult males aged

between 20 to 39 years old. The Surgeon General's Report on the Health Consequences of Smoking also emphasized on the negative effects of tobacco smoking on reproductive system (Klonoff-Cohen *et al.*, 2001). Tobacco smoking would also bring about smaller volume of ejaculate, lower sperm density and sperm with abnormal morphology (Vine *et al.*, 1996). Tobacco smoking contributes to an unknown proportion of infertility cases. Experts in the field of reproduction and infertility are also aware about the risk of infertility and treatment interference by exposure to tobacco products (Dorfman, 2008). Hence, screening for tobacco use and cessation need to be carried out in order to prevent infertility (American Society for Reproductive Medicine, 2006b).

1.4 Treatment for Male Infertility

The cause of male infertility complications need to be determined prior to appropriate treatment. Process of determining cause of male fertility starts with physical examination and detailed history of the patient such as duration of attempted conception, frequency and timing of copulation, penile erection, ejaculation, lifestyle factors and drug intake (Petrelli & Mantovani, 2002).

In order to determine the fertility status of a male subject, semen analysis serves as a tool in diagnosis processes, which can determine the cause of male subfertility (McLachlan *et al.*, 2003). Human sperm are diversified and incomparable in kind, hence a range of normal and abnormal sperm can be discovered from semen samples (Chia *et al.*, 1998). In addition, a normal spermiogram, which include physical, morphological as well as biochemical investigations of semen, will not give the full picture of an individual's fertility potential. Bound by all these restrictions in accessing semen, only 40 % of males seeking assistance on fertility problems can be accurately diagnosed of

male factor infertility (Moldenhauer *et al.*, 2003). Only when the actual cause of male infertility has been confirmed, a precise and appropriate treatment, which will eventually leads to a cure, can be carried out (Abdel Raheem & Ralph, 2011). If a patient falls under the category of idiopathic infertility and basic treatment fails, Assisted Reproductive Techniques (ART) such as artificial insemination, *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) are recommended (Abdel Raheem & Ralph, 2011). In cases where patients are diagnosed with either non-obstructive or obstructive azoospermia, which is not responsive to surgical reconstruction, surgical sperm retrieval followed by ICSI shall be carried out (Abdel Raheem & Ralph, 2011).

The birth of Louise Brown, the first ‘test-tube baby’ in 1978 marked a significant advancement in reproductive medicine and also a breakthrough in the medical field (Steptoe & Edwards, 1978). The most important requirement for IVF of the oocyte is motile sperm; hence, IVF would not yield desirable results if male factor infertility is involved (Palermo *et al.*, 1992). However, this shortcoming of IVF has been solved with the introduction of ICSI in 1992 (Palermo *et al.*, 1992). Intracytoplasmic sperm injection (ICSI) procedure required the use of ejaculated sperm when it was first introduced. However, surgically retrieved sperm either from the testis or epididymis is widely used today (Devroey *et al.*, 1994; Silber *et al.*, 1994). It is a very precise but flexible procedure. Even if a male patient is found to have a lack of germ cells and no evidence of spermatogenesis in seminiferous tubules, ICSI can still be carried out using rare spermatozoa present in the testes (Matzuk & Lamb, 2008).

However, there are arguments regarding the safety and reliability of Assisted Reproductive Technologies 30 years after the first successful birth through IVF and 16

years after ICSI was introduced (Matzuk & Lamb, 2008). To date, results of researches do not give a clear picture of side effects caused by ART and there are just mere suggestions that ICSI-conceived children are prone to congenital defects, sex chromosome defects, multiple gestation and increase risk of epigenetic syndromes (Alukal & Lamb, 2008). In addition, there are also studies regarding the delay in development and impaired neurologic status in children born through either ICSI or IVF procedures (Alukal & Lamb, 2008). Hence, alternative treatments on infertility are widely practised nowadays owing to possibly lesser side effects and relatively cheaper costs compared to ART.

1.5 Alternative Treatment for Male Infertility

Alternative medicines used for the purpose of improving fertility in animals are increasing due to the fact that animal owners nowadays, prefer natural plant products to synthetic drugs (Dada & Ajilore, 2009). Plants that have not been studied and considered to have no medicinal values are currently being studied and developed into drugs that possess little or no side effects (Adedeji *et al.*, 2006). Since ancient times, herbs had been used in treating various diseases including to enhance fertility (Ramsey, 2000). One of the herbs commonly used traditionally is *Nigella sativa* (*habbatus sauda*), a plant from the Ranunculaceae family that grows abundantly in several Middle Eastern and Southern Mediterranean countries (Tariq, 2008). Seeds of *Nigella sativa* are usually used as spice, besides being used widely as traditional medicine for various ailments (Meddah *et al.*, 2009). In addition, seeds and oils from *Nigella sativa* have antipyretic, analgesic, antihypertensive and antineoplastic properties (Ali & Blunden, 2003). Since evidence on the effects of *habbatus sauda* on sperm and testis parameters are still lacking, this study was carried out to address this matter, which leads to the objective of this study.

1.6 Objectives

The present study investigated the after effects which resulted from 100 days administration of nicotine, *habbatus sauda* and co-administration of nicotine-*habbatus sauda* on *Sprague-Dawley* male rats. Specifically, the objectives were as below:

1. To determine the body weight increment of rats
2. To measure the profile of selected blood hormone related to reproductive physiology (testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH))
3. To evaluate selected sperm parameters (motility, morphology and vitality)
4. To study changes to testis histological features

CHAPTER 2

LITERATURE REVIEW

2.1 Male Reproductive System

The male reproductive system is made up of the scrotum, testes, spermatic ducts, sex glands, and penis. The function of testes is for the production of male gametes, while sex glands produce other components of semen. The presence of these organs and structures allows the transfer of male gametes into female reproductive tract for fertilisation to take place.

2.1.1 Scrotum

The scrotum is a fibromuscular organ made of skin and muscles. It is divided by a median septum (raphe) to form two compartments that house the testes and associated structures. The structures and layers that make up the wall of a scrotum are rugated skin, superficial fascia, dartos muscle, external spermatic fascia, cremasteric fascia and internal spermatic fascia (Dogra *et al.*, 2003). The tunica albuginea which is covered by the tunica vaginalis can be divided into a visceral layer which extends over the testis and epididymis and a parietal layer which lines the wall of the scrotum. Both visceral and parietal layers of the tunica albuginea merge at the posterolateral part of the testis which is also the site of attachment between the tunica and scrotal wall. Meanwhile, the whole testis and epididymis are covered by the tunica vaginalis leaving a small area uncovered posteriorly (Dogra *et al.*, 2003).

2.1.2 Testes

Testes are the male gonads responsible for the production of sperm and testosterone. Factors, such as age and sexual development stage play significant roles in determining testicular size. Commonly, the length and width of human male testis are 1.5 cm and 1 cm, respectively, at birth, while the volume of a testis is approximately 1-2 cm³ before the age of 12 years old. When the volume of testis reaches 4cm³ or more, an individual is considered to have reached his puberty period (Dogra *et al.*, 2003). Testes of postpubertal male are symmetric, ovoid in shape and have a dimension of 5x3x2 cm (Doherty, 1991). The function of the nonstriated smooth muscle cells of the fibrous tunica albuginea is for spermatozoa transportation towards the rete testis and into the epididymis (Cook & Dewbury, 2000). Mediastinum is formed when posterior surface of tunica albuginea connects with the inner part of testis and numerous fibrous septa will extend from mediastinum into the testis forming 250-400 lobules. Each lobule consists of 1-3 seminiferous tubules containing Sertoli cells and spermatogenic cells which will eventually form sperm besides Leydig cells (LCs) responsible for testosterone production (Dogra *et al.*, 2003).

Interstitial cells started to produce androgens during puberty followed by the enlargement of the seminiferous tubules. There are 2 types of cells inside seminiferous tubules, germ cells involve in spermatogenesis and sustentacular (Sertoli) cells that support, nourish and regulate cells during spermatogenesis (Mader, 2004).

2.1.3 Epididymis

Epididymis shares the same border with the posterior part of the testis where it lies superior to the testis. Generally, epididymis has a tubular structure and can be divided into head, body and tail. The head of the epididymis (globus major) consists of 8-12

efferent ducts which will combine into a single larger duct forming the body and tail (globus minor). The same duct inside the body and tail of epididymis will form the vas deferens and extends further into the spermatic cord (Dogra *et al.*, 2003).

2.1.4 Spermatic cord and ductus deferens

Spermatic cord descends from the deep inguinal ring to the scrotum. Structures such as the vas deferens, testicular artery, cremasteric artery, deferential artery, pampiniform plexuses, genitofemoral nerve and lymphatic vessels are the building blocks of spermatic cord (Krone & Carroll, 1985; Langer, 1993).

Ductus deferens is an unconvoluted duct leading to the urethra. The ductus deferens has a thick wall structure which is further lined by pseudostratified epithelium and a thick layer of smooth muscle (Setchell & Breed, 2006). Types of cells that can be found in ductus deferens are similar to principal, narrow and basal cells of the epididymis (Hermo *et al.*, 2002).

2.1.5 Ejaculatory duct

The ductus deferens passes through the prostate and joins with the urethra at a structure known as the ejaculatory duct. The ejaculatory duct contains the ducts from the seminal vesicles as well. During ejaculation, sperm and the secretions from the seminal vesicles is expelled into the urethra through an opening of the ejaculatory duct.

2.1.6 Seminal vesicles

The seminal vesicles produce secretion known as the seminal fluid which makes up 50-80 % of total ejaculate volume (King *et al.*, 1991). Liquid produced by seminal vesicles are alkaline in nature to ensure sperm survival in vagina, which has an acidic

environment. The normal length and diameter of a typical seminal vesicle are 3.0 ± 0.8 cm and 1.5 ± 0.4 cm, respectively (Kim *et al.*, 2002). Meanwhile, seminal vesicle has a normal volume of approximately 13.7 ± 3.7 ml (Kim *et al.*, 2002). It is common to find slight asymmetry between a pair of seminal vesicles (Aboul-Azm, 1979). The seminal vesicles located posterior to the urinary bladder and distal ureters. Ejaculatory duct will be formed when seminal vesicle connects to the distal part of vas deferens and seminal fluid will pass through the verumontanum to the prostatic urethra (Kim *et al.*, 2009).

2.1.7 Urethra

Semen passes from the ejaculatory duct to the exterior of the body via the urethra, a 20 to 25 cm long muscular tube. The urethra passes through the prostate and ends at the external urethral orifice located at the tip of penis. Urine exiting the body from the urinary bladder also passes through the urethra.

2.1.8 Penis

Penis, cylindrical in shape is the male external sexual organ. Semen of mammals is transferred into the female reproductive tract through the penis. Basically, penis can be classified into the vascular penis and the fibroelastic penis (Walton, 1960). Os penis or baculum is present in many eutherians while the tip of penis is bifid in some marsupials (Biggers, 1966; Patterson & Thaeler, 1982; Woolley, 1982). Vascular penis has two corpora cavernosa merged by a septum and a single corpus spongiosum that covers the urethra. Glans, an extension of the corpus spongiosum covers the distal end of the corpora cavernosa (Setchell & Breed, 2006).

Fibroelastic penis has a thick tunica made up of dense collagen fibers and erectile tissue lined by fibroelastic tissue can only be found at the root of penis. During

erection, there is no size increment of the penis, instead, a bulge is created by relaxation of the retractor penis muscles and straightening of the sigmoid flexure (Setchell & Breed, 2006).

2.1.9 Accessory sex glands

The mesonephric duct, prostatic and penile urethra gives rise to the accessory sex glands. Mesonephric duct has an increased area of secretory epithelium due to villous infoldings that give rise to the seminal vesicles and ampullary glands. On the other hand, the proximal and distal urethra gives rise to the prostate and bulbourethral glands, respectively (Setchell & Breed, 2006). In addition, the prostate appears disseminate or lobular relying on the species (Eckstein & Zuckerman, 1956; Hamilton, 1990). The size and function of these glands change with age depending on concentrations of androgens inside the body, hence termed androgen dependent (Mann & Lutwak-Mann, 1981). In rodents, the abstractions of some of their accessory sex glands tend to reduce fertility *in vivo* (Queen *et al.*, 1981). Accessory sex glands produce secretions containing various substances which is part of semen in an ejaculate.

2.1.10 Semen

Semen, fluid produced by males for sexual reproduction and is ejaculated from the body during sexual intercourse. Semen contains sperm, the male reproductive gametes, along with a number of chemicals suspended in a liquid medium. The chemical composition of semen gives it a thick, sticky consistency and a slightly alkaline pH. These traits of semen help sperm to remain alive within the vagina after intercourse and neutralize the acidic environment of the vagina. In healthy adult human males, semen contains around 100 million sperm cells per milliliter.

2.2 Spermatogenesis

Spermatogenesis is a complex process of spermatozoa production that takes place within the testis (Johnson, 1991). Three main steps involved in spermatogenesis are spermatocytogenesis which involves spermatogonia, meiosis which involves spermatocytes and spermiogenesis which involves spermatids (Johnson *et al.*, 2000).

In rodents, a single spermatogonium cell undergoes active mitotic cell divisions either for the purpose of generating new spermatogonial stem cells or to produce differentiating spermatogonia (de Rooij, 1998). In rats, 53 days are needed for one complete spermatogenesis cycle. Final transition of spermatozoa through the epididymides requires 6-7 days (Ke & Tso, 1982). In mice, spermatogonia start to proliferate soon after parturition, at approximately 5 days post-partum. Spermatogenic differentiation generates primary spermatocytes at approximately 10 days post-partum, followed by spermatids and mature spermatozoa at 20 and 30 days post-partum, respectively (Bellve *et al.*, 1977; Zhao & Garbers, 2002). In addition, the spermatogenic cycle can be divided into various stages or cell associations based on a specific species. It was reported that spermatogenic cycle of mouse involved 12 stages while that of rat involved 14 stages (Leblond & Clermont, 1952; Oakberg, 1956).

Spermatogonia are germ cells located near the capsule of seminiferous tubule which upon mitosis produce primary spermatocytes (Mader, 2004). Primary spermatocytes will then undergo meiotic cell division to become haploid secondary spermatocytes with reduced number of chromosomes. Secondary spermatocytes will undergo another meiotic cell division and form 4 spermatids by the end of cell division (Mader, 2004; Handel & Schimenti, 2010). Spermatids then enter the process of spermiogenesis where spermatids differentiate into spermatozoa (O'Donnell *et al.*,

2011). According to Ahmed and Rooij (2009), spermiogenesis in mice could be subdivided into 16 steps where steps 1 to 8 involve round spermatids and steps 9 until 16 involve elongation of spermatids before reaching the lumen of the seminiferous tubule. Finally, the mature spermatozoa would be channeled to the epididymis (Mader, 2004).

Laboratory animals have been used extensively in studies to investigate the effects of various chemical compounds exposures on spermatogenesis during adulthood. Dosage of chemical compounds used on many experimental animals was usually higher than that of human environmental exposure under normal circumstances. Hence, the evaluation on spermatogenesis due to the effects of human environmental chemical compound exposure in such studies was not accurate (Sharpe, 2010). Studies using human subjects to determine cause and effect are not plausible compared to laboratory animal. This is partly due to fewer variables in phenotype and spermatogenesis profile of laboratory animal compared to that of human males (WHO, 1999; Sharpe, 2000). In addition, human males are exposed to various complex mixtures of environmental chemicals in daily life that might in one way or another affect spermatogenesis, while only one specific chemical is studied when using laboratory animals. These conditions make extrapolation based on animal studies onto human more challenging (Sharpe, 2009). Furthermore, the dose of environmental chemicals exposure in human varies greatly, unlike experimental animals' exposure to consistent dose under specified circumstances (Sharpe, 2010).

The main difference of spermatogenesis in laboratory animals is that it is a highly organised and efficient process involving development of germ cells in a synchronized manner. Association between germ cells and Sertoli cells in each

seminiferous tubule is homogeneous which is also known as the radial arrangement of stages in spermatogenic cell cycle (Sharpe, 1994). In human, spermatogenic stages have irregular arrangement. Other differences between human and rodent in terms of testis development and spermatogenesis were shown in Table 2.1.

Table 2.1: Comparison of features of testis development and spermatogenesis in rodents and humans

Parameter	Rodent	Human
Sertoli cell proliferation	Foetal, neonatal/prepubertal; maturation at puberty.	Foetal, neonatal/prepubertal; maturation at puberty.
Perinatal germ cell proliferation and differentiation	Synchronous proliferation and differentiation (rapid changes).	Asynchronous proliferation and differentiation (spread over foetal and early postnatal life)
Neonatal/infant period of hypothalamic-pituitary-testis hormone activity	Lasts approximately 6 hours	Lasts 4-5 months
Childhood testis “quiescence”	None	Lasts 11-14 years
Puberty	Reactivation of hypothalamic-pituitary-testis hormone activity.	Reactivation of hypothalamic-pituitary-testis hormone activity.
Spermatogenesis	Synchronous (“organised”); highly efficient.	Asynchronous (“disorganised”); inefficient.
Sperm count/sperm storage	Sperm stored in epididymis, so high sperm count can be maintained during repeated ejaculation.	Minimal/no sperm storage, so ejaculatory frequency is an important determinant of sperm count.

2.3 Sperm

Basically, sperm in testis and caput epididymides are incapable to fertilise an egg unless the sperm undergo capacitation within the female reproductive tract (Oura & Toshimori, 1990). Mature sperm can be divided into 3 parts, head covered by acrosome, middle piece as source of energy for movement and flagellum-like tail for movement (Mader, 2004). Sex chromosome contained in nucleus of haploid sperm will determine the gender of an offspring (Segal, 1985). Lifespan of sperm inside the female genital tract is approximately 48 hours or less (Mader, 2004).

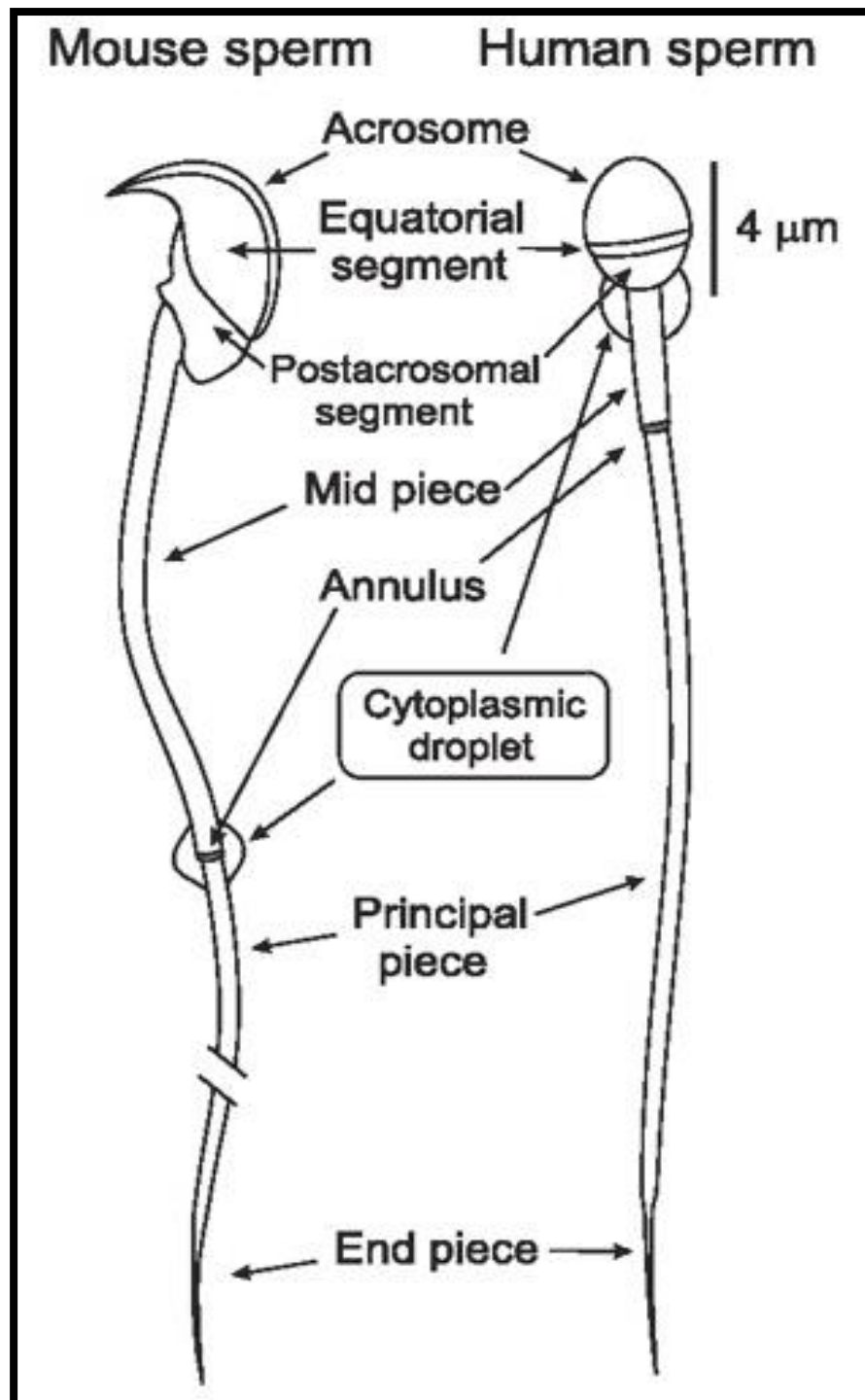


Figure 2.1: The distribution of regions on a falciform-shaped mouse sperm and human sperm (retrieved from <http://physrev.physiology.org/content/physrev/91/4/1305/F1.large.jpg>)

2.3.1 Sperm head

A typical mammalian sperm have a spatulate head containing the nucleus and acrosome surrounded by cytoskeletal components and cytoplasm. However, the sperm of some rodents exhibit falciform-shaped head where acrosome overlying convex margin of nucleus (Figure 2.1). Van der Horst *et al.* (1991) demonstrated that by considering the structure and shape of acrosome, 4 very similar species of ferret could be distinguished from one another. A separate research by Breed (2004; 2005) indicated that the structure of sperm head was linked to the phylogenetic relationships in rodents.

2.3.2 Mid-piece

The neck or mid-piece of sperm usually made up of connecting piece and the centriole (Fawcett, 1975). Axoneme and outer dense fibers which are covered by a long sheath of mitochondria can be found in the mid-piece of a typical mammalian spermatozoon. These mitochondria appear elongated and have a helical arrangement around the centre of the sperm tail. Mitochondria located in the sperm midpiece serve as the energy source for the sperm to propel. The density of these mitochondria will determine flagellar beat frequency (Cardullo & Baltz, 1991). Mid-piece of a human sperm consists of 15 mitochondrial gyres whereas that of some rodent species contains up to 300 gyres (Fawcett, 1975).

2.3.3 Flagellum/Tail

A typical mammalian sperm tail is made up of an axonemal complex consisting of two central microtubules and nine outer dense fibers which shows a 9+2 pattern (Baccetti & Afzelius, 1976). The axonemal-outer dense fiber complex of the flagellum is surrounded by fibrous sheath which is further divided into several transverse ribs along the principal piece (Fawcett, 1970). The main function of this fibrous sheath is as a

structural support for the flagellum that allows rapid propulsion, especially in the female reproductive tract across viscous medium (Fawcett, 1970; Anderson & Personne, 1975).

2.4 Sperm Parameters

In older days, quality of a semen sample in terms of percentage of motile sperm, percentage of sperm with normal morphology and concentration in a unit dose were evaluated prior to artificial insemination. The purpose of semen analyses is to achieve accurate, objective, rapid and inexpensive output of fertility assessment (Gillan *et al.*, 2005). However, common laboratory assays used in semen analyses did not meet these standard criteria and fertilising potential of sperm could not be portrayed due to the fact that sperm are complex cells that require other additional criteria to be fulfilled in order for fertilisation to occur (Gillan *et al.*, 2005). In fact, there are no specific parameters that can be evaluated to determine the fertilising capacity of sperm. Nevertheless, semen analysis can serve as a screening tool for complications of the male reproductive system, which is helpful in determining the cause of infertility. Parameters of ejaculates that are evaluated in a basic semen analysis include appearance, smell, viscosity, liquefaction, sperm concentration, volume, sperm motility and sperm vitality (NAFA and ESHRE, 2002).

2.4.1 Sperm motility

Spermatozoa are immotile *in vivo*. Motility is only acquired after ejaculation or spawning. Changes in conditions surrounding the sperm such as osmotic stimulation or ionic concentrations will induce motility (Morisawa, 1994). Apart from sperm count and morphology evaluations, sperm motility is one of the frequently evaluated parameters in researches on mammalian reproduction (Perrault, 1998). The use of automated analyser has made the measurement of sperm motility possible. Sperm

motility is defined as the percentage of moving sperm in a properly collected sample. In order to differentiate between motile and immotile sperm and between sperm and debris, parameters on the automated systems such as gate, size and intensity is calibrated accordingly (Holson *et al.*, 2006).

2.4.2 Sperm morphology

Previously, sperm concentration, motility and morphology were evaluated to determine fertility status and for the purpose of IVF (Menkveld *et al.*, 2011). Nowadays, one of the essential parameters to be considered when evaluating the quality of semen sample taken from either human or animals is sperm morphology (Henkel *et al.*, 2007; Van der Horst *et al.*, 2009). In addition, there was a well-established connection between fertility status and sperm morphology evaluation (Menkveld *et al.*, 2003).

Evaluation of sperm morphology is very difficult due to high level of variability shown by normal and abnormal sperm. However, these variables can be reduced by the introduction of standard analytical methods besides sufficient knowledge and training (Eustache & Auger, 2003). Visual assessment on sperm morphology is challenging due to the fact that it depends greatly on the mechanism of human vision and their integration in the brain, which is subjected to various limitations (Auger, 2010). Human integration of vision-brain mechanism involved in evaluating sperm morphology is more accurate in pattern recognition compared to computer assisted sperm evaluation. However, human visual assessment have limitation on measurement of sperm, for example, inter-observer variability in determining percentage of sperm with size defects was higher compared to percentage of sperm with qualitative variance (Eustache & Auger, 2003). Hence, sperm assessment in terms of sizes, shapes and textures are best to

be analysed using computer software in order to reduce level of variability and human error.

2.4.3 Sperm vitality

Sperm vitality is the percentage of dead and live sperm. The vitality of immotile sperm must be evaluated to determine the viability status of a sperm, particularly when the sperm is to be used for intracytoplasmic sperm injection (ICSI) procedure (Franken & Oehninger, 2012). A scenario where large numbers of dead immotile sperm cells are detected may indicate epididymal pathology, while a large proportion of live immotile sperm cells may indicate structural defects of the flagellum (Wilton *et al.*, 1988; Chemes & Rawe, 2003). The importance of sperm vitality determination was highlighted in semen analysis and various researches. During sperm vitality evaluation, motile sperm are considered live, while other techniques are needed to distinguish immotile live sperm from immotile dead sperm. There are various dye exclusion techniques to differentiate immotile live sperm from immotile dead sperm. Live sperm with intact cell membranes do not take in the dye and thus remain colourless, while dead sperm with disintegrated cell membranes take up the stain. Initially, a 2-step staining technique was introduced to evaluate bull sperm; eosin opal blue (bluish eosin) added to the bull semen prior to addition of nigrosin solution (Blom, 1950). This 2-step sperm staining technique is applicable to human sperm (Williams & Pollak, 1950). Later, researchers introduced other staining techniques suitable for sperm evaluation of mammals (Campbell *et al*, 1956; Dott & Foster, 1975; Dott, 1975).

2.5 Nicotine

Nicotine, which can be used as natural insecticide, is an alkaloid found mainly in plants and present in high concentration in tobacco (*Nicotiana tabacum*). A lot of studies have been conducted to understand the metabolism and effects of nicotine in numerous organisms (Hukkanen *et al.*, 2005). It can cause severe toxicity and long-term risks (Attia, 2007; da Silva *et al.*, 2010). Nicotine can be delivered to the brain quickly through smoking behavior and abuse of nicotine would lead to addiction and dependence among users (Stolerman & Shoaib, 1991; Benowitz, 1996).

Based on debates regarding the impact of smoking on various semen parameters, smoking is considered as a risk factor of infertility (Vine, 1996). Cigarette smoke consists of numerous substances such as nicotine, carbon monoxide, polonium, benzopyrene, dimethylbenzanthracene, dimethylnitrosamine, naphthalene and methnaphthalene (Wong *et al.*, 2000). Cigarette smoke can be divided into gaseous and particulate phases. The major constituents that affect health are nicotine and tar in the particulate phase and carbon monoxide in gaseous phase (Hammond *et al.*, 2006). Nicotine when degraded would produce cotinine as a major product. Cotinine possesses a longer half-life (approximately 20 hours) compared to nicotine (approximately 2 hours), based on its specificity and detectable concentration in body fluids such as serum, follicular fluid, urine and seminal plasma (Binnie *et al.*, 2004; Kapawa *et al.*, 2004).

Inside the body, nicotine would be metabolised quickly into cotinine and the concentration could reach 900 ng/ml of blood in smokers (Hukkanen *et al.*, 2005). When comparing the route of nicotine administration, nicotine level in blood would increase faster after intravenous administration compared to oral administration due to

the fact that nicotine has first-pass effect in the liver (Nesil *et al.*, 2011). Approximately, 1 mg of nicotine could be absorbed when a single cigarette was consumed (Gocze *et al.*, 1999). Nicotine was reported to exert detrimental effects on gonadal functions by inducing biochemical changes in the testes (Riesenfeld & oliva, 1988). Nicotine causes numerous changes in the testes such as tunica properia thickening, increased collagen fibers under irregular basal lamina, deterioration of junctional specialization between Sertoli cells and germ cell degeneration leading to excess cytoplasm retention in spermatids (Fávaro & Cagnon, 2006). In addition, lower epididymal sperm count and reduction in height of surface epithelial cell of epididymis and seminiferous ducts were found to be concurrent with the presence of nicotine (Wong *et al.*, 2000).

Most animal research is focused on the neurophysiological impacts of nicotine and pharmacological ailments because smoking of cigarette is done to get the nicotine effect (Balfour *et al.*, 2000; Di Chiara, 2000a). Similarly, in the attempt to reduce cigarette smoking in human, attention has been directed at the actions of nicotine (Benowitz, 1993; Rose & Corrigall, 1997).

Although there was a decrement of approximately 2 % in cigarette sales annually from the year 1998 until 2007, there was an increment in the usage of alternative tobacco products (Connolly & Alpert, 2008). However, the actual mechanism where tobacco affecting fertility and chemical agents involved are yet to be clarified (Viloria *et al.*, 2007).

2.5.1 Nicotine and infertility

Even though there are more than 4,800 chemical compounds found in tobacco, nicotine is the main alkaloid component responsible for its addictive effect (Stolerman & Jarvis,

1995). Active or passive smoking would cause the substances present in cigarette to enter the seminal plasma of smokers through diffusion and active transport (Zavos *et al.*, 1998). There is therefore high probability that smoking could have adverse effects on the male reproductive system.

Effects of smoking on the fertility of males have not been conclusively confirmed. However, there are some evidence that showed the negative impact of smoking on semen parameters, which suggested that smoking could be the risk factor for infertility (Colagar *et al.*, 2007). When the quality of sperm before and after sperm swim-up in both smoker and non-smoker males were examined, it was found that sperm count, motility and morphology of sperm were negatively affected by smoking habits. It was also showed that smoking could reduce sperm density and motility besides cause damage to sperm morphology (Stillman, 1989). Vine *et al.* (1994) reported that smokers had 13-17 % lower sperm density compared to non-smokers. According to Yamamoto *et al.* (1998), rats exposed to smoke had decreased fertilizing potential, whether through *in vivo* or *in vitro* fertilization. This was most likely due to changes in spermatogenesis and sperm maturation processes in the epididymis.

On the other hand, there were studies that denied the adverse effects of smoking on sperm quality and function, such as in studies carried out by Vogt *et al.* (1986) and Dikshit *et al.* (1987). In addition, little is known about the mechanisms of action on the sperm due to smoking (Saleh *et al.*, 2002; Kunzle *et al.*, 2003). There is a suggestion that cigarette smoke could induce the formation of phagocytic sperm that produced reactive oxygen species (ROS) and oxidised other normal sperm DNA, protein and lipids; hence, causing sperm dysfunction (Aitken & Baker, 2006). Sperm are very susceptible to ROS due to large quantities of polyunsaturated fatty acid (PUFA) present

in the membrane and low levels of scavenging enzymes in the cytoplasm (Agarwal & Prabakaran, 2005; Aitken & Baker, 2006). Excessive level of ROS in the body could induce DNA damage leading to increased rate of germ cell apoptosis which would eventually lower sperm count and could contribute to male infertility (Agarwal & Allamaneni, 2004).

It was reported that 39 % of non-smokers had normal sperm production (normozoospermic), while only 3 % of smokers were normozoospermic. Higher rate of asthenozoospermia or asthenoteratozoospermia were also observed among smokers compared to non-smokers (Gaur *et al.*, 2007). Total number of cigarettes consumed was inversely related to total sperm count, sperm motility and ejaculate volume (Ramlau-Hansen *et al.*, 2007). Another detrimental effect of cigarette smoking on sperm was the lower rate of successful assisted reproductive technique when the male subject was a smoker (Zitzmann *et al.*, 2003).

To date, there is no report on the association between smoking and sperm parameters. Investigations carried out on 517 non-smokers, 109 ex-smokers and 478 smokers nullified any relation between smoking and semen parameters, although significantly higher number of round cells and leukocytes were found in smokers (Trummer *et al.*, 2002). Similarly, it has been also reported that no significant difference detected between sperm parameters of healthy smokers and non-smokers (Belcheva *et al.*, 2004). No significant difference was also reported in terms of sperm motility when comparison was made among 522 non-smokers, 143 mild smokers (<10 cigarettes/day), 154 moderate smokers (11-20 cigarettes/day) and 70 heavy smokers (>20 cigarettes/day) (Pasqualotto *et al.*, 2006).

The accessory glands such as prostate gland, seminal vesicles and epididymis were also affected by smoking behaviour (Pakrashi & Chatterjee, 1995). Kunzle *et al.* (2003) found that smokers tend to have lower citrate concentration, which could indicate compromised prostate function. Nicotine could also disrupt normal endocrine functions, besides eliciting cytotoxicity to the testes that would in turn cause testicular atrophy, gonadal dysfunction, erectile dysfunction, and male factor infertility (Gocze & Freeman, 2000). Nicotine, which is a major compound found in cigarette, serves as a powerful oxidising agent that affects the plasma membrane of sperm and integrity of its DNA (Arabi, 2004). Yamamoto *et al.* (1998) found that administration of nicotine could lead to changes in gonadal functions, deficiency in sperm maturation and spermatogenesis besides having a negative impact on the sperm-fertilizing potential of male rats.

2.5.2 Nicotine and body weight

Association between smoking and body weight could be commonly observed even in adolescents. It was reported that adolescents especially girls made smoking a habit in order to control and lose weight (Fulkerson & French, 2003). It was reported that young adults on diet had 40 % more tendency to consume cigarettes due to the belief that smoking could reduce appetite and weight (Wee *et al.*, 2001). Generally, body weight of adult smokers are 4-5kg lesser than non-smokers, reducing the tendency of smokers to become overweight. However, smoking cessation by smokers lead to weight gain (Kruger *et al.*, 2009). Amount of weight gained from smoking cessation varies from one individual to another. Within 8 years of smoking cessation, an individual tend to gain approximately 3.2-8.6kg, while those who continue smoking gain on average 1.8-2.3kg (Lycett *et al.*, 2011). However, the actual mechanisms involved in smoking and body weight decrement is still poorly understood (Audrain-McGovern & Benowitz, 2011).

Studies had shown that smokers tend to have a lower body weight compared to non-smokers and adolescents smoke in order to control their weight (Potter *et al.*, 2004). One of the possible reasons that caused smoking relapse was the increment of weight after smoking cessation (Meyers *et al.*, 1997). Filozof *et al.* (2004) stated that there were a lot of factors that could affect the mechanisms of action between smoking and body weight such as alterations in caloric intake, activity, metabolic rate and lipogenesis. Despite all these factors, the focus was on food intake and a general assumption that nicotine would reduce food consumption, which contradicted with that observed in smokers. Perkins (1992) found that smokers had the same or higher amount of food intake compared to non-smokers although body weight of smokers was lower than non-smokers. The author also found that administration of nicotine would increase caloric intake by fasting smokers. Mannucci *et al.* (2005) also discovered that 4 times of nicotine injection daily on female mice for a period of 14 days did not significantly affect the total food intake, but there was increment in food consumption in the first 2 hours following 24 hours of starving.

Nicotine has been found responsible for most of the factors that lead to body weight changes resulting from cigarette smoking (Audrain-McGovern & Benowitz, 2011). It could affect the nicotinic cholinergic receptors in both the brain and autonomic ganglia (Benowitz, 2010). The binding of nicotine onto nicotinic receptors opened ion channels and there would be an influx of sodium and calcium, which in turn lead to the release of various neurotransmitters. In addition, this interaction also released systemic catecholamines, while in the central nervous system, dopamine, norepinephrine, serotonin, acetylcholine, glutamate, γ -aminobutyric acid, and other neurotransmitters were released (Audrain-McGovern & Benowitz, 2011).

Nicotine, the major psychoactive compound contained in tobacco could bring about a short period of anorexia besides increase energy expenditure of body leading to reduce body weight in humans (Albanes *et al.*, 1987; Klesges *et al.*, 1989) and rodents (Chen *et al.*, 2005). Hence, an increase in body weight will occur due to sudden smoking cessation (O'Hara *et al.*, 1998) or sudden termination of chronic nicotine treatment (Bishop *et al.*, 2002). Interestingly, Manoj *et al.* (2011) found that rats would develop tolerance against anorectic effect with chronic nicotine treatment. In their study, rats, which were intraperitoneally injected with nicotine, had reduced food intake for the first 6 days followed by a rise in food intake to the control level. Feeding behavior alterations during chronic nicotine treatment also affect body weights where there were body weight reductions of rats starting from day 2 of nicotine treatment (Manoj *et al.*, 2011). Other causes that bring about reduction in body weight of rodents during chronic nicotine treatment include reduced caloric intake and increased energy expenditure (Chajek-Shaul *et al.*, 1987; Frankham & Cabanac, 2003; Bishop *et al.*, 2004). In a separate study, Sparks and Pauly (1999) added saccharine sodium into nicotine solutions in order to reduce the adverse effect of nicotine. Nesil *et al.* (2011) found that there was lower volume of nicotine solution intake by rats with increasing concentration of nicotine solution and they suggested that rats change behaviour to synchronise nicotine intake.

2.5.3 Nicotine and blood hormonal level

Testosterone is an essential androgen that plays a significant role in male sexual maturation, behavior, spermatogenesis, differentiation and maintenance of accessory sex organs (Ojeda & Urbanski, 1994). Pituitary gonadotrophin hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) will regulate the synthesis and release of androgens besides playing an essential role in maintaining

testicular function and spermatogenesis. In addition, LH is the main tropic regulator of Leydig cell functions and with the absent of LH, androgen production will not take place (Huthaniemi & Toppari, 1995).

Based on previous study, more dopamine was released when nicotinic acetylcholine receptors in the neurons of mesolimbic system bound with nicotine (Di Chiara, 2000b; Watkins *et al.*, 2000). This might have in turn gave an impact on the release of gonadotropins (FSH and LH), prolactin of the anterior hypophysis, feedback mechanism of the hypothalamus-pituitary-gonadal axis and production of testosterone and inhibin B in the testes (Fuxe *et al.*, 1989; Funabashi *et al.*, 2005). The release of various anterior and posterior pituitary hormones had been shown to be stimulated by smoking behavior. Besides that, smoking also would increase levels of prolactin, growth hormone (GH), adrenocorticotrophin (ACTH) and arginine vasopressin (AVP) while concentrations of thyroid stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) in the plasma were unaffected (Seyler *et al.*, 1986). Tobacco consumption was found to disrupt endocrine system of the male hormonal profile by disrupting specifically on LH, testosterone and prolactin levels in the body (Blanco-Muñoz *et al.*, 2012). However, controversies still exist regarding the association between tobacco smoking and male reproductive hormone profile (Vine, 1996; Kapoor & Jones, 2005).

2.6 *Habbatus sauda* (*Nigella sativa*)

Habbatus sauda from the Arabic term Habat-ul-Sauda also known as Kalonji in South Asia besides having an English name Black cumin (Nadkarni, 1976). *Habbatus sauda* (*Nigella sativa*) oil was shown to contain thymoquinone, dithymoquinone, thymohydroquinone and thymol through high performance chromatographic analysis

(Omar *et al.*, 1999). Black cumin oil had been studied extensively for its pharmacological properties (Ramadan, 2007; Luther *et al.*, 2007; Lutterodt *et al.*, 2010). Essential fatty acids, bioactive phytosterols and tocopherols were found abundantly in the oil of black cumin seed (Ramadan & Moersel, 2002; Ramadan, 2007). Thymoquinone present in the seed and oil of *Nigella sativa* were found to exhibit medicinal effects as traditional medicine (Salem, 2005). The essential compound, thymoquinone, found in *Nigella sativa* is responsible for most of the pharmacological properties of the plant (Gilani *et al.*, 2004).

Nigella sativa is traditionally used to relieve chronic headaches and migraines. It is also applied externally to treat leucoderma, alopecia, eczema, freckles and pimples (Usmanghani *et al.*, 1997). *Nigella sativa* also acts as a remedy for mercury poisoning, sores and leprosy (Evans, 1996). According to Kapoor (1990), seeds obtained from *Nigella sativa* have anthelmintic and antibiotic properties.

Kawther *et al.* (2008) found that *Nigella sativa* possess anti-viral, anti-cancer, angiogenic and antioxidant properties. Studies by various researchers agreed upon the anti-viral properties of *Nigella sativa* due to the present of antimicrobial compounds, nigellon, thymoquinone and thymohydroquinone (El-Gaafarawy *et al.*, 2003; Gilani *et al.*, 2004; Hanafi *et al.*, 2005). The production of eicosanoid in leukocytes and membrane lipid peroxidation would be halted with the introduction of *Nigella sativa* oil and its derivatives (El-Dakhakhny *et al.*, 2002). Al-Jassir (1992) reported that *Nigella sativa* oil is rich in fatty acids such as oleic acid, linoleic acid and linolenic acid, besides carotene which is convertible to vitamin A. Omar *et al.* (1999) reported that the seed of *Nigella sativa* consist of eight different essential amino acids, which play the role of improving the activities of immune system. *Nigella sativa* seeds are consumed in

traditional medication in the Middle and Far East as remedy for various illnesses, such as headache, obesity, bronchial asthma, dysentery, hypertension, gastrointestinal complications and back pain (Schleicher & Saleh, 1998; Al-Rowais, 2002). Use of *Nigella sativa* in treating eczema is well-known worldwide (Goreja, 2003). Riaz *et al.* (1996) reported that *Nigella sativa* could also be used to treat hair loss, ear pain, headache, parturition complications, toothache, glandular diseases, disturbances of the digestive system, liver, spleen and eye diseases.

2.6.1 *Habbatus sauda* and fertility

A previous study reported that *Nigella sativa* could be used to improve reproductive performance of female mice (Noor, 2008). Park (2007) reported that oral administration of *Nigella sativa* oil to healthy or hyperlipidemic rats for 2 months would increase the seminal vesicle weight, testosterone level in plasma, sperm count and motility besides lower sperm abnormalities.

Mukhallad *et al.* (2009) also reported an increment in the motility of sperm obtained from the cauda epididymides of rats treated with *Nigella sativa*, possibly due to the action of oxidative phosphorylation enzymes (Azzarito *et al.*, 1996). Al-Sa'aidi *et al.* (2009) reported an increment in sperm parameters obtained from cauda epididymides, weight and size of rat testis treated with alcoholic extract of *Nigella sativa* for 53 days. Thus coincided with findings by Al-Mayali (2007).

In a different study done by Al-Sa'aidi *et al.* (2009), oral administration of oil extracted from seeds of *Nigella sativa* for a period of 53 days could improve male rat fertility. Al-Mayali (2007) and Al-Sa'aidi *et al.* (2009) reported an increment in testicular and epididymal weights for groups treated with alcoholic extract of *Nigella*

sativa. Studies have also shown that alkaloids and phenols content of the black seed stimulate the secretion of testosterone and FSH (Al-Dejlyli, 2001; Al-Sa'aidi *et al.*, 2009). The increase in level of testosterone and FSH in testicular tissue would in turn increase sperm concentration (Mclachlan *et al.*, 2002). Mukhallad *et al.* (2009) found that fertility in male albino rats was increased due to oral administration of *Nigella sativa*. Male albino rats treated with *Nigella sativa* through oral administration also showed an increase in sperm motility of its cauda epididymis (Mukhallad *et al.*, 2009), which might be due to the effects of *Nigella sativa* on oxidative phosphorylation enzymes (Azzarito *et al.*, 1996). To date, data concerning the medicinal use of *habbatus sauda* oil on reproductive performance and testicular dysfunction is still lacking.

According to Al-Sa'aidi *et al.* (2009), the increase in testis weight of *Nigella sativa* treated rats was either due to increase testosterone level or increased activity of hypothalamus-pituitary-gonadal axis. In a separate study by Mclachlan *et al.* (2002), increased testosterone and follicle stimulating hormone (FSH) levels in testicular tissue was found to lead to an increase in sperm concentration since testosterone and FSH are essential in process of spermatocytogenesis and spermiogenesis in seminiferous tubules. Testosterone on the other hand is responsible for the maturation of sperm in the epididymis. Alkaloids and phenols present in black seeds were found to stimulate the release of testosterone and FSH (Al-Dejlyli, 2001; Al-Sa'aidi *et al.*, 2009).

2.6.2 *Habbatus sauda* and body weight

To date, data on the effect of *Nigella sativa* on body weight is only available on birds, specifically broiler/chicken. There was an association between body weight gain and consumption of *Nigella sativa* in broiler's diet (Siddig & Abdelati, 2001). This association might be due to the presence of primary nutritional factors in *Nigella sativa*

such as glutamic acid (Al-Gaby, 1999), methionine and lysine (Takruri & Damch, 1998), which were essential amino acids. In addition, the seeds of *Nigella sativa* also contain linoleic acid responsible for gaining maximum body weight (Saleh, 1992). Previous researches found that birds given *Nigella sativa* tend to have a significantly higher body weight gain, weight of thigh and weight of breast besides having better feed conversion ratio (Halle *et al.*, 1999; Osman & Barody, 1999; Al-Homidan *et al.*, 2002). However, its effects on body weight of rodents and other mammals are still lacking.

2.6.3 *Habbatus sauda* and blood hormonal level

Oral administration of oil extracted from seeds of *Nigella sativa* for a period of 53 days shown improved testosterone levels in blood (Al-Sa'aidi *et al.*, 2009). *Nigella sativa* seeds exert effects on the main enzymes responsible for the metabolism and secretion of steroids in the testis, eventually leading to an increase in testosterone levels in blood (Al-Sa'aidi *et al.*, 2009).

In a separate study, male rats administered with alcoholic extract of *Nigella sativa* seeds on a daily basis for 60 days showed increase concentrations of testosterone and LH, while no significant change was observed on FSH level. The increased plasma testosterone level could bring about an increment in the number of somatic and germinal cells of the testis, which eventually increase the weight of testes and epididymides (Parandin *et al.*, 2012). This was further supported by findings on the stimulatory effects of testosterone on the growth and secretory activities of reproductive organs (Bhasin *et al.*, 1988; O'Donnell *et al.*, 1994; Singh *et al.*, 1995).

On the other hand, regulatory effects of gonadotropins such as LH and FSH on sperm parameters (sperm count, motility and viability) in mammals were proven.

Spermatogenesis is stimulated by the binding of FSH onto receptors of Sertoli cells (Parandin *et al.*, 2012). Meanwhile, Leydig cells will be stimulated to produce testosterone with the presence of LH. Testosterone promotes spermatogenesis by acting on Sertoli and peritubular cells of the seminiferous tubules (Bhasin *et al.*, 1988; O'Donnel *et al.*, 1994; Singh *et al.*, 1995). Hence, an increase level of LH in rats treated with *Nigella sativa* seed extract would increase testosterone secretion by Leydig cells (O'Donnel *et al.*, 1994; Parandin *et al.*, 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Animal Maintenance

Sprague-Dawley male rats (7-9 weeks) with average weight of 200-250 g were used in this study. Rats were reared in the animal house located at Centre for Foundation Studies in Science, University of Malaya. Water and food in the form of pellets were given *ad libitum* to the rats. Based on previous researches, it is known that female rodents had higher tolerance against nicotine compared to male rodents; hence, fertility of male rodents was more susceptible to nicotine exposure in comparison to that of females (Glander *et al.*, 2002). So, in this study male *Sprague-Dawley* rats were used instead of female rats. Wood shavings were used as bedding, covering the bottom of cages to absorb urine. The bedding would be changed on the average of every three days to maintain clean environment for the rats and reduce unnecessary infection (Figure 3.1).



Figure 3.1: Rats were kept in separate cages at the animal house

3.2 Treatment Solution Preparation

Habbatus sauda oil, corn oil, nicotine solution and saline were prepared accordingly prior to experiment. Each prepared solution was labeled and kept in different bottle to avoid contamination. Nicotine solution and saline were kept in refrigerator at 4°C while *habbatus sauda* and corn oil were kept in a cool and dry place at room temperature.

3.2.1 *Habbatus sauda* preparation

Pure *habbatus sauda* oil (Dogaci, Turkey) was obtained from the market (Figure 3.2 (a)). Dilution with corn oil was done to produce a concentration of *habbatus sauda* oil 6 μ l/100 g body weight of rat (Figure 3.2 (b)). 6 μ l *habbatus sauda* oil was diluted with 0.094 ml corn oil, which would give a final concentration of 0.1 ml diluted *habbatus sauda* oil per 100g body weight of rat (Appendix I).

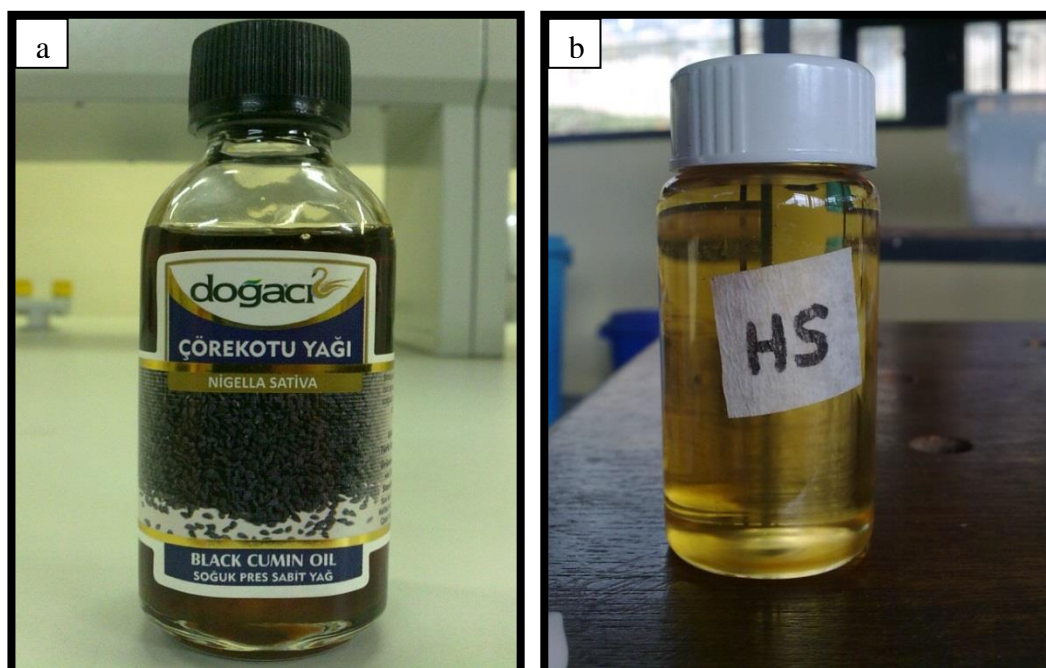


Figure 3.2: (a) Pure *habbatus sauda* oil and (b) diluted *habbatus sauda* oil.

3.2.2 Corn oil preparation

Corn oil served as a control for the *habbatus sauda* treated group. Corn oil was purchased from the local market and it was consumed without further dilution. Dosage given to the rats was 0.1 ml corn oil per 100 g body weight of rat.

3.2.3 Nicotine preparation

The concentration of nicotine (L-Nicotine, 99+ %, CAS RN: 54-11-5) used was 0.0005 ml (0.5 mg) of nicotine (99.9 %) per 100 g body weight of rats (Figure 3.3). Pure nicotine was diluted with 0.0995 ml saline, which would give a final concentration of 0.1 ml diluted nicotine solution per 100 g body weight of rat (Appendix II).

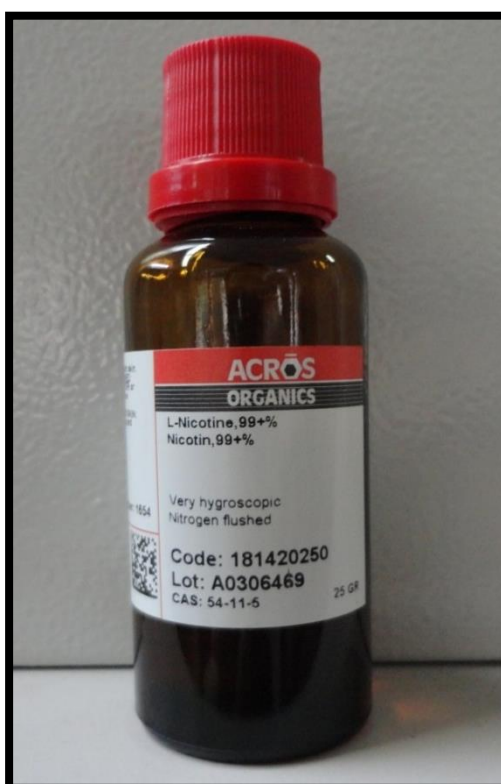


Figure 3.3: Pure nicotine (L-Nicotine, 99+ %, CAS RN: 54-11-5).

3.2.4 Saline preparation

Saline served as a control against the nicotine treated group. Saline solution was prepared by adding 100 ml of distilled water to 0.9 g sodium chloride (NaCl, SIGMA: S9625-500G) making the concentration to be 0.9 % sodium chloride solution. Dosage given to the rats was 0.1 ml of 0.9 % saline per 100 g body weight of rat (Appendix III).

3.3 Treatment Groups

Rats were randomly divided into 5 groups which were *habbatus sauda*, *habbatus sauda* control, nicotine, nicotine control and nicotine-*habbatus sauda* (Table 3.1). All groups were treated for 100 days. The protocols used on the rats were approved by the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) with the reference number of ISB/20/04/2012/DSHA (R). Rats were weighed once every three days to determine the dosages of treatment solutions to be administered and later analysed (Figure 3.4). The concentration and dosages given were as follow:

Table 3.1: Treatment groups, number of rats and treatment solution concentration

Treatment group	Number of rats	Dosage
Nicotine [N]	9	Rats were intramuscularly injected with 0.5 mg/100 g nicotine solution.
Saline (nicotine control) [NC]	9	Rats were intramuscularly injected with 0.1 ml/100 g saline.
<i>Habbatus sauda</i> [HS]	9	Rats were oral gavage with 6 μ l/100 g <i>habbatus sauda</i> oil.
Corn oil (<i>habbatus sauda</i> control) [HSC]	9	Rats were oral gavage with 0.1 ml/100 g corn oil.
Nicotine- <i>habbatus sauda</i> [NHS]	9	Rats were oral gavage with 6 μ l/100 g <i>habbatus sauda</i> oil and intramuscularly injected with 0.5 mg/100 g nicotine solution.

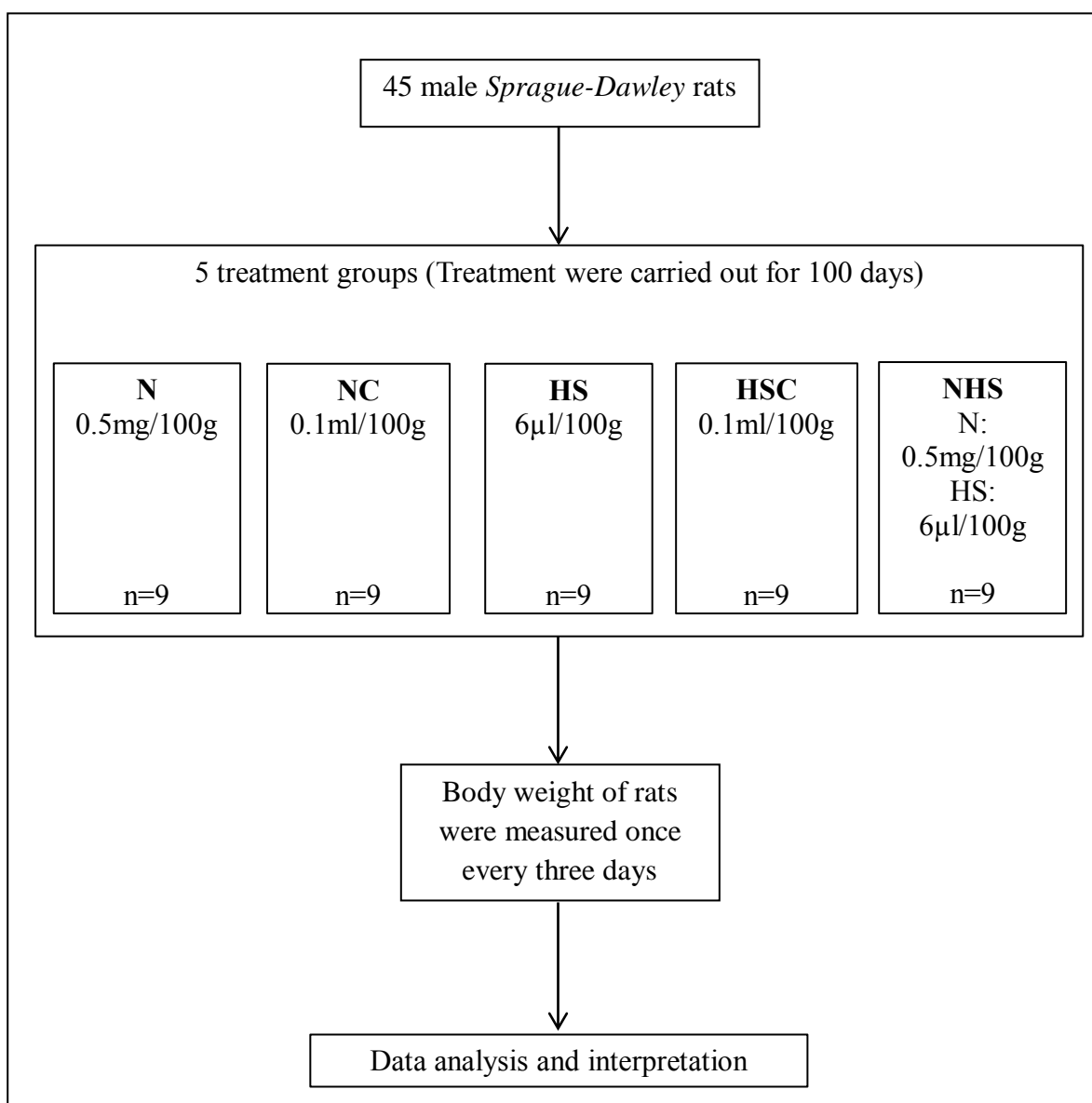


Figure 3.4: Flowchart for body weight increment of rats

N: nicotine group, NC: nicotine control group, HS: *habbatus sauda* group, HSC: *habbatus sauda* control group, NHS: nicotine-*habbatus sauda* group

3.3.1 Oral gavage

In order to reduce the risk of injury, 20 G blunt-end feeding needle attached to 1.0 ml feeding syringe was used during force feeding. The needle was washed with distilled water after each use.

3.3.2 Intramuscular injection

The solutions were administered intramuscularly through the hind legs of rats. A new sterile needle was used for each rat to avoid infection and contamination (Figure 3.5).



Figure 3.5: Intramuscular injection performed on rats

3.4 Rat Dissection

Rats were euthanized after 100 days of treatment. Dosage of 1.0 ml/100 g body weight of chloral hydrate (Chloral hydrate, UNILAB: 148-500G, Appendix IV) solution was administered intraperitoneally on the rats as an anesthesia prior to dissection. Firstly, the testes together with epididymides were excised and washed with saline. The epididymis was immersed in TYH-BSA solution prior to sperm parameters evaluation. The excised testes were then fixed in Bouin solution followed by Haematoxylin and Eosin (H&E) staining.

3.5 Enzyme-linked Immunosorbent Assay (ELISA)

Approximately, 5.0 ml of blood was collected using cardiac puncture technique. The blood was immediately transferred to centrifuge tubes and centrifuged at 4000 rpm, 25°C for 10 minutes. After centrifugation, the supernatant which consisted of blood serum was pipetted into small centrifuge tubes and kept in the deep freezer at -20°C prior to enzyme-linked immunosorbent assay (ELISA) analysis (Figure 3.6). Level of testosterone, luteinising hormone (LH) and follicle stimulating hormone (FSH) were evaluated (Figure 3.7).

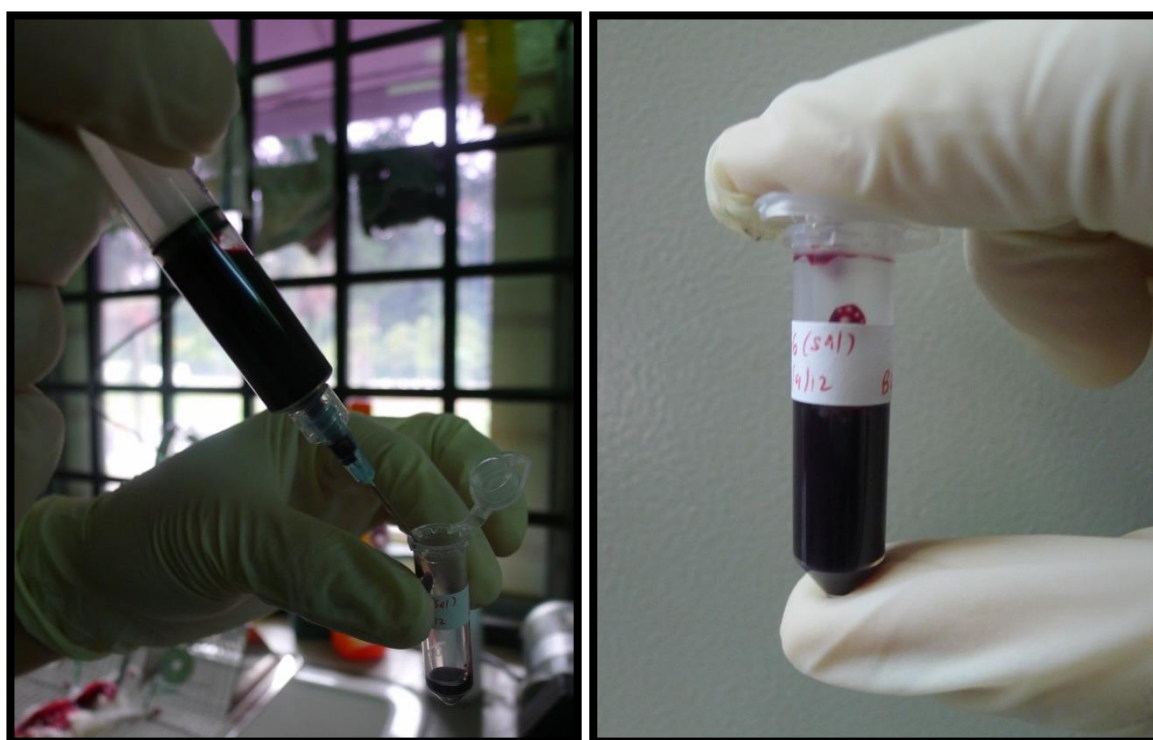


Figure 3.6: Collected blood was transferred into centrifuge tube and centrifuged for blood serum prior to storage in deep freezer at -20°C

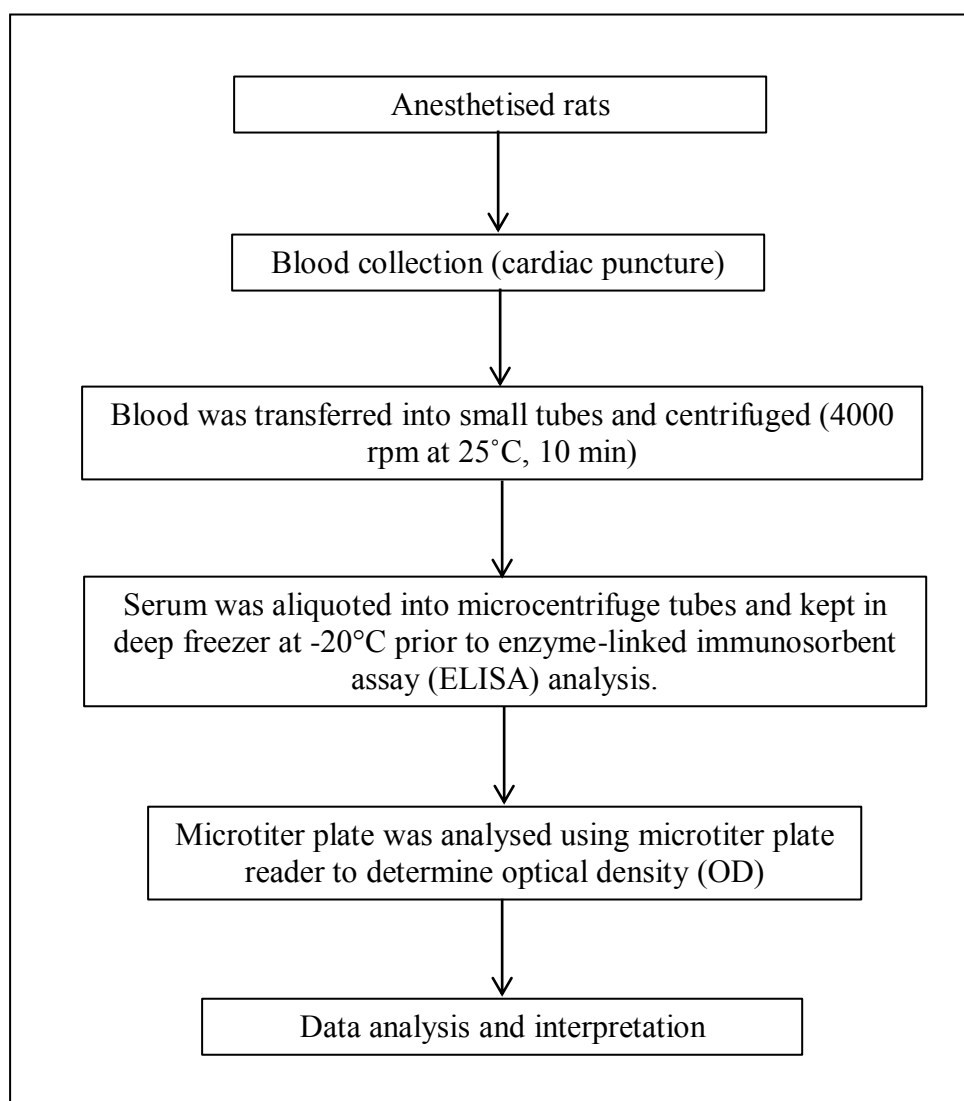


Figure 3.7: Flowchart for blood hormone analysis of rats

3.5.1 Testosterone level evaluation

The standard, control and blood serum, 25 µl each were pipetted onto the respective wells of a microtiter plate. Next, 200 µl of enzyme conjugate solution was pipetted onto each well. The microtiter plate was then covered using adhesive foil and mixed thoroughly for 10 seconds prior to incubation at room temperature for 60 minutes. After incubation, adhesive foil was removed and incubation solution in the microtiter plate was discarded. Then, each of the well was rinsed 3X using 300 µl of diluted wash buffer. Remaining solution in the wells was removed by tapping the inverted plate on a paper

towel. After that, 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) stop solution was pipetted onto each well and mixed gently. Finally, optical densities (OD) were measured within 10 minutes, using a microtiter plate reader (Tecan, infinite M200) at 450 ± 10 nm.

3.5.2 Luteinising hormone (LH) level evaluation

The standard, control and blood serum, 25 μ l each were pipetted onto the respective wells of a microtiter plate. Next, 100 μ l of enzyme conjugate solution was pipetted onto each well and mixed thoroughly for 10 seconds prior to incubation at room temperature for 30 minutes. Then, the incubation solution in the microtiter plate was discarded. Each of the well was rinsed 5X using 400 μ l of purified milli-Q water (F3CM764501, Millipore Products, UK). The remaining solution in the wells was removed by tapping the inverted plate on a paper towel. After that, 100 μ l of substrate solution was pipetted onto each well and incubated for 10 minutes at room temperature prior to the addition of 50 μ l stop solution onto each well. Finally, the optical densities (OD) of each well was measured using microtiter plate reader (Tecan, infinite M200) at 450 ± 10 nm within 10 minutes.

3.5.3 Follicle stimulating hormone (FSH) level evaluation

The standard, control and blood serum, 25 μ l each were pipetted onto the respective wells of a microtiter plate. Next, 100 μ l of enzyme conjugate solution was pipetted onto each well and mixed thoroughly for 10 seconds prior to incubation at room temperature for 30 minutes. Then, the incubation solution in the microtiter plate was discarded. Each of the well was rinsed 5X using 400 μ l of purified milli-Q water (F3CM764501, Millipore Products, UK). The remaining solution in the wells was removed by tapping the inverted plate on a paper towel. After that, 100 μ l of substrate solution was pipetted onto each well and incubated for 10 minutes at room temperature before adding 50 μ l of

stop solution onto each well. Finally, the optical densities (OD) of each well was measured using microtiter plate reader (Tecan, infinite M200) at 450 ± 10 nm within 10 minutes (Figure 3.8; 3.9).



Figure 3.8: Microtiter plate being inserted into microtiter plate reader

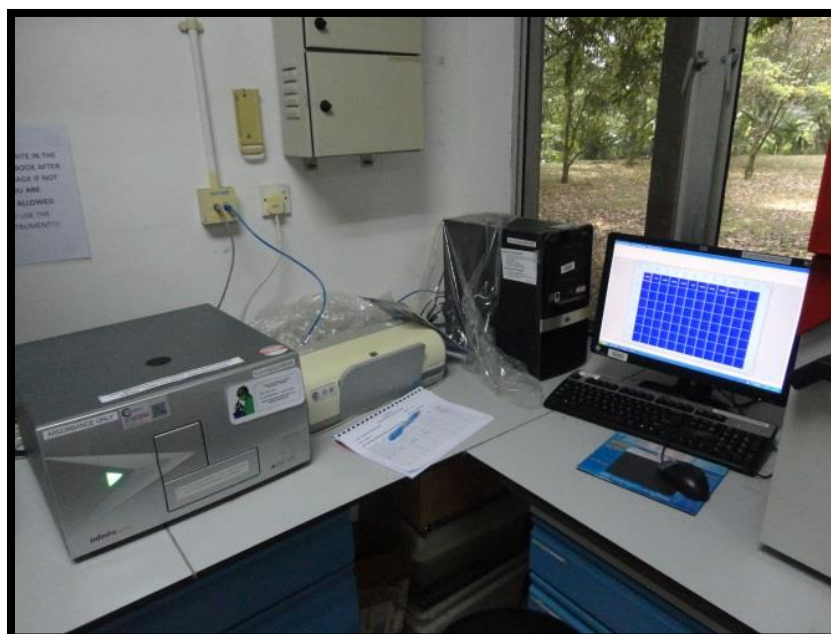


Figure 3.9: Absorbance (OD) value determination at 450 ± 10 nm wavelength using microtiter plate reader

3.6 Sperm Parameters

Toyoda-Yokoyama-Hosi (TYH) (Appendix V) solution and eosin-nigrosin dye (Appendix VI) were prepared for sperm parameter evaluation. Toyoda-Yokoyama-Hosi (TYH) solution mixed with Bovine Serum Albumin (BSA) (SIGMA, A7030-10G) was used for dilution of sperm while eosin-nigrosin was used for sperm staining. The excised cauda epididymides were transferred into 10 ml of Toyoda-Yokoyama-Hoshi (TYH) medium added with BSA and kept in CO₂ incubator (Heal Force CO₂ incubator) with 5.0 % CO₂ at 37°C. Evaluations on sperm motility, sperm morphology and sperm vitality were conducted (Figure 3.10).

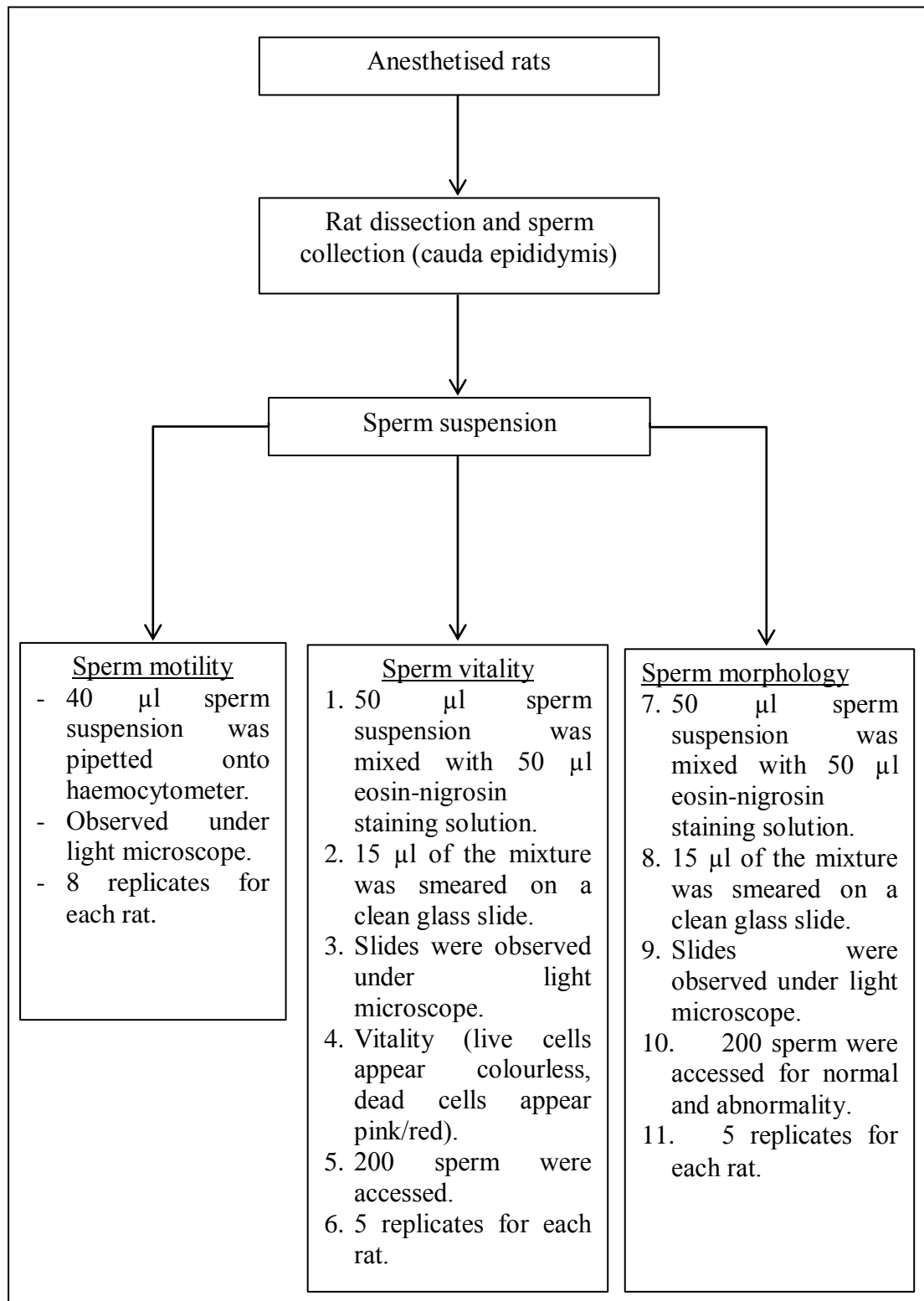


Figure 3.10: Flowchart for sperm parameter evaluation of rats

3.6.1 Sperm motility

Haemocytometer (Improved Neubauer by Hirschmann Techcolor) and coverslip were washed and wiped with 70 % alcohol. Haemocytometer was then covered with coverslip. An aliquot of 40 μ l sperm suspension was pipetted onto haemocytometer slide (Figure 3.11). The haemocytometer was then left at room temperature for 5 minutes to allow sedimentation of the sperm to the grid of the counting chamber prior to observation under light microscope (Olympus CX21FS1) with 20x (phase contrast) objective lens. Sperm motility assessment was performed in accordance to the method suggested in NAFA and ESHRE-SIGA, Laboratory Manual (2002). There were 25 small squares in each counting chamber (Figure 3.12). The number of sperm in the upper left corner square needs to be counted before determining the number of squares that needs to be counted (Table 3.2).

Table 3.2: Guidelines on counting sperm using haemocytometer.

Sperm Count	Number of squares to be counted
< 10 sperm	all the grids were counted (25 squares)
10-40 sperm	10 squares in each chamber were counted
> 40 sperm	5 squares were counted (4 corners and 1 center square)

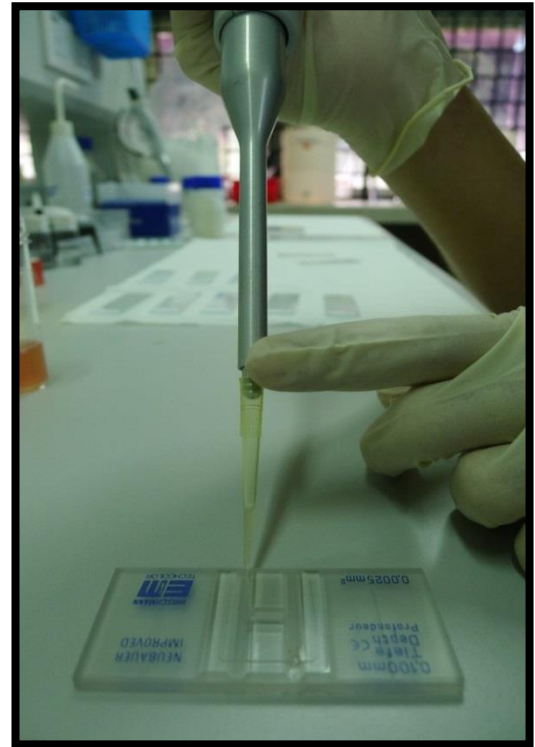
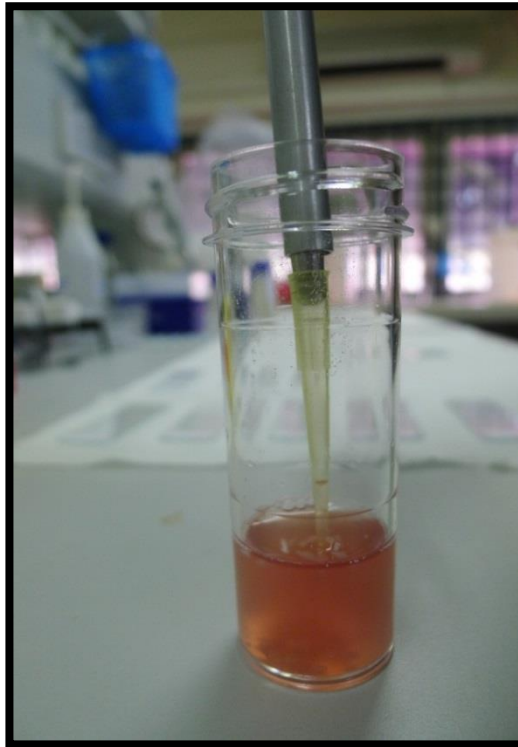


Figure 3.11: An aliquot of 40 μ l sperm suspension was pipetted onto haemocytometer

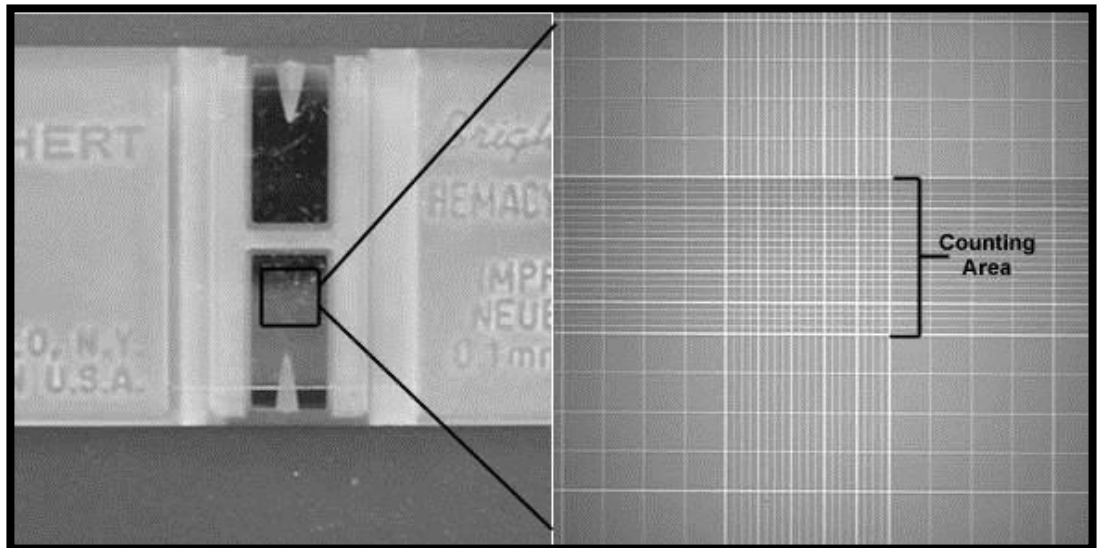


Figure 3.12: Small squares, 25 in each counting chambers of a haemocytometer

3.6.2 Sperm morphology and vitality

Vitality and morphology of sperm were analysed using eosin-nigrosine (Eosin Y: MERCK, 1.15935.0025) (Nigrosine: MERCK, 1.15924.0025) staining technique. An aliquot of 50 μ l sperm suspension was mixed thoroughly with 50 μ l of eosin-nigrosin stain on a clean petri dish. Next, 15 μ l of the stained sperm mixture was transferred onto a glass slide and five smears were made for each rat (Figure 3.13). The glass slides were then left to dry at room temperature before being observed under light microscope (Olympus CX21FS1) for vitality; live cells appeared to be colourless and dead cells appeared red or pink in colour. Approximately 200 sperm were observed for dead and live cells and percentage of dead and live cells were recorded. As for sperm morphology, another 200 sperm were accessed for normal and abnormal head and tail defects. Classification of sperm morphology and vitality were in accordance with guideline by the WHO laboratory manual (WHO, 1999).

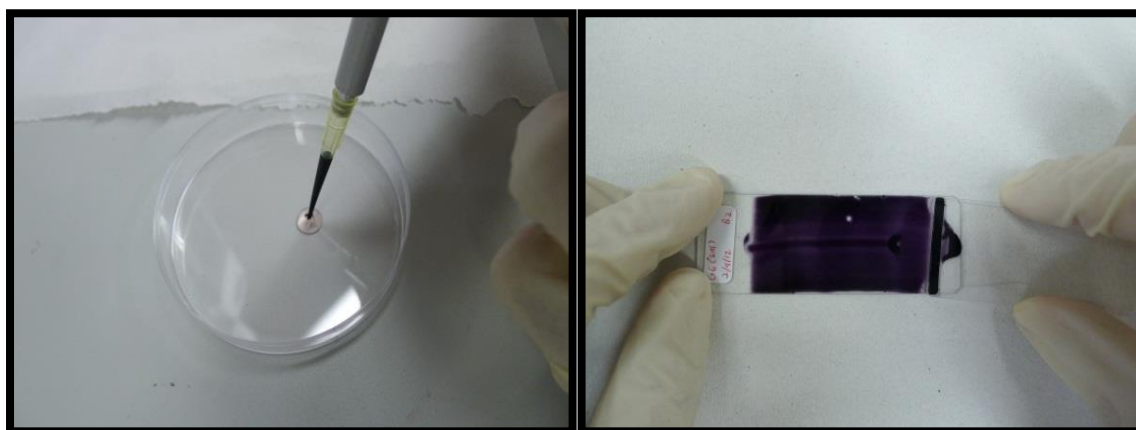


Figure 3.13: Sperm suspension, 50 μ l was mixed with 50 μ l of eosin-nigrosin stain before 15 μ l of the stained sperm mixture being smeared on a clean glass slide

3.7 Testis Morphology and Histology

Upon dissection, testes of rats were excised and transferred into Bouin solution. Fixation of the testes using Bouin solution marked the first step in histological processes. Selected testis histological features were evaluated (Figure 3.14).

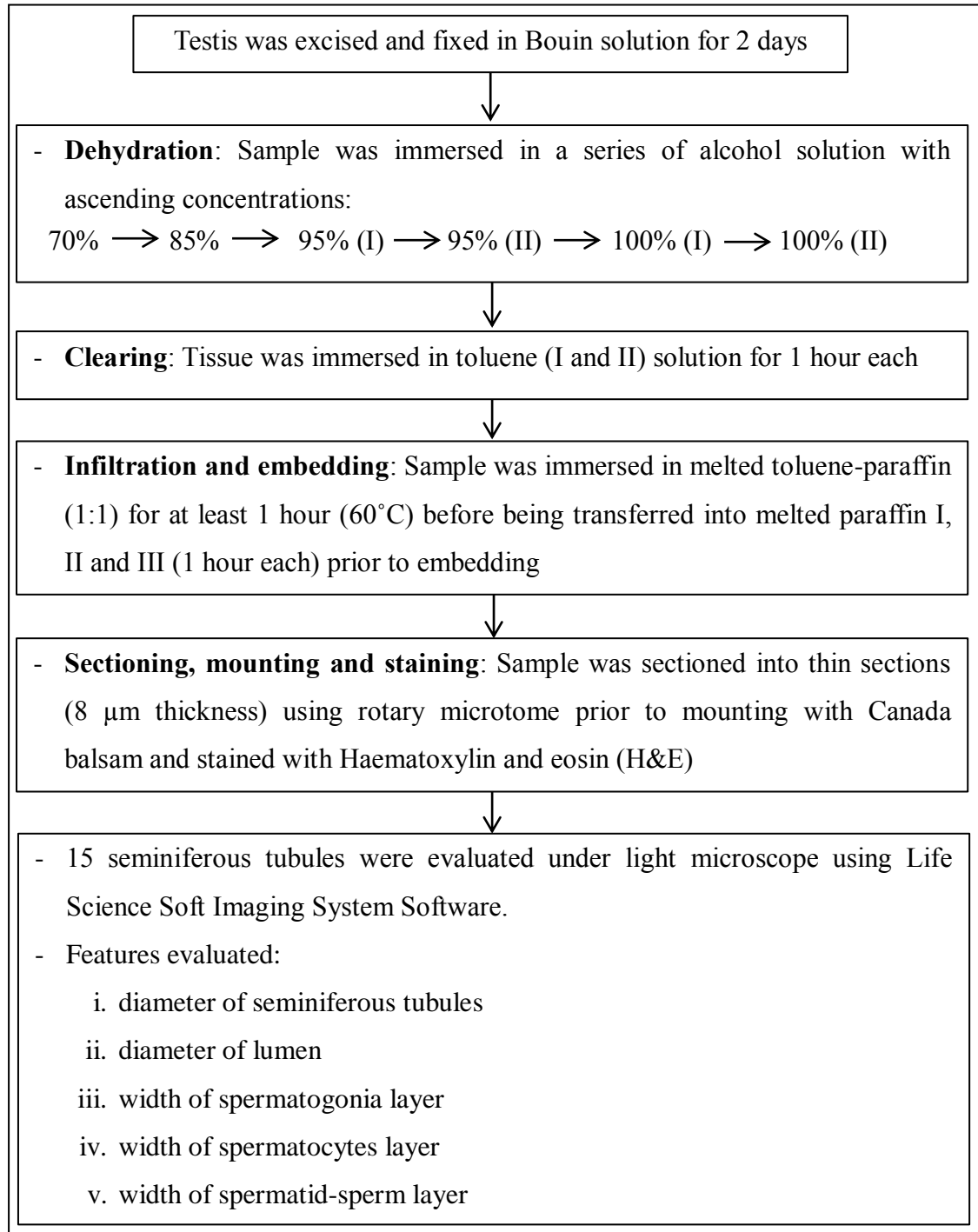


Figure 3.14: Flowchart for testis histological features analysis of rats

3.7.1 Fixation

The testes were fixed in Bouin solution for two days after being washed using normal saline (Figure 3.15) (Appendix VI). This step functions to halt biochemical reactions of the testis besides increasing mechanical strength and maintaining the condition of tissues as close as possible to normal condition. Fixation was carried out at room temperature. Extreme care was practiced when handling Bouin solution due to its hazardous properties such as being carcinogenic, target organ effect, toxic by ingestion and by skin absorption, skin sensitiser, irritant and corrosive.



Figure 3.15: The testes were fixed in Bouin solution for two days

3.7.2 Dehydration

After fixation in Bouin solution for two days, the testes were taken out and trimmed transversely into 3 parts. The trimming process was carried out in 70 % alcohol solution so as to prevent the organ from drying. Only the middle part of the trimmed testis was transferred into a small bottle containing 70 % alcohol solution to remove excessive Bouin solution. The testis tissue sample could be stored in 70 % alcohol solution for a long period of time prior to further dehydration processes (Figure 3.16).

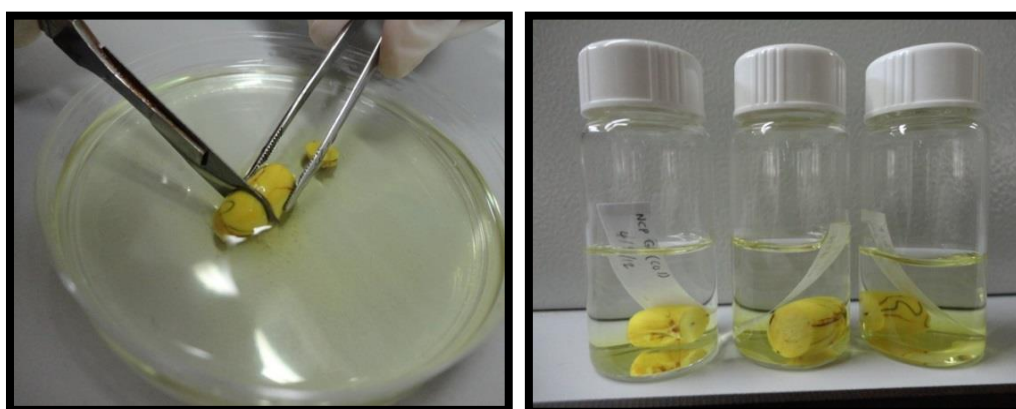


Figure 3.16: Fixed testes were trimmed prior to immersion in 70 % alcohol solution

Next, the tissue sample was immersed in a series of alcohol solution with increasing concentration and the time interval was 1 hour for each solution (Figure 3.17). The alcohol solutions used were 70 %, 85 %, 95 % and absolute alcohol (Appendix VIII).

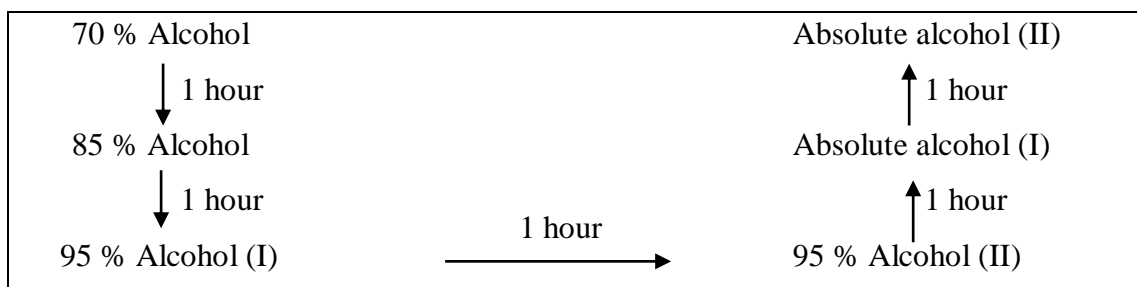


Figure 3.17: The dehydration process of tissue sample in a series of alcohol solution with increasing concentration

3.7.3 Clearing

After dehydration process, the tissue was immersed in two fresh toluene solution (toluene (I) and toluene (II)) with immersion period of 1 hour each to remove traces of alcohol from the sample (Figure 3.18).



Figure 3.18: Tissue samples being immersed in toluene (I) and toluene (II) for one hour each

3.7.4 Infiltration

After clearing, the tissue would undergo infiltration process. Infiltration process was initiated when tissue sample in toluene (II) was transferred into a plastic cassette prior to immersion of the cassette into melted toluene-paraffin (1:1) for at least 1 hour in the oven at $60^{\circ}C$. Next, the cassette was transferred into melted paraffin I, II and III with time intervals of 1 hour for each transition (Figure 3.19).



Figure 3.19: Tissue sample being infiltrated with melted paraffin I, II and III

3.7.5 Embedding

The tissue sample was then taken out from cassette and embedded in pure melted paraffin. The tissue sample was embedded in the longitudinal position with the transverse surface of the testis tissue facing upward. Finally, the tissue sample was allowed to solidify at room temperature overnight (Figure 3.20).



Figure 3.20: Tissue sample was adjusted during embedding before being allowed to solidify in water at room temperature overnight

3.7.6 Sectioning and affixing of tissue sections onto slides

Sectioning was a step where the embedded tissue sample was cut into thin sections using rotary microtome (American Optical Microtome 820). The testis paraffin block was trimmed using a blade into suitable size. The trimmed paraffin block was attached to the block stage which was then placed on the microtome holder before sectioning to obtain thin sections of approximately 8 μm thickness (Figure 3.21 (a)). A small drop of Mayer's Albumin was placed on the centre of glass slide and spread evenly using finger. A drop of distilled water was placed on the same glass slide. The tissue section was then transferred onto the glass slide and placed on the slide warmer (Electrothermal, MH 6615) at 45°C. The slide was kept in slide box prior to the staining process (Figure 3.21 (b)).

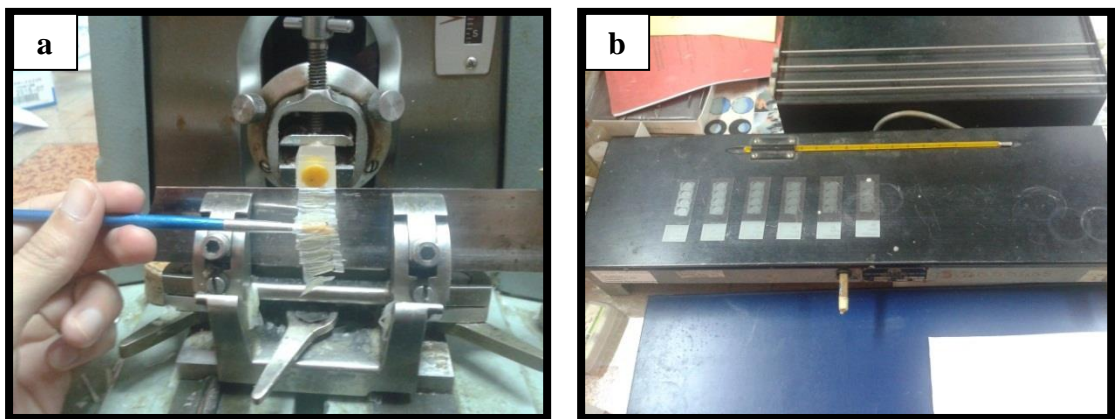


Figure 3.21: (a) Tissue sections were sectioned using rotary microtome, (b) tissue sections were affixed on clean glass slides and placed on slide warmer

3.7.7 Haematoxylin and eosin (H&E) staining technique

Haematoxylin and eosin (H&E) staining technique would stain the nucleus purple and cytoplasm pink. The steps of H&E staining technique include deparaffinisation, hydration, Haematoxylin and Eosin staining, dehydration and clearing (Figure 3.22; 3.23).



Figure 3.22: Tissue sections affixed to the glass slides were placed in a staining rack and immersed in a series of solutions in H&E staining procedure

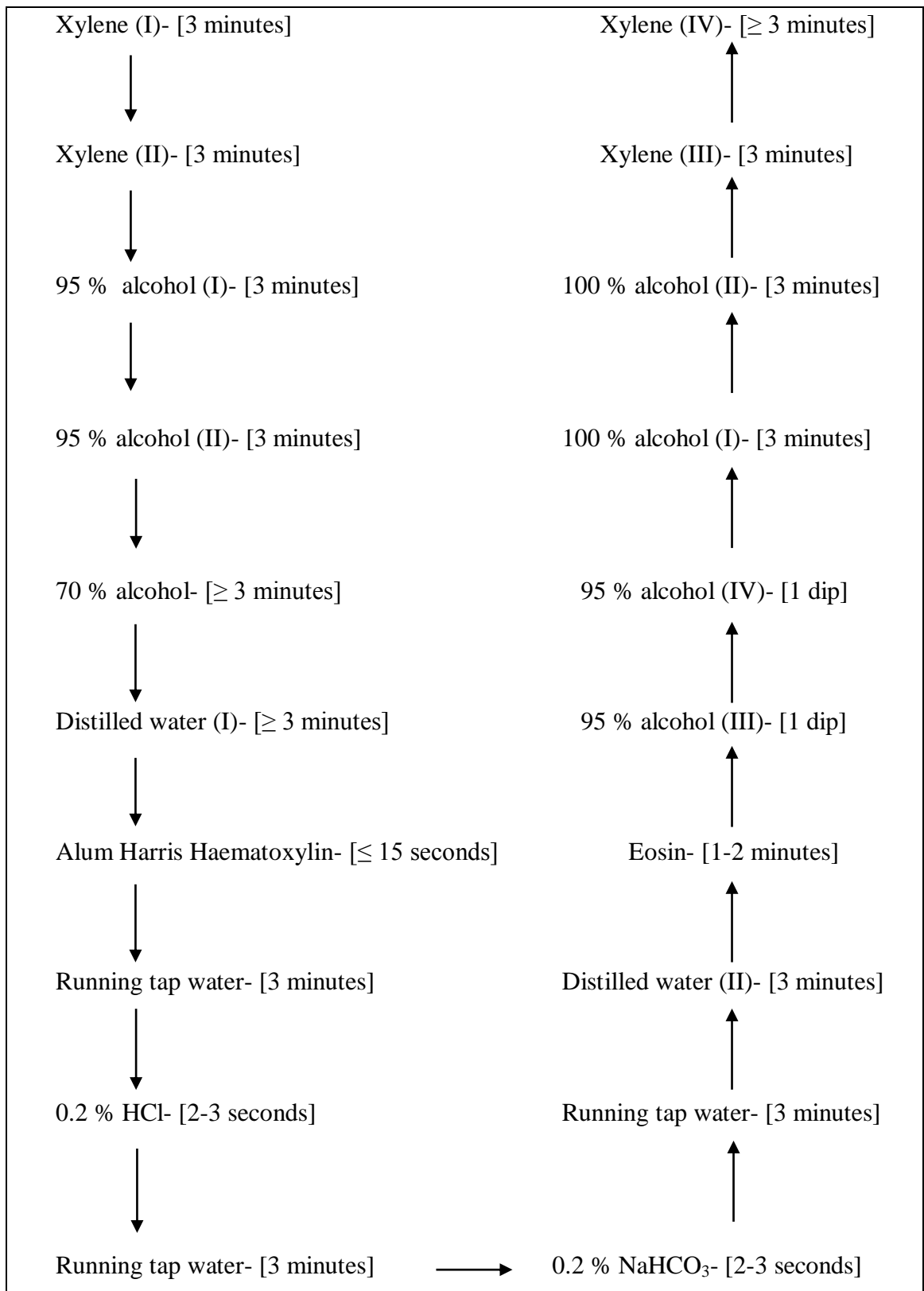


Figure 3.23: Steps and solutions in H&E staining technique

3.7.8 Permanent mounting of coverslips using Canada balsam

After the sections on the slides were stained using H&E staining technique, coverslips were permanently mounted using a mounting media, Canada balsam (MERCK, ZC882291 049). An adequate amount of Canada balsam was spread on a coverslip and placed on the stained sections. Next, the slides were placed in an oven for overnight at 42-45°C to allow solidification of Canada balsam prior to viewing (Figure 3.24).



Figure 3.24: Stained tissue sections were covered by coverslips mounted using Canada balsam

3.7.9 Testis histological features analysis

The slides were viewed under microscope and analysed using Image Analyzer Software (Life Science Soft Imaging System Software 3.0). Features evaluated were diameter of seminiferous tubules, diameter of lumen, width of spermatogonia layer, spermatocytes layer and spermatid-sperm layer (Figure 3.25).

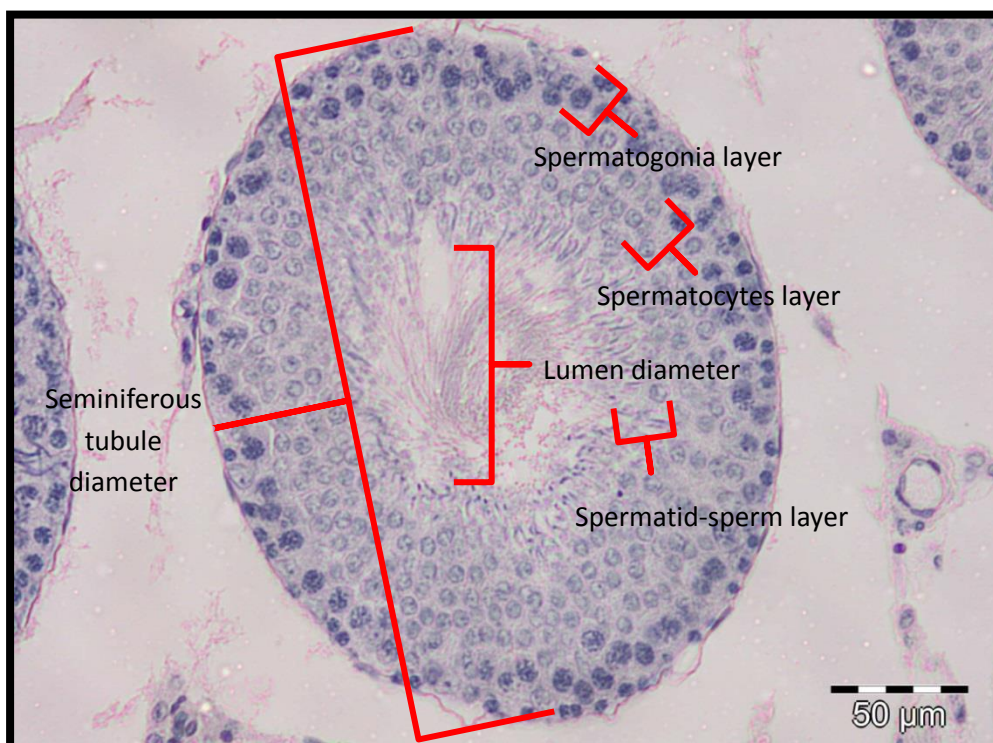


Figure 3.25: Measurements of a seminiferous tubule using Image Analyzer Software

3.8 Statistical Analysis

Data obtained were analysed using Statistical Package for the Social Sciences (SPSS) version 16 where $p < 0.05$ was considered significant. The data were analyzed using one-way analysis of variance (ANOVA) and Duncan Multiple Range Test (Duncan-MRT). Presented was mean \pm standard error (SE).

CHAPTER 4

RESULTS

The effects of 100 days specific treatment and administration were evaluated for 5 groups; nicotine, *habbatus sauda* and co-administration of nicotine-*habbatus sauda* and the controls for nicotine and *habbatus sauda*. The parameters looked at were body weight increment, reproductive blood hormonal levels, sperm parameters (motility, morphology and vitality) and testis histological features.

4.1 Body Weight Increment of Rats

The analyses of variance indicated there were no significant body weight increments in all five treatment groups (Table 4.1). Hence, the treatments itself, intramuscular injection or force feeding did not disturb the feeding habit of the animals.

Table 4.1: Mean square from analyses of variance for body weight increment of rats

Source of variation	d.f.	Mean square
		Weight increment (g)
Treatment	4	5631.52 ^{n.s}
Error	40	2271.84
Total	44	

^{n.s.} non-significant

4.2 Blood Hormonal Level Analysis

All three reproductive hormones evaluated, testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) was not affected by the treatments.

4.2.1 Effects of treatments on concentration of testosterone

The analyses of variance indicated that treatments administered did not significantly affect the testosterone level of all five groups ($p \geq 0.001$) (Table 4.2).

Table 4.2: Mean square from analyses of variance for testosterone level of rats

Source of variation	d.f.	Mean square
		Testosterone level (ng/ml)
Treatment	4	5.14 ^{n.s}
Error	40	3.68
Total	44	

^{n.s.} non-significant

4.2.2 Effects of treatments on concentration of Follicle Stimulating Hormone (FSH)

The analyses of variance indicated that treatments administered did not significantly affect FSH level of all five groups ($p \geq 0.001$) (Table 4.3).

Table 4.3: Mean square from analyses of variance for FSH level of rats

Source of variation	d.f.	Mean square
		FSH level (mIU/ml)
Treatment	4	0.09 ^{n.s}
Error	40	0.06
Total	44	

^{n.s.} non-significant

4.2.3 Effects of treatments on concentration of Luteinizing Hormone (LH)

The analyses of variance indicated that treatments administered did not significantly affect LH level of all five groups ($p \geq 0.001$) (Table 4.4).

Table 4.4: Mean square from analyses of variance for LH level of rats

Source of variation	d.f.	Mean square
		LH level (mIU/ml)
Treatment	4	0.09 ^{n.s}
Error	40	0.06
Total	44	

^{n.s.} non-significant

4.3 Sperm Parameters

Effects of the treatments on three parameters of the sperm were evaluated: (a) sperm motility, (b) sperm morphology, and (c) sperm vitality. Sperm motility means the percentage of all motile sperm in a semen sample. Sperm morphology means the percentage of normal sperm, sperm with tail defect and sperm with head defect. Sperm vitality means the percentage of live and dead sperm in a semen sample.

4.3.1 Effects of treatments on sperm motility

The analyses of variance for sperm motility indicated that treatments had high significant effects on this parameter ($p < 0.001$) (Table 4.5).

The least-square means and the Least Significant Difference (LSD) test indicated that intramuscular administration of 0.5 mg/100 g nicotine for a period of 100 days negatively affected sperm motility of rats (Table 4.6). Administration of nicotine gave significantly lower sperm motility ($1.04 \pm 0.04 \times 10^6$ sperm/ml) compared to NC ($1.35 \pm 0.03 \times 10^6$ sperm/ml), HS ($1.40 \pm 0.04 \times 10^6$ sperm/ml), HSC ($1.29 \pm 0.04 \times 10^6$ sperm/ml) and NHS ($1.29 \pm 0.04 \times 10^6$ sperm/ml) groups ($p < 0.05$). However, no significant differences were detected in term of sperm motility for rats among NC, HS, HSC and NHS groups ($p \geq 0.05$).

Table 4.5: Mean square from analyses of variance for sperm motility of treated rats

Source of variation	d.f.	Mean square
		Sperm motility ($\times 10^{10}$)
Treatment	4	134.10**
Error	355	10.06
Total	359	

**
p<0.001

Table 4.6: Sperm motility of treated rats

Parameter Treatment	Motility ($\times 10^6$ sperm/ml) (mean \pm SE)
N (n=72)	1.04 \pm 0.04 ^a
NC (n=72)	1.35 \pm 0.03 ^b
HS (n=72)	1.40 \pm 0.04 ^b
HSC (n=72)	1.29 \pm 0.04 ^b
NHS (n=72)	1.29 \pm 0.04 ^b

Different superscripts within the same column indicate significant differences (p<0.05)

N: nicotine group, NC: nicotine control group, HS: *habbatus sauda* group, HSC: *habbatus sauda* control group, NHS: nicotine-*habbatus sauda* group.

4.3.2 Effects of treatments on sperm morphology

The analysis of variance for sperm morphology indicated that treatments had high significant effects on normal, abnormal head and abnormal tail (p<0.001) (Table 4.7).

The N group (82.05 \pm 0.02 %) had significantly lower percentage of normal sperm compared to NC (89.36 \pm 0.01 %), HS (90.69 \pm 0.01 %), HSC (86.52 \pm 0.01 %) and NHS

(88.52±0.02 %) groups ($p<0.05$) (Table 4.8). Percentage of normal sperm for the NC (89.36±0.01 %) group was significantly higher compared to the HSC group (86.52±0.01 %) ($p<0.05$). The HS group (90.69±0.01 %) had significantly higher percentage of normal sperm compared to other groups, except for NC group (89.36±0.01 %) ($p<0.05$). The HSC group (86.52±0.01 %) had significantly lower percentage of normal sperm compared to the NC (89.36±0.01 %) and HS (90.69±0.01 %) groups, but significantly higher compared to N group (82.05±0.02 %) ($p<0.05$). As for the NHS group (88.52±0.02 %), it had significantly higher percentage of normal sperm than the N group (82.05±0.02 %), but significantly lower compared to the HS group (90.69±0.01 %) ($p<0.05$) (Table 4.8).

The N group had significantly higher percentage of head defect sperm (4.24±0.01 %) compared to NC (1.87±0.01 %), HS (2.12±0.00 %), HSC (2.89±0.00 %) and NHS (2.72±0.01 %) groups ($p<0.05$) (Table 4.8). Percentage of head defect sperm for the NC group (1.87±0.01 %) was significantly lower compared to N (4.24±0.01 %), HSC (2.89±0.00 %) and NHS (2.72±0.01 %) groups ($p<0.05$). Percentage of head defect sperm for the HS group (2.12±0.00 %) was also significantly lower compared to N (4.24±0.01 %), HSC (2.89±0.00 %) and NHS (2.72±0.01 %) groups ($p<0.05$). Percentage of head defect sperm for the HSC group (2.89±0.00 %) was significantly higher compared to NC (1.87±0.01 %) and HS (2.12±0.00 %) groups, but significantly lower compared to N group (4.24±0.01 %) ($p<0.05$). Percentage of head defect sperm for the NHS group (2.72±0.01 %) was significantly higher compared to NC (1.87±0.01 %) and HS (2.12±0.00 %) groups, but significantly lower compared to N group (4.24±0.01 %) ($p<0.05$) (Table 4.8).

The N group had significantly higher percentage of tail defect sperm (13.47 ± 0.01 %) compared to NC (8.50 ± 0.01 %), HS (7.06 ± 0.01 %), HSC (10.40 ± 0.01 %) and NHS (8.55 ± 0.01 %) groups ($p < 0.05$) (Table 4.8). Percentage of tail defect sperm for the NC group (8.50 ± 0.01 %) was significantly lower compared to N (13.47 ± 0.01 %) and HSC (10.40 ± 0.01 %) groups ($p < 0.05$). Percentage of tail defect sperm for the HS group (7.06 ± 0.01 %) was also significantly lower compared to N (13.47 ± 0.01 %) and HSC (10.40 ± 0.01 %) groups ($p < 0.05$). Percentage of tail defect sperm for the HSC group (10.40 ± 0.01 %) was significantly higher compared to NC (8.50 ± 0.01 %), HS (7.06 ± 0.01 %) and NHS (8.55 ± 0.01 %) groups but significantly lower compared to N group (13.47 ± 0.01 %) ($p < 0.05$). Percentage of tail defect sperm for the NHS group (8.55 ± 0.01 %) was significantly lower compared to N (13.47 ± 0.01 %) and HSC (10.40 ± 0.01 %) groups ($p < 0.05$) (Table 4.8). Hence, those receiving *habbatus sauda* oil has the highest percentage for normal sperm and least for head and tail defects sperm. As for the combined treatments, the values of this group were intermediate between HS and N groups for normal and head defect sperm. Examples of sperm morphology defect (normal, tail defect and head defect) are shown in figure 4.1.

Table 4.7: Mean square from analyses of variance for sperm morphology of treated rats

Source of variation	d.f.	Mean square		
		Sperm morphology		
		Normal	Abnormal head	Abnormal tail
Treatment	4	359.55**	109.44**	246.79**
Error	220	18.51	7.05	17.11
Total	224			

**
p<0.001

Table 4.8: Sperm morphology of treated rats

Parameter Treatment	Morphology (%) (mean \pm SE)		
	Normal	Abnormal	
		Head defect	Tail defect
N (n=45)	82.05 \pm 0.02 ^a	4.24 \pm 0.01 ^c	13.47 \pm 0.01 ^c
NC (n=45)	89.36 \pm 0.01 ^{c,d}	1.87 \pm 0.01 ^a	8.50 \pm 0.01 ^a
HS (n=45)	90.69 \pm 0.01 ^d	2.12 \pm 0.00 ^a	7.06 \pm 0.01 ^a
HSC (n=45)	86.52 \pm 0.01 ^b	2.89 \pm 0.00 ^b	10.40 \pm 0.01 ^b
NHS (n=45)	88.52 \pm 0.02 ^{b,c}	2.72 \pm 0.01 ^b	8.55 \pm 0.01 ^a

Different superscripts within the same column indicate significant differences (p<0.05)

N: nicotine group, NC: nicotine control group, HS: *habbatus sauda* group, HSC: *habbatus sauda* control group, NHS: nicotine-*habbatus sauda* group.

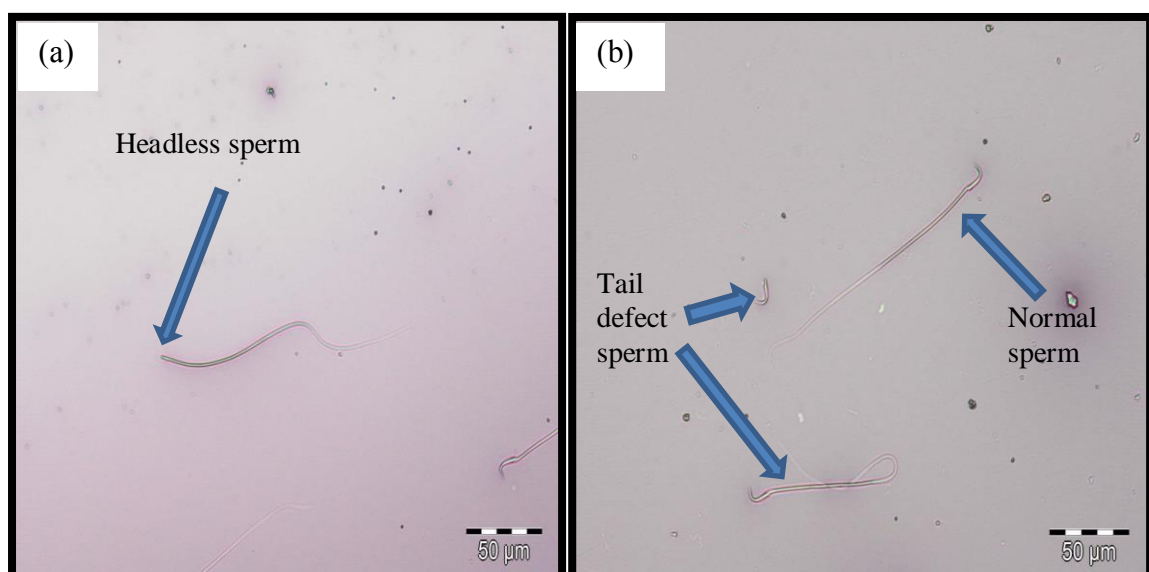


Figure 4.1: Sperm at 200X magnification for (a) head defect and (b) normal and tail defect.

4.3.3 Effects of treatments on sperm vitality

The analyses of variance for sperm vitality indicated that treatments had high significant effects on both live and dead sperm ($p < 0.001$) (Table 4.9). N group had significantly lower percentage of live sperm (93.45 ± 0.01 %) compared to NC (95.88 ± 0.00 %), HS (96.80 ± 0.00 %) and NHS (95.96 ± 0.01 %) groups ($p < 0.05$) (Table 4.10). Percentage of live sperm for NC group (95.88 ± 0.00 %) was significantly higher compared to N (93.45 ± 0.01 %) and HSC (94.07 ± 0.01 %) groups, but significantly lower compared to HS group (96.80 ± 0.00 %) ($p < 0.05$). Percentage of live sperm for the HS group (96.80 ± 0.00 %) was the highest compared to the other groups ($p < 0.05$). Percentage of live sperm for the HSC group (94.07 ± 0.01 %) was significantly lower compared to NC (95.88 ± 0.00 %), HS (96.80 ± 0.00 %) and NHS (95.96 ± 0.01 %) groups ($p < 0.05$). Percentage of live sperm for the NHS group (95.96 ± 0.01 %) was significantly higher than N (93.45 ± 0.01 %) and HSC (94.07 ± 0.01 %) groups but significantly lower than the HS group (96.80 ± 0.00 %) ($p < 0.05$) (Table 4.10).

Percentage of dead sperm for N group (6.55 ± 0.01 %) was significantly higher compared to NC (4.12 ± 0.00 %), HS (3.19 ± 0.00 %) and NHS (4.04 ± 0.01 %) groups ($p < 0.05$) (Table 4.10). Percentage of dead sperm for NC group (4.12 ± 0.00 %) was significantly lower compared to N (6.55 ± 0.01 %) and HSC (5.93 ± 0.01 %) groups, but significantly higher compared to HS group (3.19 ± 0.00 %) ($p < 0.05$). Percentage of dead sperm for HS group (3.19 ± 0.00 %) was the lowest compared to other groups ($p < 0.05$). Percentage of dead sperm for HSC group (5.93 ± 0.01 %) was significantly higher compared to NC (4.12 ± 0.00 %), HS (3.19 ± 0.00 %) and NHS (4.04 ± 0.01 %) groups ($p < 0.05$). The NHS group had significantly lower percentage of dead sperm (4.04 ± 0.01 %) compared to N (6.55 ± 0.01 %) and HSC (5.93 ± 0.01 %) groups, but significantly higher compared to HS group (3.19 ± 0.00 %) ($p < 0.05$) (Table 4.10).

Table 4.9: Mean square from analyses of variance for sperm vitality of treated rats

Source of variation	d.f.	Mean square	
		Sperm vitality	
		Live	Dead
Treatment	4	160.48**	160.48**
Error	220	9.34	9.34
Total	224		

**
p<0.001

Table 4.10: Sperm vitality of treated rats

Parameter Treatment	Vitality (%) (mean \pm SE)	
	Live	Dead
N (n=45)	93.45 \pm 0.01 ^a	6.55 \pm 0.01 ^c
NC (n=45)	95.88 \pm 0.00 ^b	4.12 \pm 0.00 ^b
HS (n=45)	96.80 \pm 0.00 ^c	3.19 \pm 0.00 ^a
HSC (n=45)	94.07 \pm 0.01 ^a	5.93 \pm 0.01 ^c
NHS (n=45)	95.96 \pm 0.01 ^b	4.04 \pm 0.01 ^b

Different superscripts within the same column indicate significant differences (p<0.05)

N: nicotine group, NC: nicotine control group, HS: *habbatus sauda* group, HSC: *habbatus sauda* control group, NHS: nicotine-*habbatus sauda* group.

4.4 Testis Histological Features

Evaluations were conducted on selected features such as diameter of seminiferous tubules and lumen and width of spermatogonia, spermatocytes and spermatid-spermatozoa layers. Measurements were made based on histologically processed testis

tissue sections. Measurement techniques and units of measurement were explained in Figure 3.25.

4.4.1 Effects of treatments on diameter of seminiferous tubules and lumen

The analyses of variance indicated that treatments had high significant effects on lumen diameter of seminiferous tubules ($p < 0.001$) but not significant for diameter of seminiferous tubules ($p \geq 0.001$) (Table 4.11). Figures 4.1; 4.2; 4.3 illustrates these findings.

Lumen diameter for the N group ($100.74 \pm 1.75 \mu\text{m}$) was significantly bigger compared to NC ($78.02 \pm 1.79 \mu\text{m}$), HS ($66.65 \pm 1.57 \mu\text{m}$), HSC ($94.26 \pm 1.81 \mu\text{m}$) and NHS ($67.41 \pm 1.92 \mu\text{m}$) groups ($p < 0.05$) (Table 4.12). Lumen diameter of NC group ($78.02 \pm 1.79 \mu\text{m}$) was significantly smaller than N ($100.74 \pm 1.75 \mu\text{m}$) and HSC ($94.26 \pm 1.81 \mu\text{m}$) groups, but significantly larger compared to HS ($66.65 \pm 1.57 \mu\text{m}$) and NHS ($67.41 \pm 1.92 \mu\text{m}$) groups ($p < 0.05$). The HS group had significantly smaller lumen diameter ($66.65 \pm 1.57 \mu\text{m}$) compared to other groups except for NHS group ($67.41 \pm 1.92 \mu\text{m}$) where no significant difference was detected. The HSC group had significantly larger lumen diameter ($94.26 \pm 1.81 \mu\text{m}$) compared to other groups except for N group ($100.74 \pm 1.75 \mu\text{m}$) where it was significantly smaller ($p < 0.05$). The NHS group ($67.41 \pm 1.92 \mu\text{m}$) had smaller lumen diameter compared to the other groups, except for HS group where there was no significant difference detected (Table 4.12).

Table 4.11: Mean square from analyses of variance for diameter of seminiferous tubules and lumen of treated rats

Source of variation	d.f.	Mean square	
		Diameter	
		Seminiferous tubules	Lumen
Treatment	4	563.07 ^{n.s}	32543.38**
Error	670	256.35	422.53
Total	674		
**p<0.001		n.s. non-significant	

Table 4.12: Lumen diameter of seminiferous tubules of treated rats

Parameter	Diameter (μm) (mean ± SE)
Treatment	Lumen
N (n=135)	100.74±1.75 ^d
NC (n=135)	78.02±1.79 ^b
HS (n=135)	66.65±1.57 ^a
HSC (n=135)	94.26±1.81 ^c
NHS (n=135)	67.41±1.92 ^a

Different superscripts within the same column indicate significant differences (p<0.05)

N: nicotine group, NC: nicotine control group, HS: *habbatus sauda* group, HSC: *habbatus sauda* control group, NHS: nicotine-*habbatus sauda* group.

4.4.2 Effects of treatments on width of spermatogonia, spermatocytes and spermatid-spermatozoa layers

The analyses of variance indicated that treatments had high significant effects on the width of spermatogonia, spermatocytes and spermatid-spermatozoa layers ($p<0.001$) (Table 4.13). Figures 4.1; 4.2; 4.3 illustrate the differences.

The N group had significantly thicker spermatogonia layer ($19.51\pm0.29\ \mu\text{m}$) compared to NC ($18.08\pm0.25\ \mu\text{m}$), HS ($17.59\pm0.25\ \mu\text{m}$) and NHS ($16.12\pm0.24\ \mu\text{m}$) groups ($p<0.05$) (Table 4.14). Spermatogonia layer of NC group ($18.08\pm0.25\ \mu\text{m}$) was significantly thinner compared to N ($19.51\pm0.29\ \mu\text{m}$) and HSC ($18.92\pm0.25\ \mu\text{m}$) groups, but significantly thicker compared to NHS ($16.12\pm0.24\ \mu\text{m}$) group ($p<0.05$). The HS group ($17.59\pm0.25\ \mu\text{m}$) had significantly thinner spermatogonia layer compared to N ($19.51\pm0.29\ \mu\text{m}$) and HSC ($18.92\pm0.25\ \mu\text{m}$) groups, but significantly thicker compared to NHS group ($16.12\pm0.24\ \mu\text{m}$) ($p<0.05$). The HSC group ($18.92\pm0.25\ \mu\text{m}$) had significantly thicker spermatogonia layer compared to other groups, except for N group. The NHS group ($16.12\pm0.24\ \mu\text{m}$) showed significantly thinner spermatogonia layer compared to the other groups ($p<0.05$) (Table 4.14). There seemed to be a deleterious effect of nicotine on the more matured spermatogens, hence probably causing increase production of spermatogonia.

The N group had significantly thinner spermatocyte layer ($32.56\pm0.41\ \mu\text{m}$) compared to HS ($34.56\pm0.40\ \mu\text{m}$) and NHS ($35.94\pm0.39\ \mu\text{m}$) groups ($p<0.05$) (Table 4.14). Spermatocyte layer of NC group ($33.66\pm0.43\ \mu\text{m}$) was significantly thinner compared to NHS group ($35.94\pm0.39\ \mu\text{m}$) ($p<0.05$). The spermatocyte layer of HS group ($34.56\pm0.40\ \mu\text{m}$) was intermediate in its width compared to N ($32.56\pm0.41\ \mu\text{m}$) and NHS ($35.94\pm0.39\ \mu\text{m}$) groups ($p<0.05$). The HSC group had significantly thinner

spermatocyte layer ($33.62 \pm 0.38 \mu\text{m}$) compared to NHS group ($35.94 \pm 0.39 \mu\text{m}$) ($p < 0.05$). The NHS group ($35.94 \pm 0.39 \mu\text{m}$) showed significantly thicker spermatocyte layer compared to the other groups ($p < 0.05$) (Table 4.14).

The N group had significantly thinner spermatid-spermatozoa layer ($21.51 \pm 0.51 \mu\text{m}$) compared to the other groups ($p < 0.05$) (Table 4.14). Spermatid-spermatozoa layer of NC group ($29.91 \pm 0.82 \mu\text{m}$) was significantly thicker compared to N ($21.51 \pm 0.51 \mu\text{m}$) and HSC ($26.24 \pm 0.61 \mu\text{m}$) groups, but significantly thinner compared to HS ($38.05 \pm 0.61 \mu\text{m}$) and NHS ($37.84 \pm 1.22 \mu\text{m}$) groups ($p < 0.05$). The HS group ($38.05 \pm 0.61 \mu\text{m}$) had significantly thicker spermatid-spermatozoa layer compared to the other groups, but non-significantly thicker when compared to NHS group ($37.84 \pm 1.22 \mu\text{m}$) ($p < 0.05$). The HSC group ($26.24 \pm 0.61 \mu\text{m}$) had significantly thicker spermatid-spermatozoa layer compared to N group ($21.51 \pm 0.51 \mu\text{m}$), but significantly thinner compared to NC ($29.91 \pm 0.82 \mu\text{m}$), HS ($38.05 \pm 0.61 \mu\text{m}$) and NHS ($37.84 \pm 1.22 \mu\text{m}$) groups ($p < 0.05$). The NHS group ($37.84 \pm 1.22 \mu\text{m}$) showed significantly thicker spermatid-spermatozoa layer compared to the other groups, except for HS group where no significant differences detected (Table 4.14).

Table 4.13: Mean square from analyses of variance for width of spermatogonia, spermatocytes and spermatid-spermatozoa layers of treated rats

Source of variation	d.f.	Mean square		
		Width		
		Spermatogonia layer	Spermatocytes layer	Spermatid-spermatozoa layer
Treatment	4	230.99**	215.08**	7092.50**
Error	670	8.94	21.71	85.32
Total	674			

**
p<0.001

Table 4.14: Width of spermatogonia, spermatocytes and spermatid-spermatozoa layers
of treated rats

Parameter Treatment	Width (μm) (mean \pm SE)		
	Spermatogonia Layer	Spermatocyte Layer	Spermatid-spermatozoa Layer
N (n=135)	19.51 \pm 0.29 ^c	32.56 \pm 0.41 ^a	21.51 \pm 0.51 ^a
NC (n=135)	18.08 \pm 0.25 ^b	33.66 \pm 0.43 ^{a,b}	29.91 \pm 0.82 ^c
HS (n=135)	17.59 \pm 0.25 ^b	34.56 \pm 0.40 ^b	38.05 \pm 0.61 ^d
HSC (n=135)	18.92 \pm 0.25 ^c	33.62 \pm 0.38 ^{a,b}	26.24 \pm 0.61 ^b
NHS (n=135)	16.12 \pm 0.24 ^a	35.94 \pm 0.39 ^c	37.84 \pm 1.22 ^d

Different superscripts within the same column indicate significant differences ($p < 0.05$)

N: nicotine group, NC: nicotine control group, HS: *habbatus sauda* group, HSC: *habbatus sauda* control group, NHS: nicotine-*habbatus sauda* group.

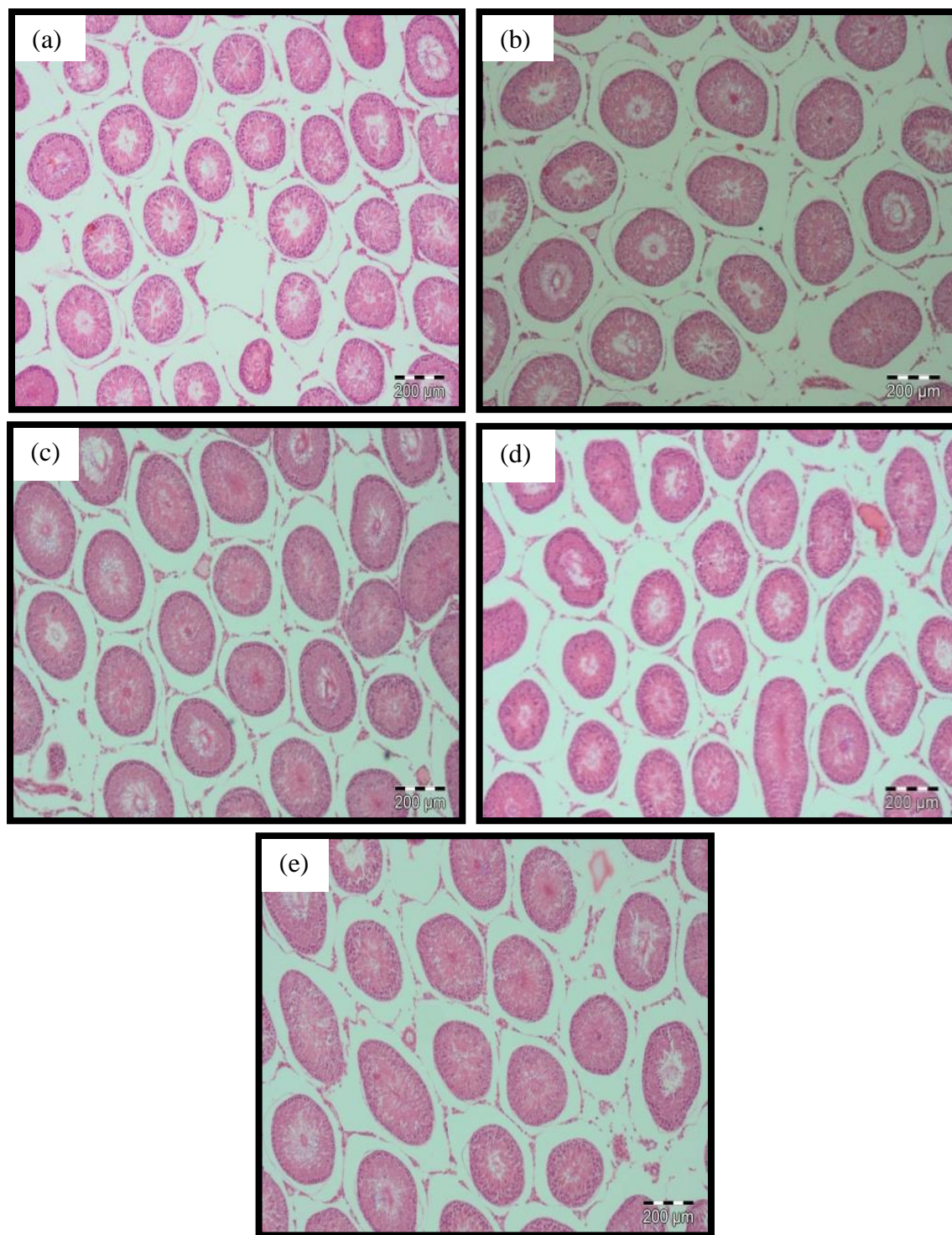


Figure 4.2: Seminiferous tubule at 40X magnification for (a) N (nicotine), (b) NC (nicotine control), (c) HS (*habbatus sauda*), (d) HSC (*habbatus sauda* control), and (e) NHS (nicotine-*habbatus sauda*) groups

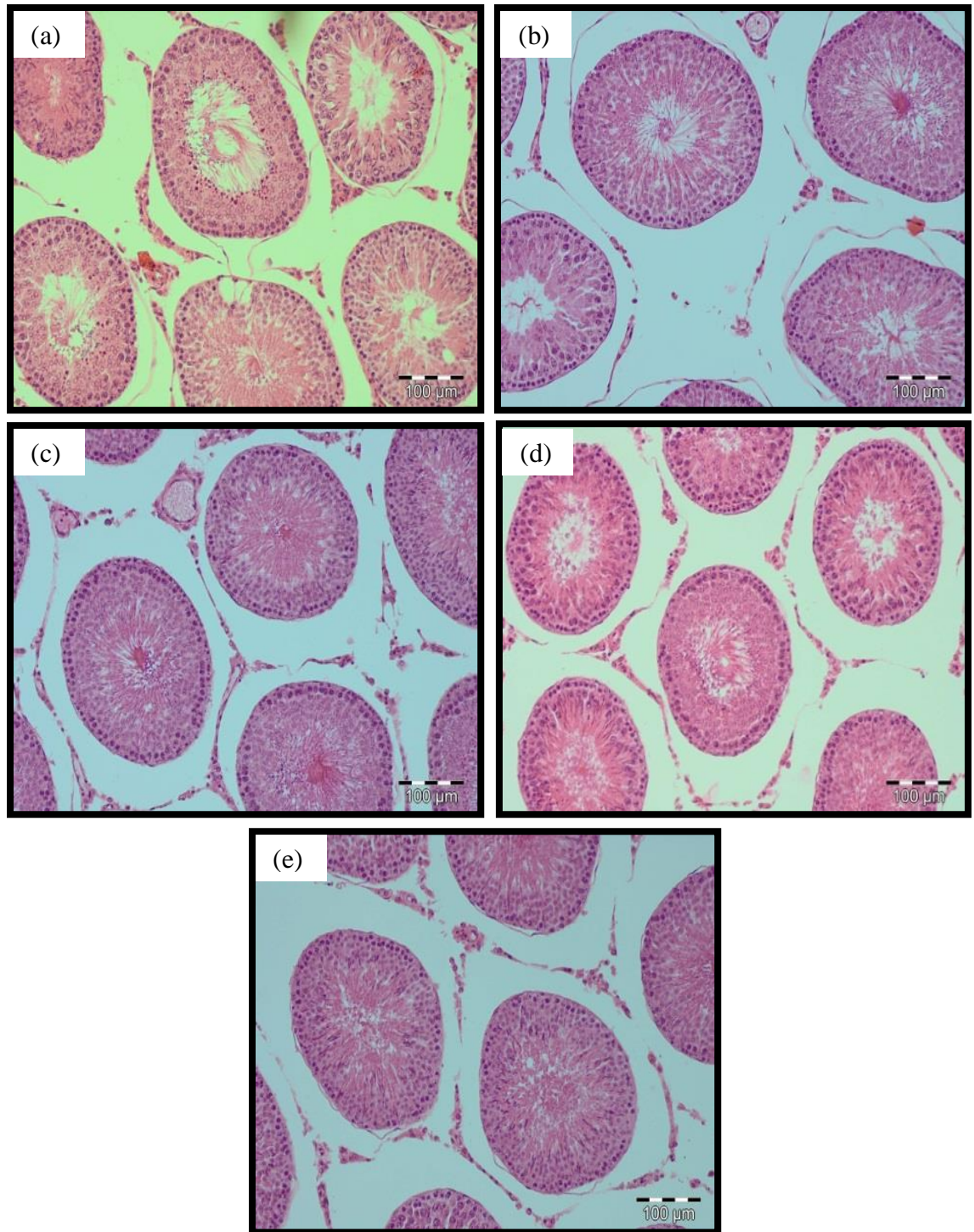


Figure 4.3: Seminiferous tubule at 100X magnification for (a) N (nicotine), (b) NC (nicotine control), (c) HS (*habbatus sauda*), (d) HSC (*habbatus sauda* control), and (e) NHS (nicotine-*habbatus sauda*) groups

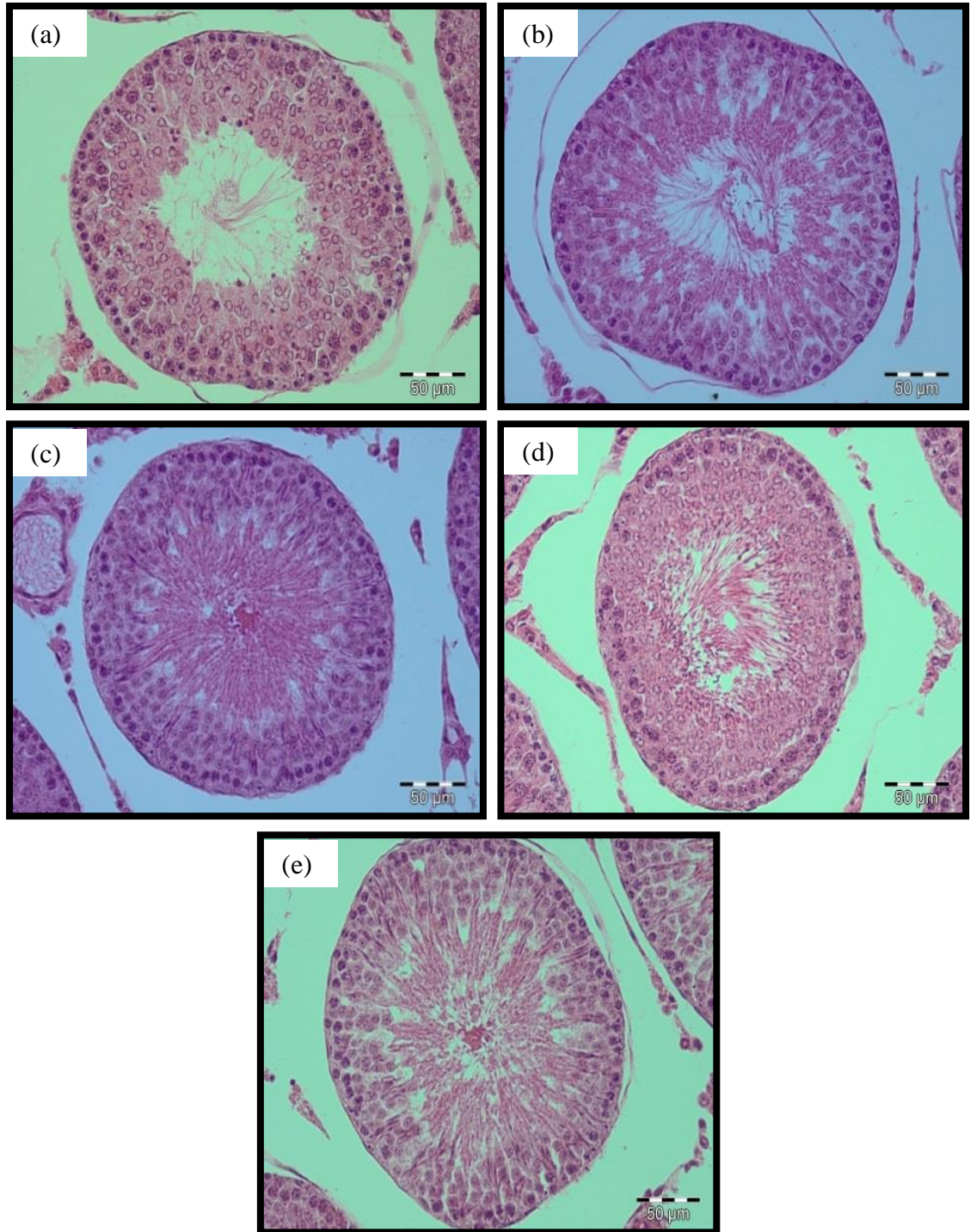


Figure 4.4: Seminiferous tubule at 200X magnification for (a) N (nicotine), (b) NC (nicotine control), (c) HS (*habbatus sauda*), (d) HSC (*habbatus sauda* control), and (e) NHS (nicotine-*habbatus sauda*) groups

CHAPTER 5

DISCUSSION

5.1 Effects of Treatments on Body Weight Increment

Based on results obtained from present study, there were no significant changes in term of body weight increment of rats being administered with nicotine (0.5 mg/100 g body weight) for 100 days. In contrast, a study by Grunberg *et al.* (1987) discovered that there was decrement in food consumption by female rats but not by male rats after nicotine exposure (12 mg/kg body weight) for 16 days.

There were studies which reported that adult mice (*Mus musculus*) injected with nicotine subcutaneously (1mg/kg body weight) for 6 weeks showed decreased body weight and food intake compared to saline treated mice (Sharif *et al.*, 2012). In addition, Audi *et al.* (2006) reported similar findings in rats administered with cigarette smoke (1 hour/day) for 2 months. Decreased body weight and food intake in nicotine treated subjects might be due to the effects of nicotine on neuroregulatory substances, involved in food intake mechanism (Wack & Rodin, 1982).

Eric *et al.* (2011) observed differences in food intake between free-feeding and food-restricted individuals due to nicotine intake. These results were based on 2 different effects with different temporal dynamics. The first effect was that nicotine increased satiety and lowered meal size (Bellinger *et al.*, 2003; Bellinger *et al.*, 2005), while the second effect was that nicotine enhanced the reinforcing properties of food.

Nicotine was found to suppress appetite of rodents through activation of melanocortin-4 receptors expressed on hypothalamic pro-opiomelanocortin (POMC) neurons that signals satiety (Huang *et al.*, 2011; Mineur *et al.*, 2011). In other animal studies, it was found that hypothalamic neuropeptide Y (NPY) being a potent orexigenic neuropeptide responsible for increased food intake might be enhanced either through acute or 7-week nicotine treatment (Chen *et al.*, 2007; Huang *et al.*, 2011). Conversely, hypothalamic neuropeptide Y (NPY) was suppressed after 12 weeks of nicotine treatment leading to reduced food intake (Huang *et al.*, 2011). Rodents treated with nicotine also showed increase orexins, another hypothalamic hormone that enhanced food intake (Huang *et al.*, 2011). These findings demonstrated possible mechanism of increased food intake in acute nicotine treatment and decreased intake in chronic nicotine treatment. In the current study, food intake was not investigated.

On the other hand, *Nigella sativa* oil (6 µl/100 g body weight) administered on rats in the present study also showed no significant changes in terms of body weight increment. This is in agreement with work by Parandin *et al.* (2012) where oral administration of alcoholic extract of *Nigella sativa* with doses of 200 and 400 mg/kg body weight in male rats for 60 days did not affect body weight of male rats. In contradiction, oral administration of 0.5 and 1.5 g/kg body weight of alcoholic extract of *Nigella sativa* for 53 days on rats led to an increase in body weight (Al-Sa'aidi *et al.*, 2009). It was also found that diabetic male Wistar rats given 2 % *Nigella sativa* for 30 days had increased body weight (Ghल्ली *et al.*, 2012). Body weight increment of animals treated with *Nigella sativa* could be due to the abundance of primary nutritional factors such as amino acid, glutamic acid and other essential amino acids in the black seed (Takruri & Damch, 1998).

5.2 Effects of Treatments on Testosterone, Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH)

Past studies showed varied results after nicotine treatment. The present study investigated the effects of nicotine on the levels of reproductive hormones (testosterone, LH and FSH) in adult male rats, which are all male androgen markers. In addition, LH stimulates Leydig cells to produce testosterone. It was also suggested that quality of sperm might be positively affected by the increment in testosterone and FSH levels due to the fact that these two hormones are responsible for spermatocytogenesis and spermiogenesis in seminiferous tubules, while testosterone is responsible for epididymal function in maturation of sperm (McLachlan *et al.*, 2002).

Results obtained from present studies are similar to a cross sectional study involving 255 men that showed no significant association between smoking and male androgen markers such as testosterone, sex-hormone-binding globulin (SHBG), LH and FSH (Halmenschlager *et al.*, 2009). Meanwhile, in another population-based cross sectional study involving 3427 men, smokers were found to exhibit significantly higher levels of total and free testosterone compared to non-smokers (Svartberg & Jorde, 2007). There was a study that showed an increase in total but not free testosterone, SHBG and LH in smokers compared to non-smokers (Wu *et al.*, 2008).

In vitro studies showed that LH-stimulated steroidogenesis in isolated mouse Leydig cells was inhibited by presence of nicotine (Patterson *et al.*, 1990). The quantity of testosterone produced was directly proportional to the amount of LH available, since, LH stimulates Leydig cells to produce testosterone (Guyton & Hall, 2000). In the present study, there was no significant difference in testosterone level which in turn suggested that LH level was not significantly changed in rats treated with nicotine.

Furthermore, nicotine is a known central nervous system depressors that can inhibit neural stimulus essential for the release of pituitary gonadotrophins (Reddy *et al.*, 1998).

It is interesting to note that adult mice (*Mus musculus*) injected with nicotine subcutaneously (1mg/kg body weight) for 6 weeks compared to 100 days in the current study, showed a decreased testosterone level (Sharif *et al.*, 2012). This finding was further supported by reports that nicotine tended to have adverse effects on gonad functions in males, besides lowering serum testosterone levels (Kavitharaj & Vijayammal, 1999). Presence of nicotine would also reduce LH and FSH secretions besides testosterone (Wang *et al.*, 2005). Previous researches showed that prostaglandin production was inhibited by the cytotoxic effects of nicotine, resulting in reduced testosterone levels (Favaro & Cagnon, 2006; Ahmadnia *et al.*, 2007). Low levels of testosterone as seen in smokers (chronic treatment) might be due to increased activity of liver hydroxylase, which is responsible in increasing testosterone metabolism (Mittler *et al.*, 1983). It has also been reported that nicotine induced some biochemical changes in the testis that in turn affected gonadal functions (Riesenfeld & Oliva, 1988).

Present results also indicated no significant changes in LH level of nicotine treated rats. Similar to present result, 100 men aged 20-60 years old showed that biologically active LH in smokers (chronic treatment) was not significantly different from non-smokers (Ahmed & AbdElkarim, 2014). In contrast, rats administered with different concentrations of nicotine (0.25 and 0.50mg/kg) daily for 8 weeks (acute treatment) showed decreased serum testosterone level, increased circulating levels of androgen binding protein (ABP) and increased FSH for rats treated with higher dose of nicotine. Besides that, level of LH was not significantly affected by nicotine when compared to the control group (Osama *et al.*, 2013). Contradictorily, there were reports

showing linear correlation between tobacco smoking and LH levels. These variation of results might be due to time factor of sample collection. It had been reported that there was a significant increase in level of LH within 14 minutes after cigarette smoking which remained higher than baseline for 40 minutes, after which LH level did not fluctuate from baseline level (Mendelson *et al.*, 2003).

Studies have reported that sex hormone-binding globulins (SHBG) and free testosterone levels of smokers were higher compared to non-smokers (Field *et al.*, 1994; English *et al.*, 2001). In contrast, other reports showed reduction in testosterone level of smokers (Shaarawy & Mahmoud, 1982; Ahmed & Abdelkarim, 2014). These findings suggested that effects of smoking on testosterone level could greatly depend on changes in plasma-binding capacity and not the direct effect of nicotine on testosterone (Ahmed & Abdelkarim, 2014). However, there are still uncertainties since there was an increment in testosterone level with smoking after adjustments were made on SHBG level (Svartberg *et al.*, 2003).

Cigarette smoking stimulates the release of several anterior and posterior pituitary hormones. It also contributes to the increase of certain hormone levels in the blood such as growth hormone (GH), adrenocorticotrophin (ACTH) and arginine vasopressin (AVP). However, there was a report that plasma level of thyroid stimulating hormone (TSH), luteinizing hormone (LH) and follicle stimulating hormone (FSH) remain unaffected by smoking behaviour (Seyler *et al.*, 1986). It had been reported that tobacco consumption affect levels of LH, testosterone and prolactin in male (Blanco-Muñoz *et al.*, 2012). Until now, the effect of cigarette smoking on reproductive hormones in men remains controversial (Vine, 1996; Kapoor & Jones, 2005).

In comparison to similar work on rodents, results obtained from the present study contradicted with the one done by Oyeyipo *et al.* (2013) who found significant reduction in serum testosterone level of rats treated with two different doses of nicotine. The reduction in serum testosterone level could be due to the adverse effect of nicotine on cytoarchitecture of the testis (Oyeyipo *et al.*, 2010). Consequently, Leydig cells inside the testis which produce testosterone would be adversely affected by nicotine leading to lower serum testosterone level (Osama *et al.*, 2013). Pituitary gonadotrophins which consist of FSH and LH would regulate the synthesis and release of androgens. Hence, no significant changes in testosterone level observed in present study might be due to non-significant changes in levels of LH, being the main tropic regulator of Leydig cell function. It was also reported that presence of both FSH and LH were needed for regulation of normal testicular function and spermatogenesis (Huthaniemi & Toppari, 1995).

In the present study, rats in HS and NHS group showed no significant changes in blood hormonal level. Contradictorily, rats orally administered with 0.5 and 1.5 g/kg alcoholic extract of *Nigella sativa* for 53 days showed increased testosterone hormone concentration (Al-Sa'aidi *et al.*, 2009). *Nigella sativa* was found to counteract the antiandrogenic effect of cimetidine via stimulation of Leydig cells in the testes. The Leydig cells were stimulated by the effects of *Nigella sativa* on steroid hormone production and coenzymes involved in metabolic pathways. Besides that, *Nigella sativa* could convert cholesterol into pregnenolone prior to testosterone production, which eventually counteracts the cimetidine effect (Al-Zamely, 2008). Rats treated with *Nigella sativa* also showed decrease cholesterol level, which suggested that steroidogenesis was active with cholesterol processed into hormones (Al-Sa'aidi *et al.*, 2009). It was found that *Nigella sativa* was able to maintain the normal mechanism of

testosterone homeostasis in alloxan-induced diabetic rats. This was made possible by the stimulation of interstitial cell receptors by *Nigella sativa*, which in turn would stimulate the secretion of testosterone (Kamal, 2005). This was supported by study where rats treated with *Nigella sativa* oil of 250 mg/kg for 4 weeks showed an ameliorative effect on induced testicular toxicity. This ameliorative effect was seen as increased testicular weight, improved quality and quantity of semen and also increased level of testosterone (Hala & Wahba, 2011).

Androgens are known to regulate the weight, size and secretory function of the testis, epididymis, prostate, seminal vesicle and vasa deferentia (Choudhary & Steinberger, 1975; Agrawal *et al.*, 1986). There was a report with findings on the correlation between increment in androgen level and significant increase in number of Leydig cells (Mukhallad *et al.*, 2009). The number of spermatocytes and spermatids were also increased in *Nigella sativa* treated rats based on similar study done by Mukhallad *et al.* (2009). It was reported that spermatocytes and spermatids were fully dependent on androgen (Dym *et al.*, 1979). Therefore, increase number of spermatocytes and spermatids reflected the increase level of androgen.

In agreement with the present study, 1.5 g of powdered *Nigella sativa* administered orally twice a day for three months did not significantly affect blood testosterone level of human. Besides that, no side effects were reported in subjects treated with *Nigella sativa* (Najmi *et al.*, 2008).

5.3 Effects of Treatments on Sperm Parameters

It was reported that reproductive function of males was negatively affected by cigarette smoking behavior. Cigarette smoking was associated with deterioration in semen

quality including sperm concentration, sperm motility, sperm morphology, semen volume and acidity (Zhang *et al.*, 2000; Pasqualotto *et al.*, 2006). Other researches reported the effects of cigarette smoking on sperm density, sperm motility and sperm morphology (Gaur *et al.*, 2007; Ramlau-Hansen *et al.*, 2007). In a study by Colagar *et al.* (2007), sperm count, motility and normal morphology were adversely affected by cigarette smoking. It was also reported that sperm obtained from smokers have lower fertilising capacity and produced lower implantation rates of embryos after fertilisation (Soares *et al.*, 2007; Ramlau *et al.*, 2008). In addition, association was found between cigarette smoking and sperm quality which included lower total sperm count and increase abnormal morphology (Robert *et al.*, 2003). Cigarette smokers exhibit higher cotinine level in blood. Cotinine is the major metabolite of nicotine (Sofikitis *et al.*, 1995).

In a separate study involving cigarette smoke exposed rats, it was found that nicotine and cotinine levels in the rat serum were increased, leading to impaired spermatogenesis, epididymal sperm content, sperm motility and fertilizing potential (Aydos *et al.*, 2001). These findings were similar to that of the present study, where reduction in quality of sperm and testis parameters were recorded. Since nicotine and cotinine could be detected in the seminal plasma of smokers, it would be expected that other harmful constituents of tobacco smoke could also pass through the blood-testis barrier (Vine *et al.*, 1993).

A meta-analysis which involved 27 different studies showed that smoking caused detrimental effects on sperm parameters. A 13 % reduction in sperm density, 10 % reduction in sperm motility and 13 % reduction of normal-shaped sperm was seen (Vine *et al.*, 1996). When comparisons were made between smokers and non-smokers,

smokers had significantly lesser motile and viable sperm (Zavos *et al.*, 1998). It was also reported that adverse effects such as lower sperm density, lower number of sperm and lower sperm motility could be attributed to cigarette smoking (Kunzle *et al.*, 2003). In one study, male albino Wistar rats treated with 20mg/kg and 30mg/kg aqueous extract of *nicotiana tobacum* for 21 days showed decreased sperm count and sperm motility (Ibraheem *et al.*, 2013). In addition, male *Sprague-Dawley* rats administered orally with 0.5 mg/kg and 1.0 mg/kg nicotine per body weight for a period of four weeks (acute treatment) significantly decreased the progressive sperm motility and sperm vitality compared to the control group, which was in agreement with present results (Oyeyipo *et al.*, 2011). There was linear association between reductions in progressive sperm motility with sperm vitality due to the fact that immotile sperm were considered dead after being stained using eosin-nigrosin stain (Oyeyipo *et al.*, 2011). Furthermore, lower sperm motility and vitality could also be due to the spermatotoxic effect of nicotine on maturing or matured sperm in the epididymis (Pacifici *et al.*, 1995). In idiopathic infertile subject, cigarette smoking was associated with sperm head defect, immature sperm and altered sperm chromatin and DNA integrities (Elshal *et al.*, 2009). There was also statistically significant correlation between concentration of cotinine in seminal plasma and percentage of abnormal sperm morphology (Smith *et al.*, 2002). Such results strongly support present findings that the percentage of head and tail defect sperm were significantly higher in rats treated with nicotine compared to the control group.

Findings in the present study were also partially reflected in other studies, which showed that cigarette smoking was found to be associated with lower sperm count, lower sperm motility and higher number of abnormal sperm (Close *et al.*, 1990; Sofikitis *et al.*, 1995; Saleh *et al.*, 2002). However, some studies failed to detect any

association between smoking and sperm parameters (Vogt *et al.*, 1986; Dikshit *et al.*, 1987). A few mechanisms of action of smoking on sperm parameters were suggested by researchers. One of the mechanisms implicated, ROS induced by seminal oxidative stress as the cause of negative effects on sperm quality and function. It was reported that oxidative stress levels in semen of smokers was higher compared to the non-smokers (Saleh *et al.*, 2002).

Less scavenging enzymatic activities have been detected in the cytoplasm, making it vulnerable to damage induced by excessive ROS (Agarwal & Prabakaran, 2005; Aitken & Baker, 2006). Besides causing negative effects on the fluidity and function of sperm plasma membrane, excessive ROS can also disrupt DNA integrity inside the sperm nucleus (Agarwal & Saleh, 2002; Agarwal & Prabakaran, 2005). It was reported that DNA damage caused by ROS might bring about a chain reaction whereby the process of germ cell apoptosis was activated, leading to lower sperm count and male infertility (Aitken & Krausz, 2001; Agarwal & Allamaneni, 2004). Researches also found direct associations between levels of ROS and sperm motility (Armstrong *et al.*, 1999). Thus, reduction in sperm motility of nicotine treated rats in present study could be due to peroxidative damage of ROS on the membrane and axonemal proteins of sperm.

Mahanem *et al.* (2006) found that various sperm parameters of male adult *Sprague-Dawley* rats were adversely affected as a result of nicotine treatment. Similar to different toxicants described in other studies, nicotine also caused deleterious effects by lowering sperm count (60 % reduction), lower sperm motility and lower percentage of normal sperm compared to the control (Bustos-Obrego & Gonzalez-Hormazabal, 2003; Sarkar *et al.*, 2003). Generally, nicotine treatment was known to increase lipid

levels and lipid peroxidation products in serum and testis of rats (Latha *et al.*, 1993; Ashakumary & Vijaya, 1997). It was also reported that nicotine administration disrupted the components of free radical defence system and tended to exert oxidative stress in germ cells (Bui *et al.*, 1995; Yildiz *et al.*, 1998). Free radicals produced would lead to cellular injury. The structure and fluidity of cell membrane would be altered when membrane phospholipids were disintegrated, marked by the release of unsaturated fatty acid from membrane phospholipids (Slater, 1984; Bagchi *et al.*, 1993).

Based on present results, decrease in sperm and testis histological features qualities observed in nicotine treated rats was probably due to increased oxidative degradation of phospholipids. Concentration of lipid peroxidation (LPO) products has been reported to be increased in nicotine treated rats, which could be attributed to decrease activity of scavenging enzymes such as catalase and superoxide dismutase (Helen *et al.*, 2000). In addition, it has been proven that administration of nicotine could halt antioxidant properties of scavenging enzymes, while supplementation of compounds with antioxidant properties, such as vitamin E could lower the levels of LPO products (Ashakumary & Vijayammal, 1996). Therefore, it could be expected that *habbatus sauda* oil supplementation, also considered as a source of antioxidant compounds, could lower LPO products in nicotine treated rats and counteract the adverse effects posed by nicotine.

Nigella sativa oil possesses antioxidant properties and act as a free radical scavenger. Antioxidant properties of *Nigella sativa* oil was shown in hyperlipidemic rats where damage caused by hyperlipidemia-induced free radicals on nephrons was ameliorated by *Nigella sativa* oil supplementation (Badary *et al.*, 2000). Besides that, administration of *Nigella sativa* oil was found to increase high-density lipoprotein

(HDL) level in rats (Samir Bashandy, 2007). HDL is one of the five major groups of lipoproteins and it is the densest because it contains the highest proportion of protein to lipids. Since HDL is the most abundant lipoprotein in tissue fluid, HDL might counteract the effects of lipid peroxidation in the tissue fluid and may possibly protect cell membranes (Eisenberg, 1984). HDL also possesses antioxidant properties and its concentration was increased in rats treated with *Nigella sativa* oil, suggesting the improvement of antioxidant activities (Idzior *et al.*, 2004).

Nigella sativa administered orally on normal and hyperlipidemic rats for 2 months showed significant decrease in levels of cholesterol, triglycerides and LDL, while HDL level was increased. Reductions in lipid concentrations might be caused by hypolipidemic effects of unsaturated fatty acids (oleic and linoleic acids) contained in *Nigella sativa* oil (Allman-Farinelli *et al.*, 2005; Wendel & Belury, 2006). Improvements were also reported in terms of reproductive efficiency, seminal vesicle weight, testosterone level, sperm motility and sperm quality of *Nigella sativa* treated rats. These beneficial outcomes on fertility observed in hyperlipidemic rats might be due to antioxidant and hypolipidemic properties of *Nigella sativa* oil (Samir Bashandy, 2007). In a different study, similar findings of favorable effects of *Nigella sativa* oil on abnormal sperm parameters have also been reported. It may be due to the unsaturated fatty acids content of *Nigella sativa* oil (Nickavar *et al.*, 2003). This hypothesis was further supported by research findings on the correlation between unsaturated fatty acid supplementation and enhanced sperm count, motility, and normal morphology in infertile men (Nissen & Kreysel, 1983; Safarinejad 2011). It is known that unsaturated fatty acids play an important role in sperm maturation, for example shark liver oil supplementation containing unsaturated fatty acids significantly increased boar sperm motility (Mitre *et al.*, 2004).

Similar to findings from present study, Mukhallad *et al.* (2009) found increment in sperm motility collected from cauda epididymis of male albino rats treated with *Nigella sativa* (300mg/kg body weight) for 60 days. Similarly, *Nigella sativa* oil being administered orally on rats for a period of 6 weeks showed increase epididymal sperm concentration and sperm motility (Sherif *et al.*, 2013). Another study also reported improvements in semen quality of diabetic male Wistar rats in terms of sperm motility and quantity after being supplemented with 2 % *Nigella sativa* diet for 30 days (Ghल्ली *et al.*, 2012).

Male Wistar rats being fed orally with *Nigella sativa* oil for 60 consecutive days showed a decrease in the number of abnormal sperm. This which might be due to a reduction in lipid peroxidation. The positive effects on sperm quality could be attributed to the antioxidant property of *Nigella sativa* oil that inhibits excessive free radical generations (Sherif *et al.*, 2013). This finding could be attributed to the effects of *Nigella sativa* on oxidative phosphorylation enzymes (Azzarito *et al.*, 1996). It was suggested that dietary supplementation of powdered *Nigella sativa* could inhibit oxidative stress caused by oxidized corn oil in rats (Al-Othman *et al.*, 2006).

Positive effects of *Nigella sativa* on male fertility are closely associated with the chemical composition of this plant (Parandin *et al.*, 2012). Phytochemical analysis carried out on the seeds of *Nigella sativa* showed presence of high concentrations of unsaturated fatty acids such as linoleic acid (55.6 %), oleic acid (23.4 %), palmitic acid (12.5 %), stearic acid (3.4 %), and others (Nickavar *et al.*, 2003). This was further supported by a study where rats fed with oils rich in polyunsaturated fatty acids (PUFAs) showed improved reproductive functions (Fellner *et al.*, 1995). The unsaturated fatty acids were found responsible for the stimulation of 17 β -hydroxysteroid dehydrogenase

activities, which was an important enzyme in the testosterone biosynthesis pathway (Gromadzka Ostrowska *et al.*, 2002). Testosterone is responsible for the regulation of the male reproductive organ.

Present study discovered that sperm parameters of rats co-administered with nicotine and *habbatus sauda* oil showed improvements when comparisons were made against rats treated with only nicotine or *habbatus sauda*. Ghlissi *et al.* (2012) reported that the improvements of reproductive function in diabetic male rats were due to the protective effect of *Nigella sativa*. Constituents of *Nigella sativa* seeds such as proteins, vitamins, minerals, alkaloids and phenols were believed to contribute to this protective effect (Al-Dejly, 2001; Kanter *et al.*, 2005). Ghlissi *et al.* (2012) also found increase antioxidant activity besides lower measurement of malonaldehyde in reproductive system of rats treated with *Nigella sativa*. Salem (2005) also demonstrated antioxidant properties of black cumin oil; attributed to its active compound, thymoquinone (TQ), which was responsible to enhance scavenger system leading to antitoxic effects. Other researches also reported that *Nigella sativa* tended to decrease lipid peroxidation product level and liver enzymes, leading to increase activity of antioxidant defense system (Kanter, 2008). Under normal circumstances, ROS would be neutralized by antioxidants present in ejaculatory fluid (Lamirande & Gagnon, 1999). Any changes to these circumstances, such as reduction of antioxidant property or increased ROS production in semen, would in turn increase the level of oxidative stress which would be detrimental to sperm parameters (Aitken & Krausz, 2001; Schulte *et al.*, 2010). It was also reported that ROS levels in semen of infertile patients were elevated (Zini *et al.*, 1993).

Salem (2005) found that both the oil of black cumin and its active ingredients, in particular thymoquinone, gave reproducible antioxidant effects through enhancing the oxidant scavenger system. Consequently, this led to antitoxic effects against detrimental effects induced by several insults. Furthermore, Parveen and Shadabig (2011), indicated that generation of reactive oxygen species (ROS) as a result of oxidative stress due to any toxic agent was depressed by the antioxidant effects of *Nigella sativa* seeds. The result was consistent with Ragheb *et al.* (2009) and Uzma *et al.* (2012), who showed the importance of thymoquinone as a pharmacologically active quinone with antioxidant effect.

Thymoquinone (TQ) being a potent antioxidant has proven its protective effects in studies against nephrotoxicity (Badary *et al.*, 1997), cardiotoxicity (Al-Shabanah *et al.*, 1998) and hepatotoxicity (Al-Gharably *et al.*, 1997) in mice and rats. It was also found to be responsible for inhibiting eicosanoid generation in leucocytes and lipid peroxidation in ox brain (Houghton *et al.*, 1995). Another major compound isolated from *Nigella sativa* seed is Nigellone, a carbonyl polymer of TQ. Toxicity of Nigellone is significantly lower compared to TQ. However, its pharmacologic properties are similar to that of TQ. Some of the properties of Nigellone include antioxidant, antispasmodic and antihistaminic (El Dakhakhny *et al.*, 2002).

In a different study involving men with abnormal semen parameters, oral intake of 5ml *Nigella sativa* oil for 2 months showed improvements in sperm count, sperm morphology, sperm motility, semen volume and pH (Kolahdooz *et al.*, 2014). Results on the effects of *Nigella sativa* oil supplementation on abnormal semen parameters in present study also shared similar findings with other studies where *Nigella sativa* oil administration caused positive outcomes on male fertility in normal rats (eg. Samir

Bashandy, 2007). Mechanisms of these effects are not fully understood. However, it was opined that antioxidant properties of *Nigella sativa* oil played a big role which led to these findings. In support to this finding, thymoquinone being the major active compound in *Nigella sativa* oil with antioxidative properties could neutralize ROS in semen leading to improvement of sperm parameters (Al-Wafai, 2013). Similarly, study using other compounds with antioxidant properties such as vitamin E, selenium and other nutritional supplements showed clear improvements of sperm motility in infertile men (Suleiman *et al.*, 1996; Vézina *et al.*, 1996; Sinclair, 2000).

5.4 Effects of Treatments on Histological Parameters of Seminiferous Tubule

Testis consists of seminiferous tubules where spermatogenesis takes place. Cross sectional histological sections of the testis in this study also showed that seminiferous tubules consisted of spermatogenic cells at various stages of development.

Based on results from present study, nicotine treated rats showed reduced quality in terms of testis histological features. It was reported that nicotine could exert toxic effects on gonadal functions in the testes through a series of nicotine-induced biochemical changes (Riesenfeld & Oliva, 1988). The testicular tissues were directly affected by nicotine, subsequently leading to impaired spermatogenesis (Zavos *et al.*, 1999). The testis tissues of mice administered with nicotine were damaged either by the direct cytotoxic effects of nicotine on spermatogenic cells or inhibition of prostaglandin production, which was important for the maintenance of the mouse reproductive system (Polyzos *et al.*, 2009). Male albino Wistar rats treated with 20mg/kg and 30mg/kg aqueous extract of *nicotiana tobacum* for 21 days showed decrease spermatogenic cell numbers in testis histological sections as compared to the control group (Ibraheem *et al.*, 2013).

Observations made on the testicular sections from male Swiss albino mice treated with nicotine for a period of one and two weeks showed reduction in spermatogenic stages, reduction in seminiferous tubule sperm count, scattered nuclear pyknotic change in basal cell layers and obvious degenerative changes on seminiferous tubules (Gawish *et al.*, 2010). These observations could be attributed to vascular insufficiency from nicotine toxicity (Ahmadnia *et al.*, 2007). Besides that, the testis histological sections of nicotine treated rats showed tubule vacuolation and shedding of immature germ cells into the lumen. Current study also showed that the lumens of nicotine treated rats were significantly large. Sertoli cells and germ cells inside the tubules were shrunken and spermatogenesis was adversely affected in the nicotine treated rats (Mahanem *et al.*, 2006). It was proven that damaged Sertoli cell structure leads to disrupted spermatogenesis (Born *et al.*, 1988).

Histomorphometrical examinations on testis tissue of nicotine treated mice showed significantly reduced number of interstitial Leydig cells (LCs) compared to mice in the control group (Gawish *et al.*, 2010). Decrease number of interstitial Leydig cells of nicotine treated mice could be due to nicotine-induced apoptosis inside the testis (Kim *et al.*, 2005). It was also suggested that nicotine induced specific intracellular death-related pathways (Cohen, 1997). In present study, abnormal testicular histology observed in nicotine treated rats included obvious spaces in between the tubules, possibly causing reduced number of LCs. The Abnormal testicular histology could be due to increased ROS generation and decreased antioxidant enzyme activity in the testes.

Spermatogenesis and function of reproductive organs are greatly dependent on androgen. Male albino rats treated with *Nigella sativa* with dose of 300 mg/kg body weight for 60 days showed increased numbers of mature Leydig cells. Previous study

reported that Leydig cells were associated with androgen levels. Therefore, administration of *Nigella sativa* would indirectly increase androgen level, thus improving spermatogenesis (Mukhallad *et al.*, 2009). The increased number of spermatocytes and spermatids also confirmed the increase in androgen levels, since spermatocytes and spermatids are completely androgen dependent (Dym *et al.*, 1979).

Clear improvements on testis histological features were observed in rats administered with *Nigella sativa* oil in the present study. Testis histological study performed on white male rats treated with alcoholic extract of *Nigella sativa* for 53 days showed increased diameter and wall thickness of the seminiferous tubules (made up of spermatogonia, primary and secondary spermatocytes, as well as spermatid) and free spermatozoa in the lumen of seminiferous tubules (Al-Sa'aidi *et al.*, 2009). Other histological studies on spermatogenesis showed the same findings that spermatogenesis was clearly improved in the animals treated with aqueous and alcoholic extracts of *Nigella sativa* (Al-Helali, 2002; Al-Mayali, 2007).

Male adult albino rats co-administered orally for 12 weeks with colchicine and aqueous suspension of powdered *Nigella sativa* seeds showed improvements in histopathological parameters of the testis. There was improved germ cell lining of seminiferous tubule, normal interstitial cells of Leydig and basement membrane besides positive reaction of Periodic acid Schiff (PAS) stain (Said *et al.*, 2013). The group suggested that protective effect of *Nigella sativa* seeds against testicular toxicity caused by colchicine might be due to its antioxidant properties and free radical scavenger. Thymoquinone (TQ) was reported to be the main constituent in *Nigella sativa* seeds with analgesic, anti-inflammatory and antioxidant properties (Chandra *et al.*, 2009). In addition, reactive oxygen species (ROS) due to oxidative stress of any toxic agent can

be counteracted by *Nigella sativa* seeds which contains TQ as a major antioxidant (Parveen & Shadabig, 2011). Supplementation of TQ or vitamin E during heat stress could facilitate the recovery of the seminiferous tubules structure. The recovery was indicated by normal epithelium with a regular arrangement of germinal cells with most phases of spermatogenesis including differentiation phase of spermatids (Al-Zahrani *et al.*, 2012). These results could be attributed to the role of vitamin E or TQ in improving the antioxidant defense system (Sahin *et al.*, 2001; Ragheb *et al.*, 2009).

In some other studies, vitamin E which possesses antioxidant properties (Suleiman *et al.*, 1996; Helen *et al.*, 2000) demonstrated protective effects against nicotine based on sperm analysis and testis histopathology of rats (Mahanem *et al.*, 2006). Hence, the improvements on testis histological features observed in the NHS treated rats could be due to antioxidant properties of thymoquinone present in *Nigella sativa* oil. Thus, assumption of TQ being an important antioxidant, which could counteract oxidative stress caused by nicotine administration can also be made based on present study.

Other previous studies also showed benefit of TQ against oxidative stress conditions. For example, mice suffering from testicular torsion/detorsion (TD) showed lower Malondialdehyde (MDA) level, which was the secondary product of ischemia-reperfusion (I/R) injury when treated with TQ; demonstrated inhibition of lipid peroxidation (Filho *et al.*, 2004; Avlan *et al.*, 2005). Higher total oxidative stress (TOS) level in the serum of mice with ischemia-reperfusion (I/R) injury could be significantly lowered with TQ treatment (Gökçe *et al.*, 2010).

In the current study, the effect of nicotine, reportedly a potent oxidant, was chosen to be evaluated using *Nigella sativa*. Findings on the protective effect of TQ on testicular injury caused by oxidative stress was rather similar to present study; better results of testis histological features were observed in rats treated with both nicotine and *habbatus sauda*, compared to nicotine treatment alone.

Other previous studies looking specifically at detrimental effects of cisplatin-based chemotherapy on testicular function also indicated protective effect of TQ (de Mora *et al.*, 2003; Cherry *et al.*, 2004; Ishikawa *et al.*, 2004). Co-administration of cisplatin and 0.5ml/kg body weight of *Nigella sativa* oil for 21 days on rats showed that histopathological changes of the testes appeared similar to those of control group (Awadalla, 2012).

5.5 Future Studies

To date, studies regarding the protective or healing effects of *Nigella sativa* oil on nicotine treated male rat reproductive system and body weight increment are still lacking which made it hard to clarify results obtained from this study.

Body weight of treated rats did not show any significant increment in the present study. Hence, future studies on body weight increment of treated rats should be given different variables in duration and route of administration of treatment on rats. Duration of treatment could be changed to acute treatment instead of chronic treatment, since rats might develop tolerance against treatment particularly nicotine, over time. Tolerance against nicotinic effects could also manifest in other parameters such as reproductive hormone (testosterone, FSH and LH) levels in blood.

Reproductive hormone levels analysed were not significantly different when comparison were made among groups. Other types of test kit could be used to determine the concentration of particular hormone in blood; hence kits such as radioimmunoassay (RIA) kit could be used in future studies to double check the presence and concentration of hormonal level measured. Apart from that, it is important to note that LH stimulates Leydig cells to produce testosterone (Guyton & Hall, 2000). Mosbah *et al.* (2015) also observed reduction in testosterone levels as well as reduced presence of Leydig and Sertoli cells in the testes of nicotine treated rats, which in turn suggested disrupted androgen production. Thus, Leydig and Sertoli cell counts could be carried out in future to determine number of Leydig cells for each particular treatment group.

As for sperm parameters, there were clear changes in terms of sperm motility, morphology and vitality of treated groups. Hence, more detailed studies on sperm ultrastructure should be carried out. For instance, mitochondria count could suggest the potential influence on sperm motility. Apart from that, ROS levels of treated rats should also be determined in order to get a clearer picture on effects of treatment on ROS levels. Mosbah *et al.* (2015) found that nicotine treatment tended to increase the indicators of oxidative stress in rats. It is significant to determine ROS levels, since ROS were known to cause detrimental effects on sperm and also testicular cells. As an additional supportive data for present study, the concentration of LPO products and activity levels of scavenging enzymes in the nicotine treated rats could be determined in future study.

Based on literature search, there were no similar studies on the effects of treatments on testis histological studies. Hence, further studies are needed to shed light on the actual mechanism that leads to the difference in thickness of spermatogenic cell layers as observed in present study. Since Sertoli cells were closely associated with both Leydig and germ cells in maintaining regular spermatogenesis, future studies should take into consideration of observing effects of nicotine on these cells as well.

Present study was done particularly on rats which shared a lot of similarity with human in terms body physiology. However, further studies are needed to elucidate the exact mechanisms of action besides to determine whether the results obtained in present or future studies may be applied or extrapolated to humans.

CHAPTER 6

CONCLUSION

This study was carried out for 100 days to examine the effects of chronic administration of nicotine, *habbatus sauda* and co-administration of nicotine-*habbatus sauda* on *Sprague-Dawley* male rats. Aspects being investigated included body weight, sperm parameters (motility, morphology and vitality), testis histological features and blood hormone profile. Research objectives of this study were fulfilled and it was found that:

- nicotine and *habbatus sauda* administration performed on rats did not significantly affect their body weight pre and post treatment.
- nicotine administration adversely affect sperm parameters and testis histological features of rats.
- *habbatus sauda* oil supplementation could improve sperm parameters and testis histological features of rats.
- *habbatus sauda* oil may have the potential ameliorative/protective effect against nicotinic effect on sperm and testis histological parameters.

6.1 Effects of Nicotine on Sperm Parameters, Testis Histological Features, Blood Hormonal Level and Body Weight Increment

The present investigation showed that nicotine reduced fertility in adult male rats based primarily on sperm and testis parameters studied. Chronic nicotine administration on rats did not produce any significant effect on the concentration of reproductive hormones (testosterone, LH and FSH) which could also serve as an indication on fertility status. Based on results obtained from the study, this research suggested that nicotine tended to reduce quality of sperm and adversely affected the arrangement of

spermatogenic cell layers. However, further investigation is needed to confirm the role and mechanisms of action of nicotine on male infertility.

In terms of body weight increment, chronic nicotine administration performed on rats for a period of 100 days did not significantly affect their body weight pre and post treatments.

6.2 Effects of *Habbatus sauda* on Sperm Parameters, Testis Histological Features, Blood Hormonal Level and Body Weight Increment

Chronic consumption of *habbatus sauda* oil extract for 100 days had produced positive effects on sperm morphology and vitality of rats, while, sperm motility remained unaffected. There were clear improvements on spermatogenesis, as evidenced by the improvements on spermatogenic cell layers. Although there were no significant effects detected on reproductive hormones (testosterone, LH and FSH) of *habbatus sauda* treated rats, it could be deduced that *habbatus sauda* oil administration performed on rats could bring positive effects on fertility of male rats in some aspects.

In terms of body weight increment, *habbatus sauda* oil administration performed on rats for a period of 100 days did not significantly affect their body weight pre and post treatments.

6.3 Effects of Co-administration of Nicotine and *Habbatus sauda* on Sperm Parameters, Testis Histological Features, Blood Hormonal Level and Body Weight Increment

Dosage of nicotine used in this study (0.5mg/100g body weight) was found to have negative effects on fertility of rats. Contradictorily, rats treated with *habbatus sauda* oil

with dosage of 6µl/100g body weight showed improvements in sperm and testis histological parameters. Present findings of this study indicated that co-administration of nicotine and *habbatus sauda* oil performed on rats for 100 days could reduce the detrimental effect caused by nicotine administration alone.

Based on previous literature *habbatus sauda* oil possessed antioxidant properties, which may act on excessive ROS produced by presence of nicotine, leading to reduced severity of sperm and testis damage. Besides that, it was found that rats administered with both nicotine and *habbatus sauda* oil did not significantly alter body weight and blood hormonal level (testosterone, LH and FSH). Similarly rats in N and HS groups also did not show any significant changes in terms of body weight and blood hormonal levels.

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APPENDICES

Appendix I: *Habbatus sauda* dilution

- 6 μ l of pure *habbatus sauda* oil/100 g body weight of rat
- 1000 μ l = 1 ml
6 μ l = 0.006 ml

Assumption: 20 rats treated daily (body weight: 200 g)

Diluted *habbatus sauda* solution (diluted with corn oil-0.1 ml/100 g body weight of rats)
= 0.1 ml x 2 x 20 rats = 4.0 ml diluted *habbatus sauda*

Pure *habbatus sauda* oil needed

= 0.006 ml x 2 x 20 rats = 0.24 ml pure *habbatus sauda* oil

Corn oil needed

= 4.0 ml – 0.24 ml = 3.76 ml corn oil

Appendix II: Nicotine dilution

- 5.0 mg of nicotine/kg body weight of rat
= 5.0 mg of nicotine/1000 g body weight of rat
= 0.5 mg of nicotine/100 g body weight of rat
- 25 g nicotine = 25 ml nicotine
25 g nicotine = 2500 mg nicotine = 25 ml nicotine
0.5 mg nicotine = 0.005 ml nicotine (5×10^{-3} ml nicotine)
- 5×10^{-3} ml nicotine/100 g body weight of rat

Assumption: 20 rats treated daily (body weight: 200 g)

Nicotine solution (diluted with saline-0.1 ml/100 g body weight of rats)
= 0.1 ml x 2 x 20 rats = 4.0 ml diluted nicotine

Pure nicotine needed

= 5×10^{-3} ml nicotine x 2 x 20 rats = 0.2 ml pure nicotine

Saline needed

$$= 4.0 \text{ ml} - 0.2 \text{ ml} = 3.8 \text{ ml saline}$$

Appendix III: Normal saline solution preparation (0.9 % NaCl) (100ml)

Distilled water 100 ml

NaCl 0.9 g

Appendix IV: Chloral hydrate solution preparation (100 ml)

Distilled water 100 ml

Chloral hydrate 3.5 g

Appendix V: Toyoda Yokoyama Hosi (TYH) solution preparation

List of chemicals:

Chemicals	Formula	Catalog number	Weight (g/100ml)
Sodium chloride	NaCl	S9625-500G	0.5140
Potassium chloride	KCl	P4504-500G	0.0358
Calcium chloride dihydrate minimum 99 %	CaCl ₂ ·2H ₂ O	C7902-500G	0.0251
Potassium phosphate monobasic, >= 99.0 %	KH ₂ PO ₄	P5655-100G	0.0162
Magnesium sulfate heptahydrate	MgSO ₄ ·7H ₂ O	M1880-500G	0.0293
Sodium bicarbonate	NaHCO ₃	S5761-500G	0.2108
Sodium pyruvate	C ₃ H ₃ NaO ₃	P2256-100G	0.0055
D - (+) - Glucose	C ₆ H ₁₂ O ₆	G6152-100G	0.1000
Penicillin G potassium	C ₁₆ H ₁₇ KN ₂ O ₄ S	P7794-10MU	0.0075
Streptomycin sulfate salt	2C ₂₁ H ₃₉ N ₇ O ₁₂	S9137-25G	0.0050
Phenol red	C ₁₉ H ₁₄ O ₅ S	114529-5G	0.0010
L-Glutamine	C ₅ H ₁₀ N ₂ O ₃	G3126-100G	0.0146
Taurine, minimum 99 %	C ₂ H ₇ NO ₃ S	T0625-10G	0.0125
Albumin from bovine serum. Bovine albumin *BSA	-	A7030-10G	3 mg/ml (0.03g/ml)

Appendix VI: Eosin-nigrosin solution preparation (100 ml)

Chemicals	Weight (g)
Eosin Y	0.67 g
Nigrosin	10.00 g
Sodium chloride (NaCl)	0.90 g

Procedures involved in eosin-nigrosin staining solution preparation:

1. 0.67 g Eosin Y and 0.90 g NaCl were dissolved in 100 ml distilled water under gentle heating.
2. 10 g of nigrosin was added to the solution.
3. Solution was boiled and allowed to cool down in room temperature.
4. Solution was filtered with filter paper into a glass bottle and sealed prior to storage in room temperature.

Appendix VII: Bouin solution preparation

Saturated picric acid	75 ml
Formalin	25 ml
Glacial acetic acid	5 ml

Appendix VIII: 70 % and 85 % alcohol solution preparation

70 % alcohol solution (100 ml)

95 % alcohol	70 ml
Distilled water	25 ml

85 % alcohol solution (100 ml)

95 % alcohol	85 ml
Distilled water	10 ml

LIST OF PUBLICATIONS AND PAPERS PRESENTED

2014

Cho Ping N, Hashida NH and Durriyyah Sharifah HA. (2014). Effects of *Nigella sativa* (*Habbatus sauda*) oil and nicotine chronic treatments on sperm parameters and testis histological features of rats. *Evidence-Based Complementary and Alternative Medicine*. 2014:7 pages. Article ID 218293. (ISI-Cited Publication).

2013

Cho Ping N, Hashida NH and Durriyyah Sharifah HA. Effects of *Nigella sativa* (*Habbatus sauda*) oil on sperm parameters of nicotine treated rats. 5th Global Summit on Medicinal and Aromatic Plants (GOSMAP-5), Miri, Sarawak, Malaysia. 8th-12th December 2013. (Proceedings).

Cho Ping N, Hashida NH and Durriyyah Sharifah HA. Preliminary study on the effect of *Nigella sativa* (*Habbatus sauda*) oil on sperm parameters of rats. International Conference on Natural Products (ICNP), Shah Alam Convention Centre (SACC), Shah Alam, Malaysia. 4th-6th March 2013. (Proceedings).

2012

Cho Ping N, Hashida NH and Durriyyah Sharifah HA. The effects of *Habbatus sauda* oil and nicotine on testis histological features of male *Sprague-Dawley* rats. 21st Scientific Conference of the Microscopy Society Malaysia, Kota Bharu, Kelantan, Malaysia. 22nd-24th November 2012. (Proceedings).

Cho Ping N, Hashida NH and Durriyyah Sharifah HA. (2012). Preliminary study on the effect of *Nigella sativa* (*Habbatus sauda*) oil on body weight of nicotine treated male *Sprague-Dawley* rats. *Journal of Community Health*. (1):75. (non-ISI Cited Publication).

2011

Noor Juliyana R, Cho Ping N, Fatma AR, Hashida NH and Durriyyah Sharifah HA. Preliminary study on the effect of *Nigella sativa* (*Habbatus sauda*) oil on testis histological features. 1st AKEPT Young Researchers Conference and Exhibition (AYRC X3 2011), Putra World Trade Centre (PWTC), Kuala Lumpur, Malaysia. 19th-20th December 2011. (Proceedings).