ROLE OF NIGELLA SATIVA OIL ON HISTOLOGICAL FEATURES AND ANDROGENICITY OF NICOTINE TREATED MALE SPRAGUE DAWLEY REPRODUCTIVE SYSTEM

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ABSTRACT

Tobacco is a plant from which nicotine, the alkaloid in cigarette is known to have detrimental effects on male reproductive system, can be sourced. As for *Nigella sativa* is a plant which has been used in culinary and also as traditional medicine since the antiquity by worldwide society. The remedial value of *Nigella sativa* has been attributed to its antioxidant properties. This study was conducted to investigate the protective effects of *Nigella sativa* oil on spermatogenic, leydig and sertoli cell count, histological features and androgenicity of nicotine treated male rat reproductive system. Thirty male *Sprague dawley* rats, aged 7 – 9 weeks, with 150 – 250g body weight were divided into five groups; saline (S), nicotine (N), corn oil (CO), *Nigella sativa* (NS) or nicotine-*Nigella sativa* (NNS). The S and N groups were intramuscularly (i.m.) injected with 0.1ml/100g saline and 0.5mg/100g nicotine, respectively. The CO and NS groups were force-fed with 0.1ml/100g corn oil and 6µl/100g *Nigella sativa* oil, respectively. The NNS group received both nicotine and *Nigella sativa* oil with similar dosage and mode of administration to the N and NS groups. The rats were then sacrificed after 100 days of treatment. The testes, seminal vesicles and prostate glands were extracted and fixed in 10% formalin solution prior to histological and immunohistochemical studies. The NS (34.98±2.12) and NNS (30.35±1.93) groups showed a significant higher number of spermatogonia compared to the N group ($p<0.05$). The number of spermatocytes (57.77±1.76) and spermatid (176.23±5.12) of the NS group were significantly higher compared to the N and NNS groups. In addition, a significant higher number of spermatozoa was observed in the NNS (69.20±3.02) compared to the N (46.22±2.68) and NS (60.22±3.34) groups. The cells counts of the NS group were significantly higher for Sertoli (20.83±0.87) and Leydig (42.77±1.77) cells than the N and NNS groups. The histoarchitecture of the testes, prostate gland and seminal vesicle were noted to be disrupted in the N group. However, it was found to be similar to the S group with co-

administration of *Nigella sativa* oil as seen in the NNS group. The NS and NNS groups also exhibited high intensity of staining for the androgen receptor in all of the tissues examined as opposed to the N group. In brief, this study suggested that nicotine caused damage to the male reproductive system while *Nigella sativa* oil was shown to have protective properties on the detrimental effects caused by the nicotine.

ABSTRAK

Tembakau adalah tumbuhan di mana nikotin, alkaloid di dalam rokok yang dikenalpasti mempunyai kesan buruk ke atas sistem pembiakan jantan. *Nigella sativa* adalah tumbuhan yang telah digunakan sebagai bahan masakan serta juga sebagai ubatan tradisional sejak zaman dahulu oleh masyarakat dunia. Nilai perubatan *Nigella sativa* telah disumbangkan oleh ciri-ciri antioksidannya. Kajian ini dijalankan untuk mengkaji kesan perlindungan minyak *Nigella sativa* ke atas bilangan sel spermatogenik, leydig dan sertoli, ciri-ciri histologi dan androgenisiti sistem pembiakan tikus jantan yang didedahkan kepada nikotin. Tiga puluh, tikus jantan *Sprague dawley*, berumur 7 – 9 minggu, dengan 150 – 250g berat badan dibahagikan kepada 5 kumpulan; salin (S), nikotin (N), minyak jagung (CO), *Nigella sativa* (NS) and nikotin-*Nigella sativa* (NNS). Kumpulan S and N, masing-masing disuntik secara intramuskular dengan 0.1ml/100g salin dan 0.5mg/100g nikotin. Kumpulan CO and NS, masing-masing disuap paksa dengan 0.1ml/100g minyak jagung dan 6µl/100g minyak *Nigella sativa*. Kumpulan NNS menerima kedua-dua nikotin dan minyak *Nigella sativa* dengan dos dan mod administrasi yang sama seperti kumpulan N and NS. Tikus-tikus kemudian dikorbankan selepas 100 hari rawatan. Testis, vesikel seminal dan kelenjar prostat diekstrak dan diawetkan di dalam 10% larutan formalin sebelum kajian histologi dan immunohistokimia. Kumpulan NS (34.98±2.12) dan NNS (30.35±1.93) menunjukkan bilangan sel spermatogonia yang bersignifikasi tinggi berbanding kumpulan N ($p<0.05$). Bilangan spermatosit (57.77±1.76) dan spermatid (176.23±5.12) bagi kumpulan NS bersignifikasi tinggi berbanding kumpulan N and NNS. Tambahan lagi, bilangan sel spermatozoa yang bersignifikasi tinggi diperhatikan di dalam kumpulan NNS (69.20±3.02) berbanding kumpulan N (46.22±2.68) dan NS (60.22±3.34). Bilangan sel untuk kumpulan NS adalah bersignifikasi tinggi untuk sel Sertoli (20.83±0.87) dan Leydig (42.77±1.77) berbanding kumpulan N dan NNS. Histoarkitektur testis, kelenjar prostat dan vesikel seminal adalah

terganggu di dalam kumpulan N. Walau bagaimanapun, ianya didapati sama seperti kumpulan S dengan pemberian bersama minyak *Nigella sativa* seperti yang diperhatikan di dalam kumpulan NNS. Kumpulan NS dan NNS juga menunjukkan intensiti pewarnaan yang tinggi untuk reseptor androgen di dalam kesemua tisu yang dikaji berbanding dengan kumpulan N. Secara ringkas, kajian ini mencadangkan bahawa nikotin mengakibatkan kerosakan pada sistem perbiakan jantan, manakala minyak *Nigella sativa* telah menunjukkan mempunyai ciri-ciri perlindungan pada kesan buruk yang diakibatkan oleh nikotin.

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LIST OF SYMBOLS AND ABBREVIATIONS

1^o	Primary
2^o	Secondary
^oC	Celcius
%	Percent
<	Less than
±	Plus minus
α	Alpha
β	Beta
µl	microlitre
µm	micrometer
µg	microgram
ABP	Androgen binding protein
AR	Androgen receptor
ART	Assisted reproductive technology
BPA	Bisphenol A
cm	Centimetre
CO	Corn oil
DAB	3,3’ diaminobenzidine
DHEA	Dehydroepiandrotesrone
DHT	Dihyrotestosterone
DNA	Deoxyribonucleic acid
DPX	Dibutylephtahlate polystyrene xylene
ER	Oestrogen receptor
g	Gram
GnRH	Gonadotrophin releasing hormone

H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
HRP	Streptavidin-horseradish peroxidase
i.m	Intramuscular
i.p	Intraperitoneal
ICSI	Inctracytoplasmic sperm injection
IUI	Intrauterine insemination
IVF	*In vitro* fertilisation
kg	Kilogram
LD_{50}	Lethal dose
LDL	Low-density lipoprotein
LH	Luteinising hormone
LPPKN	*Lembaga Penduduk dan Pembangunan Keluarga Negara*
M	Molar
m	Meter
MDA	Malionaldehyde
mg	Milligram
MIS	Mullerian inhibiting substance
ml	Millilitre
N	Nicotine
NNS	Nicotine-*Nigella sativa*
NS	*Nigella sativa*
OS	Oxidative stress
PBS	Phosphate buffer solution
pH	pH
PUFA	Polyunsaturated fatty acids

ROS	Reactive oxygen species
S	Saline
TBS	Tris buffered saline
TFR	Total fertility rates
TQ	Thymoquinone
Type Ad	Type A dark cell
Type Ap	Type A pale cell
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

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CHAPTER 1:
INTRODUCTION

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1.1 STATEMENT OF INFERTILITY

1.1.1 Worldwide Scenario

Family is defined as a group consisting of one or two parents and their children (Oxford Advanced Learner`s Dictionary of Current English, 2000) which is similar to how the public of our society understands. Thus, for most married couples, having a child is considered as the most desired goal to them virtually all across the world and cultures. However, this could be devastating news for married couples who dream of having a child, yet facing with infertility.

Layman understanding of infertility is unable to have babies or produce young (Oxford Advanced Learner`s Dictionary of Current English, 2000). The World Health Organisation (WHO) defines infertility as the inability of a couple to conceive after 12 months of regular, unprotected intercourse (WHO, 1995). American Society for Reproduction Medicine states that infertility is a disease defined by the failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse (Practice Committee of the American Society for Reproductive Medicine, 2008). A clinical definition for infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild *et al.,* 2009). Although, many studies gave different definitions for infertility, definition from WHO is the most recommended for both clinical and research studies (Larsen, 2005).

The global estimation for infertility is approximately 72.4 million couples (Boivin *et al.,* 2007) which affect approximately 10 – 15% of the reproductive-aged worldwide (Evers, 2002; Deka and Sarma, 2010; Saner-Amigh and Halvorson 2011).

In mid-1970s, Portugal, Spain, Italy and Greece were among the European countries with high fertility rate (Serbanescu *et al.,* 2004). However, for the past thirty years the European countries had experienced dramatic changes in their demographic, causing the total fertility rates (TFR) to decline below the replacement rate of 2.1 children per woman (Frejka and Sobotka, 2008).

Similar scenario can be seen in developing countries where the fertility problem is more prominent among countries that lie in the "infertility belt". These include Cameroon, Central African Republic, Gabon, Democratic Republic of the Congo, Togo, Sudan, Kenya and Tanzania, where one-third of couples are unable to conceive (Bowa and Kachimba, 2012). A similar scenario is also seen in developed countries, where the estimated prevalence of infertility ranges from 3.5 – 16.7%. As for the less-developed nation, it ranges from 6.9 – 9.3% (Boivin *et al.,* 2007).

1.1.2 Scenario in Malaysia

A similar trend has been reported in the WHO publication for the total fertility rate (TFR) in Malaysia where it had fallen from 3.6 in 1993 to 2.9 in 2003 (WHO, 2005). The former Malaysian Minister of Health, Yang Berbahagia Dato' Sri. Liow Tiong Lai had stated that infertility affected about 10 – 15% of couples in Malaysia which was similar to that reported in other developed countries including United Kingdom (UK) and the United State of America (USA) (*Setiausaha Akhbar Menteri,* 2011).

The World Fertility Data in 2008 indicated a constant decreasing trend in Malaysia total fertility rate (TFR) with 4.67 in 1970 to 2.43 in 2004 (United Nations, 2008). Another latest publication in the World Health Statistics indicated a drop in the total fertility rate in Malaysia from 3.7 in 1990 to 2.5 in 2009, respectively (WHO, 2011).

This data was supported by a survey conducted by National Population and Family Development Board (LPPKN), Malaysia. The survey showed that the TFR in Malaysia had declined from 3.4 in 1994 to 2.8 in 2004, a drop of 0.6 within 10 years (Indramalar and Wong, 2006). One of the local newspapers, Sin Chew reported the fertility rate in Malaysia continued its downward trend with 2.6 in 2000 to 2.2 in 2008 (MySinChew, 2010).

A statistical study was conducted by the Department of Statistic, Malaysia also indicated similar trend of fertility rate of 2.8 in 2001 to 2.2 in 2010. In 2001, the highest fertility rate was monopolised by the Malay ethic group, followed by other Bumiputera, Indians, and Chinese. However, over the years, the TFR for those ethnic groups showed a declining pattern except for others ethnicity group which showed an increment from 2001 to 2010 (Table 1.1) (Vital Statistics, 2010).

Table 1.1: Total fertility rate by ethnic groups in Malaysia from 2001 to 2010. (Adapted from Vital Statistics, 2010)

Ethnic group	Total fertility rate (per woman aged 15–49 years)									
	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010[p]
Total	2.8	2.6	2.5	2.4	2.4	2.3	2.3	2.3	2.3	2.2
Malay	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.8	2.8	2.6
Other Bumiputera	3.0	2.7	2.6	2.6	2.6	2.4	2.4	2.5	2.5	2.4
Chinese	2.2	2.1	2.0	1.9	1.9	1.8	1.8	1.8	1.7	1.5
Indians	2.4	2.2	2.2	2.1	2.0	1.9	1.9	1.9	1.9	1.7
Others[1]	1.3	1.0	1.2	1.4	1.3	1.4	1.5	1.7	2.0	2.0

[p] Preliminary figures

[1] Includes non-Malaysian citizens

Another study done by Zarinah (2011) reported a similar decline in the TFR. The author also stated that majority of Malaysian whom are Malay bumiputera registered as the highest TFR and played a significant role to the overall TFR in Malaysia. The TFR for Malay bumiputera had declined from 5.0 in 1970 to 4.5 in 1980 but later increased to 4.9 in 1985. The climbing up trend however did not last long, as it decreased again in 2010 with the TFR as low as 2.8. The TFR for Malaysian Chinese and Indian, on the other hand continuously showed a downward trend reaching 1.8 TFR for the Chinese and 2.0 TFR for the Indian in 2010 (Figure 1.1) (Zarinah, 2011).

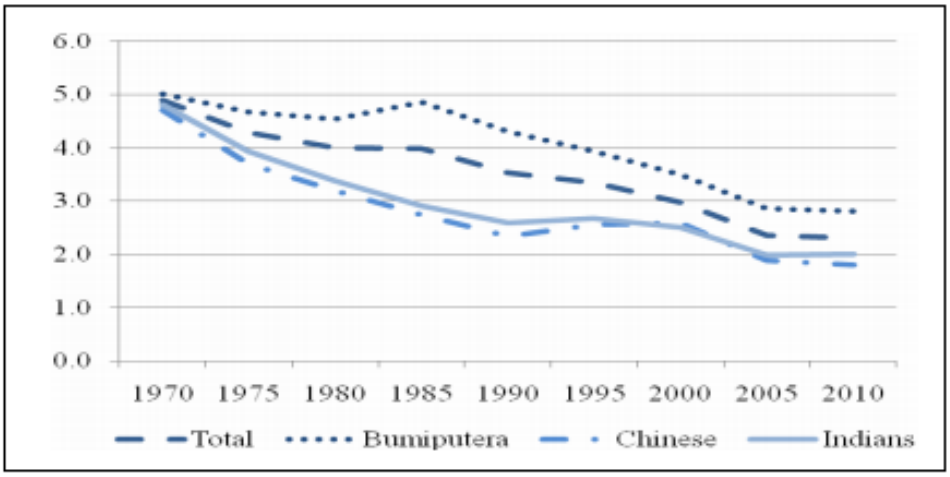

Figure 1.1: Total fertility rate by ethnic in Malaysia from 1970 to 2010. (Adapted from Zarinah, 2011).

1.1.3 Smoking and Male Infertility

Infertility can be further divided into primary and secondary infertility. Primary infertility indicates no prior pregnancies while the latter defines as infertility following at least one of prior conception (Saner-Amigh and Halvorson, 2011). Both male and female can contribute to infertility. However, half of the known cause for infertility is due to male factor (Oyeyipo *et al.*, 2011) with 25% of infertile men had idiopathic cause (Sharlip *et al.*, 2002; Marbut *et al.*, 2011).

Deterioration of reproductive health from male factor may be due to environmental, dietary, occupational and even lifestyle such as cigarette smoking (Kumar *et al.,* 2009). Cigarette smoking is widely recognised as health hazard and is a crucial risk factor for many diseases (Saleh *et al.,* 2002; Calogero, 2009) mainly lung cancer, coronary heart disease, respiratory disease and chronic obstructive pulmonary disease (Bonnie *et al.,* 2007).

According to the WHO (1997), one-third of world population aged more than fifteen years old smokes cigarette every day and approximately 5.4 million premature death are due to tobacco smoking worldwide (WHO, 2008). Moreover, studies showed that cigarette smoking could have grave effects on male reproductive health though less documented (Peate, 2005; Gaur *et al.,* 2010).

It has been estimated that more than 4000 chemicals constituents are present in cigarette smoke during combustion (Calogero, 2009; National Institute on Drug Abuse, 2012). Since, male reproductive system is highly sensitive and extremely vulnerable to both drugs legal (nicotine and ethanol) and illegal (opiates, cocaine and cannabinoids) (Sadeu *et al.,* 2010) thus smoking may cause detrimental effects to the reproductive system (Zenzes, 2000; Oyeyipo *et al.,* 2011).

The chemical compounds that are found in the cigarette smoke such as nitrosamines, heavy metal (cadmium) and alkaloid (nicotine) act differently on human reproductive system (Dechanet *et al.,* 2011; National Institute on Drug Abuse, 2012). Although, smoking is not always interchangeable with nicotine administration, but the toxicity effect of cigarette is often being correlated with nicotine content that is present in the cigarette (Iranloye and Bolarinwa, 2009). Previous studies that showed correlation

between nicotine and infertility where nicotine inhibited the luteinising hormone (LH) secretion in males (Funabashi *et al.,* 2005; Olayemi, 2010).

Besides, smoking also caused oxidative stress (OS) by interfering with the antioxidant balance by increasing the production of ROS (Fraga *et al.,* 1996; Kunzle *et al.,* 2003). High OS with low antioxidant capacity in seminal plasma may cause toxicity environment to the sperm resulting in oxidative damage (Yeni *et al.,* 2010). Study showed that cigarette smoking induced ROS causing reduction in both sperm quantity and quality (Hosseinzadeh Colagar *et al.,* 2007; Makker *et al.,* 2009). It was also shown that OS level in semen of smokers are significantly higher than those non-smoker men (Saleh *et al.,* 2002).

Previous studies showed that cigarette smoke caused detrimental effect such as oxidative DNA damage in sperm (Bosler and Wiczyk, 2010) as smoking increased the oxidant content and reduced antioxidant (Lanzafame *et al.,* 2009). Different study found that smoking also has negative effects on the male accessory sex gland, where the parameters assessed for seminal vesicle (total phosphate) and prostate gland (acid phosphatase) were decreased significantly in smokers (Pakrashi and Chatterjee, 1995). Moreover, another study showed that seminal mast cells in smokers were more abundant compared to non-smokers suggesting an indirect relationship between smoking and infertility (El-Karaksy *et al.,* 2007).

1.1.4 Alternative Treatment on Male Infertility

Infertility needs to be managed and treated, since infertility may indirectly lead to psychological stress affecting emotional and physically. Emotional stress such as depression, anxiety, marital problems and feeling worthless among parents are effects of infertility among partners (Deka and Sarma, 2010). Besides, the untreated infertility may cause partners to become more anxious which subsequently may lead to sexual dysfunction, as well as social isolation (Deka and Sarma, 2010). Though, women tend to show higher stress levels among infertile couple, men also showed an identical respond as women if infertility was associated with male factor (Deka and Sarma, 2010). Thus, treating men infertility is as important as treating fertility problem in women.

Men infertility can be treated either with modern technologies or alternative medicine. Nowadays, male infertility can be treated using modern technologies such as assisted reproductive treatment (ART) (Hsiao *et al.*, 2011). The ART main purpose in treating male infertility is to maximise the fertilisation probability by bringing male gametes closer to the female gametes (oocyte) allowing some functional shortfalls of sperm to be omitted (Tournaye, 2012; McLachlan and Krausz, 2012). The techniques used in treating male infertility using ART include intrauterine insemination (IUI), *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) programmes (Hsiao *et al*, 2011; Tournaye, 2012). Combined treatment of IVF and ICSI is carried out in order to elevate the success rate of fertilization (Tournaye, 2012).

Assisted reproductive treatment (ART) is generally safe (Macaluso *et al.*, 2010). However, discontinuation of treatment among patient was most likely due to poor prognosis or unaffordable treatment cost (Macaluso *et al.*, 2010; Boivin *et al.*, 2012). Thus, providing ART to treat infertility may be challenging for health centers in low-

income society, leaving the infertility untreated (Safarinejad and Safarinejad, 2012). Moreover, study had indicated that certain lifestyles such as smoking might also decrease the success of ART treatment (Stewart, 2006).

Though modern treatments are widely accessible nowadays, herbal medicines still remain as an option for alternative treatment for male infertility especially in developing and undeveloped countries (Safarinejad and Safarinejad, 2012). This is due to its antiquity history in improving health and curing diseases as well as its cultural values (Mukherjee *et al*., 2010, Yan *et al.*, 2008). Moreover, herbal medicine is easily accessible especially in countries that have wide variety of plant species including the well-developed traditional medicinal systems (Afsana *et al.*, 2011). The undying popularity of herbal medicine is also attributed to the unlimited components presence in the herbal medicinal that have wide range of bioactivities (Yan *et al.*, 2008).

Rai *et al.* (2000) quoted on the report by World Health Organization (WHO) that approximately 4000 million people in developing countries consumed herbal medicine frequently and accredited its efficiency. Another study showed that approximately 64% of total global population still dependent on herbal medicine as their healthcare need (Cotton, 1996). Some example of medicinal herbs that are used to treat fertility problems are the seeds of *Peganum harmala* which are recorded to enhance sexual activities and *Raphanus sativus* which are recommended in treating infertility both male and female (Alzweiri *et al.*, 2011). Other herbs that are recommended due to its capability in treating sexual weakness include *Trigonell foenum-graecum, Salvia triloba* and also *Nigella sativa* (*Habbatus sauda*) (Oran and Al-Eisawi, 1998; Alzweiri *et al.*, 2011).

Nigella sativa was chosen for this study due to its antioxidant capacity and ability to significantly reduce free radicals released by the nicotine. It was manifested in previous study that administration of *Nigella sativa* showed improvement in the spermatogenesis with increased sperm count and hormone production (Paradin *et al.*, 2012; Menezo *et al*., 2014a; Menezo *et al.,* 2014b). Therefore, in current study, the ameliorating effects of *Nigella sativa* was tested on nicotine treated male rat testes, seminal vesicle and prostate gland. Testis were selected as it to evaluate the effects of treatment on the spermatogenesis. Since seminal vesicles and prostate gland are extremely sensitive to androgen levels, changes on the histoarchitecture and presence of androgen receptor can also help to indicate the effects of treatment.

1.2 THESIS OBJECTIVES

1.2.1 General Objective

The general objective of present study was conducted to investigate the protective effect of *Nigella sativa* oil on nicotine treated male *Sprague dawley* reproductive system.

1.2.2 Specific Objectives

The present study was conducted on *Sprague dawley* rats:

1. To observe the effects of *Nigella sativa* oil on histological features of the testes, seminal vesicle and prostate gland of the nicotine treated male rats.
2. To determine the effects of *Nigella sativa* oil on spermatogenic, Leydig and Sertoli cells counts of the testes in nicotine treated male rats
3. To observe the effects of *Nigella sativa* oil on presence of androgen receptor in the testes, seminal vesicle and prostate gland of the nicotine treated male rats.

CHAPTER 2:
LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 MALE REPRODUCTIVE SYSTEM

Reproduction involves an extensive range of physiological processes with association of behaviours and anatomical structures which are vital to ensure the birth of the next generation of species in humans, domestic, wild, as well as in laboratory vertebrates.

The male reproductive system consists of testes, ductus (vas) deferens, epididymis, accessory ducts and glands, and penis (Starr and Mcmillan, 2010). Its function generally includes production, nourishment and temporary storage of haploid male gametes (spermatozoa) via spermatogenesis, introduction of semen containing spermatozoa into female genital system and production of androgens and oestrogen through steroidogenesis (Stevens and Lowe, 2005).

2.2 TESTES

2.2.1 Structure

A mature adult testis is a solid oval-shaped organ at approximately 4cm long and 2.5cm width in size. Its location in the scrotum enables its temperature to be maintained at about 2 – 3^{o}C below body temperature (Stevens and Lowe, 2005; Saladin, 2008; Marieb and Hoehn, 2010). Each testis weighs approximately 11 – 17g with the right testis generally slightly larger and heavier as compared to the left testis (Stevens and Lowe, 2005).

It is surrounded by tunica vaginalis which is a saccular extension of the peritoneum. Underneath the tunica vaginalis, is tunica albuginea which forms the white fibrous capsule of the testis (Figure 2.1) (Stevens and Lowe, 2005; Saladin, 2008; Marieb and Hoehn, 2010). Tunica albuginea is thickened posteriorly assembling mediastinum of

the testis from which fibrous septa penetrate into the testis and divide into approximately 200 – 300 wedge-shaped lobules. Each testicular lobule contains one to four tightly-coiled seminiferous tubules where sperm are being produced (Stevens and Lowe, 2005; Saladin, 2008; Marieb and Hoehn, 2010).

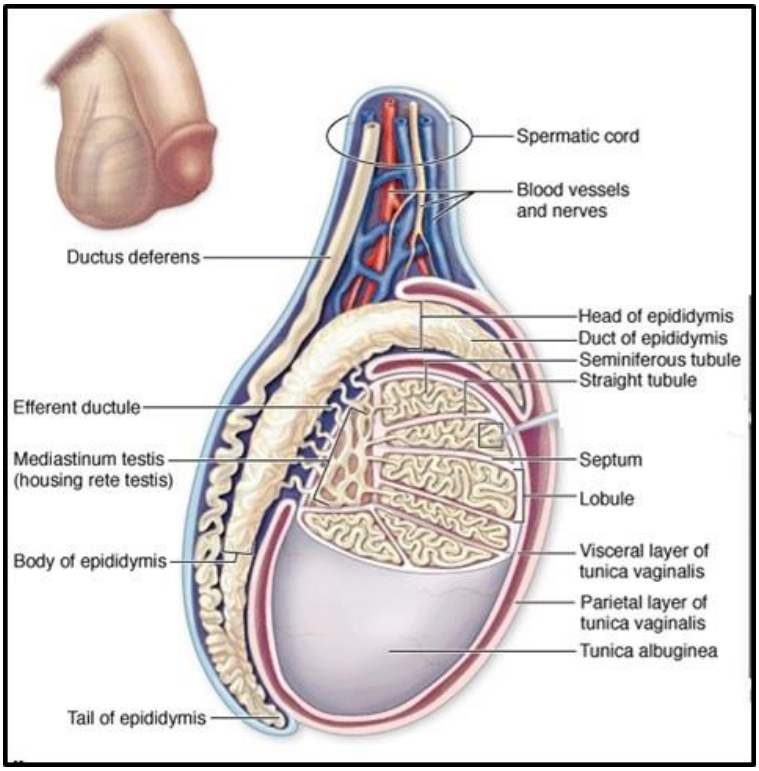

Figure 2.1: Structure of the testis. (Adapted from Marieb and Hoehn, 2010).

2.2.2 Seminiferous Tubules

Seminiferous tubules are long, convoluted tubules in the testes and each is measured approximately 70cm long and 150µm in diameter. The combined length of the tubules in a single testis is about 300 – 900m (Stevens and Lowe, 2005; Saladin, 2008). In a sexually matured male adult, each seminiferous tubule is lined by germinal epithelium. The epithelium contains two types of cells which are proliferating spermatogenic cells that develop into sperm and non-proliferating supporting cells which are known as sustentacular (Sertoli) cells (Eroschenko, 2008). The Sertoli cells are columnar cells

found scattered among the spermatogenic cells lining the tubules (Stevens and Lowe, 2005).

Surrounding the seminiferous tubules are three to five layers of myoid cells or myofibroblast which are smooth muscle-like cells (Stevens and Lowe, 2005). Blood vessels, loose connective tissue cells and interstitial (Leydig) cells are found in the interstitial spaces between the adjacent seminiferous tubules (Eroschenko, 2008).

2.2.3 Function

Two main functions of the testes are production of male gamete in the seminiferous tubule and secretion of testosterone by the interstitial cells (Leydig cells) (Mescher, 2010). The process of producing male gamete is called spermatogenesis and it is characterised by sequential processes involving spermatocytogenesis, meiosis and spermiogenesis (Eroschenko, 2008; Mescher, 2010).

Spermatogenesis starts during puberty with production of primitive germ cell known as spermatogonium. The cell is a rather small rounded cell with a diameter of approximately 12µm and is found in the epithelium adjacent to basement membrane (Mescher, 2010). The spermatogonium undergoes several mitotic division processes to produce replacement stem and other spermatogenic cells (Eroschenko, 2008). Ultimately, three types of spermatogonia cells are produced and grouped according to their nuclear appearances which are type A dark (Ad) cell, type A pale (Ap) cell, and type B cell (Figure 2.2) (Stevens and Lowe, 2005).

Ad cell is known as the precursor cell which undergoes mitosis to produce new Ad cells and some Ap cells. The Ap cells will produce type B cells from a mitosis

division. Type B cells undergo several steps of maturation to produce primary spermatocytes which subsequently mark the end of spermatocytogenesis (Stevens and Lowe, 2005).

Primary spermatocytes are the largest cells found in the spermatogenic lineage and these cells are characterised based on presence of partially condensed chromosomes (Figure 2.3) (Mescher, 2010). Compared to spermatogonia, primary spermatocytes are not in contact with the basement membrane of the seminiferous tubules (Stevens and Lowe, 2005). Primary spermatocytes enter a prophase phase of meiosis. It is the longest phase that occurs about 22 days before forming into diploid secondary spermatocytes. Within few hours, the secondary spermatocytes undergo a second meiotic division giving rise to haploid spermatids before the latter develop into spermatozoa (Stevens and Lowe, 2005; Mescher, 2010).

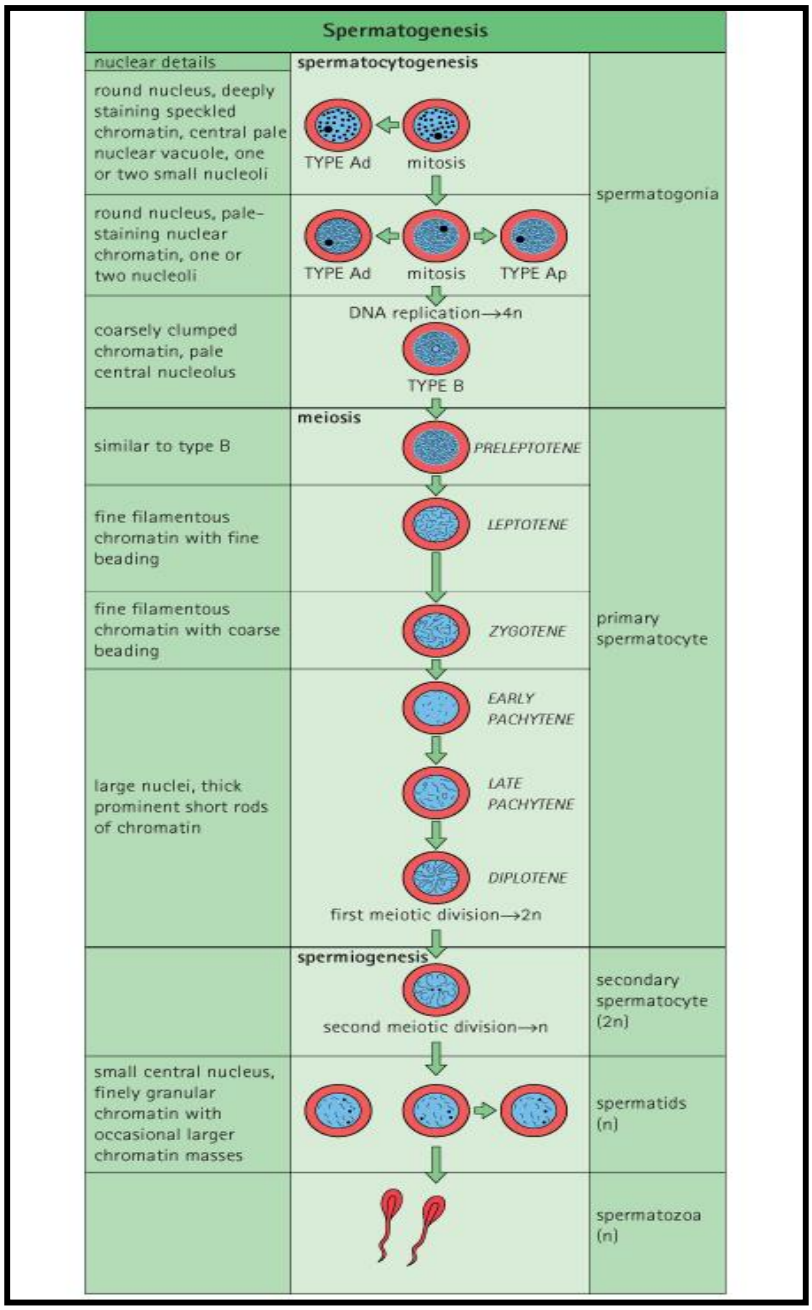

Figure 2.2: Clonal nature of spermatogenesis. Spermatocytogenesis begins with development of spermatogonia where three types of cells are recognised as type A dark (Ad) cells, type A pale (Ap) cells and type B cells. (Adapted from Stevens and Lowe, 2005).

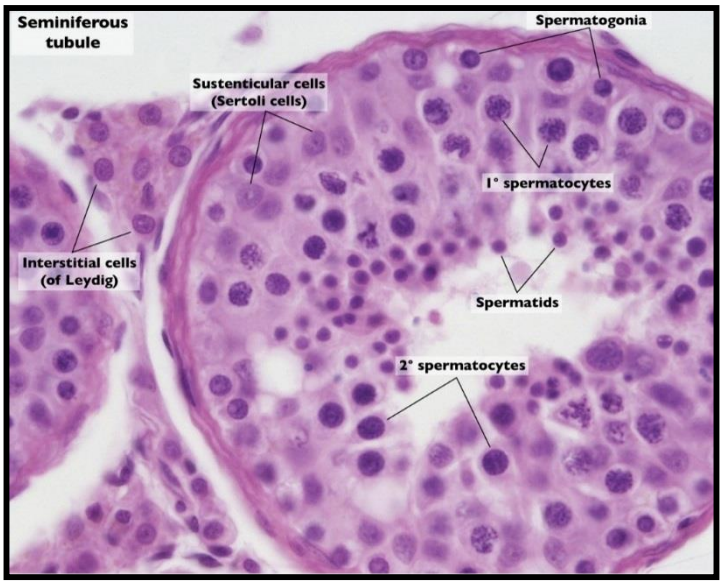

Figure 2.3: Testicular cells. (Adapted from http://rowdy.msudenver.edu/~raoa/rao/docs/Seminiferous-tubules-testis.jpg).

Sertoli cells are named after a scientist, Enrico Sertoli who first demonstrated the function of the cells (Mescher, 2010). The cells act as supporting cells, where each of it is able to support up to 30 – 50 germ cells at different stages of development. In addition, it also provides protection to the germ cells. Together with the myoid cells, Sertoli cells formed a barrier called 'blood-testis barrier' or 'Sertoli cell barrier' which prevents free exchange of large molecules between the blood and intercellular fluid within the seminiferous tubules from entering the germ cells (Saladin, 2008). Sertoli cells also carried out other specific functions which include: 1) providing nutrition for the developing spermatogenic cells; 2) phagocytosis of residual cytoplasm of the spermatids and 3) exocrine and endocrine functions (Mescher, 2010).

The exocrine function of Sertoli cells is to secrete androgen-binding protein (ABP) under influence of follicle stimulating hormone (FSH) which concentrates testosterone (Stevens and Lowe, 2005; Eroschenko, 2008; Mescher, 2010). In endocrine manner, Sertoli cell secretes inhibin which plays a part in the feedback loop in suppressing

the synthesis and release of FSH. In the male embryo, during the 8th and 9th week of foetal development, Sertoli cell secretes mullerian inhibiting substance (MIS) to suppress further development of embryonic mullerian (paramesonephric) ducts. Absence of MIS may cause to mullerian ducts formation to persist and subsequently forms parts of female reproductive system (Stevens and Lowe, 2005; Mescher, 2010).

Leydig cells are endocrine cells (Stevens and Lowe, 2005; Saladin, 2008; Marieb and Hoehn, 2010). It produces testosterone, male hormone pivotal in the development of secondary male sex characteristics, spermatogenesis and accessory gland function (Stevens and Lowe, 2005; Eroschenko, 2008; Mescher, 2010). Testosterone synthesis starts during puberty simultaneously with the secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus. Testosterone secretion by Leydig cell is prompted by luteinising hormone (LH) (Figure 2.4) (Mescher, 2010).

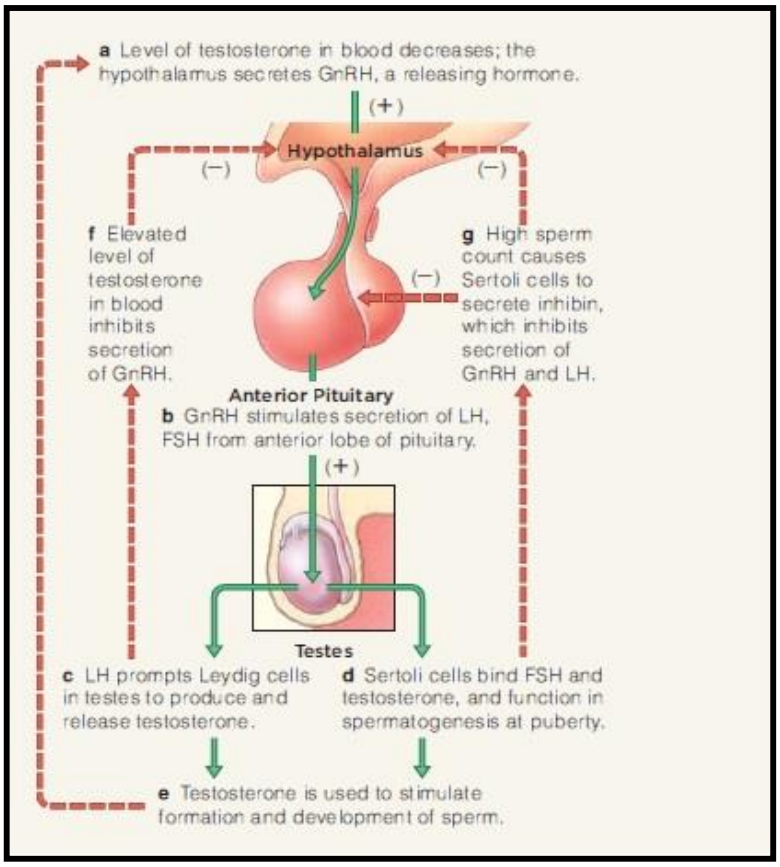

Figure 2.4: Negative feedback loops regulate the release of male reproductive hormones. (Adapted from Starr and McMillan, 2010).

2.3 PROSTATE GLAND

2.3.1 Structure

The prostate gland is one of the accessory glands in the male reproductive system. It is a dense secreting gland surrounding the urethra inferior to the bladder (Saladin, 2008; Mescher, 2010; Marieb and Hoehn, 2010). The diameter of prostate gland is approximately 2x3x4cm and its weight is approximately 20g (Saladin, 2008; Mescher, 2010).

It is made up of 30 – 50 tubuloalveolar glands that are embedded in a supporting dense fibromuscular stroma (Figure 2.5) (Saladin, 2008; Mescher, 2010; Marieb and Hoehn, 2010). Epithelial lining of the tubuloalveolar glands varies from simple or

columnar to pseudostratified epithelia and is supported by lamina propria (Stevens and Lowe, 2005; Eroschenko, 2008; Mescher, 2010).

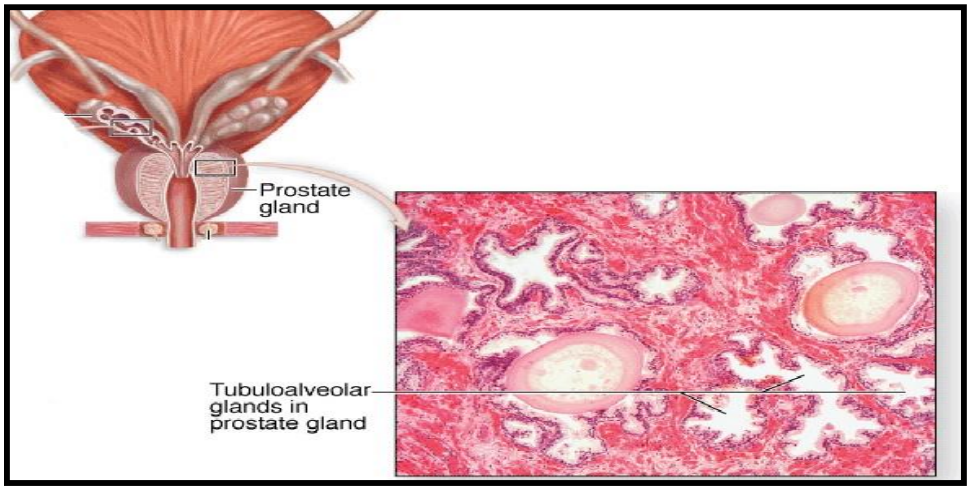

Figure 2.5: Photomicrograph shows the characteristic of individual tubuloalveolar glands of the prostate. (Adapted from Mescher, 2010).

The prostate gland is enclosed in a connective tissue capsule which penetrates into the gland as septa dividing the gland into indistinct lobes (Stevens and Lowe, 2005; Mescher, 2010; Marieb and Hoehn, 2010). In rodents, the prostate gland consists of dorsal, ventral and lateral lobes (Hayashi *et al.*, 1991; Favaro and Cagnon, 2006). The location of the dorsal lobe can be found inferior and posterior to the urinary bladder, which is below and behind to the attachment of both the seminal vesicles and coagulating glands (Hayashi *et al.*, 1991). The ventral lobes are located anterior to urethra just below the urinary bladder while the lateral lobes are located immediately below both seminal vesicles and coagulating glands (Figure 2.6) (Tlachi-Lopez *et al*., 2011).

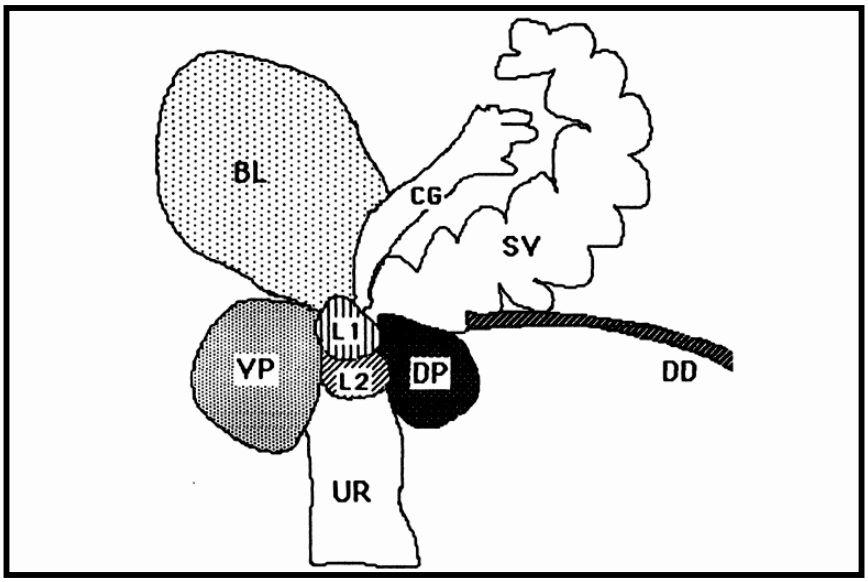

Figure 2.6: Schematic lateral view of the rat male pelvic organ. CG: coagulating gland, VP: ventral prostate, L1: lateral type 1 prostate, L2: lateral type 2 prostate, DP: dorsal prostate, BL: urinary bladder, SV: seminal vesicle, DD: ductus deferens, UR: urethra. (Adapted from Hayashi *et al.,* 1991).

2.3.2 Function

Prostatic secretion makes up about 75% of the seminal fluid and is slightly acidic (pH 6.6) (Kumar and Majumder, 1995). It is rich in citric acid and hydrolytic enzymes, especially fibrinolysin enzyme. Fibrinolysin helps to liquefy coagulated semen after it had been deposited in the female genital tract. Furthermore, albumin in prostatic secretion facilitates and enhances sperm motility while acid phosphates are involved in providing the nutrition of spermatozoa (Walsh *et al.,* 1992). Moreover, the prostatic zinc acts as antibacterial agent in the seminal fluid (Fair and Wehner, 1976).

2.4 SEMINAL VESICLE

2.4.1 Structure

Another male secondary sex organ is the seminal vesicle which is an elongated gland located on the posterior side of bladder (Eroschenko, 2008; Akinsola *et al.,* 2012). They

are highly convoluted glands of approximately 10 – 15cm long (Mescher, 2010; Akinsola *et al.,* 2012). The excretory duct of seminal vesicle adheres to the ampulla of vas deferens and forms ejaculatory duct which enters the prostate gland (Eroschenko, 2008; Akinsola *et al.,* 2012).

The lumen of seminal vesicle is lined by thin and complex mucosal lining. The mucosal layer is made up of columnar epithelial cells that are supported by fibroelastic lamina propria and surrounded by circular and outer longitudinal smooth muscle layers (Figure 2.7) (Stevens and Lowe, 2005; Eroschenko, 2008; Mescher, 2010).

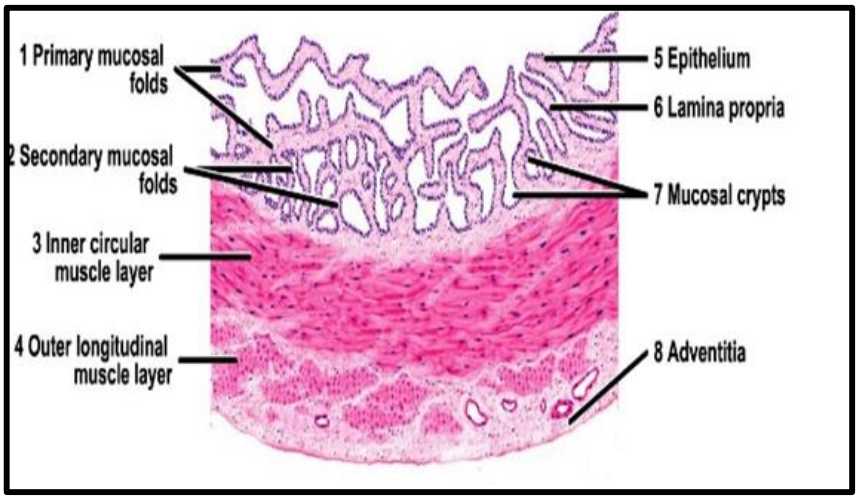

Figure 2.7: The seminal vesicle is surrounded with muscle layers. (Adapted from Eroschenko, 2008).

2.4.2 Function

The seminal vesicles are essential in assisting the male fertility processes (Gonzales, 2001). It is an androgen-dependent sex gland that produces and secretes approximately 50 – 80% of seminal plasma during ejaculation (Kierszenbaum, 2002; Kim *et al.,* 2009; Noorafshan and Karbalay-Doust, 2012).

The secretion from the seminal vesicle contains amino acids, citrate, enzymes, flavins, fructose, phosphorylcholine, proteins, vitamin C and prostaglandins (Pang *et al.,*

1979; Gonzales, 2001; Thomson and Marker, 2006). This alkaline secretion helps to neutralise the acidity of vaginal tract, subsequently expanding the lifespan of sperm (Thomson and Marker, 2006; Akinsola *et al.,* 2012). Seminal vesicle secretion in semen also helps to raise the stability of sperm chromatin (Noorafshan and Karbalay-Doust, 2012). Besides, spermatozoa obtain their main energy source from the fructose found in the seminal secretion (Thomson and Marker, 2006; Noorafshan and Karbalay-Doust, 2012). Presence of prostaglandins in the seminal secretion also helps to prevent any immune response in the female reproductive tract towards the semen (Pang *et al.,* 1979; Gonzales, 2001).

2.5 NICOTINE

Nicotine is an alkaloid found in the tobacco plant. Its biosynthesis occurs in the plant's root while the accumulation takes place in its leaves. Nicotine constitutes about 0.6 – 3.0% of the tobacco dry weight (Egesie *et al.,* 2013). Each cigarette was reported to contain approximately 10 – 14mg of nicotine (Carballosa, 2012). In average smoker takes in 1 – 2mg of nicotine by inhaling the tobacco smoke (National Institute on Drug Abuse, 2012). At this low concentration, the absorbed nicotine acts as stimulant. The stimulant's effect is the main reason for its dependency. On the other hand, at a high concentration, which is approximately at 30 – 60mg, it can be fatal (Egesie *et al.,* 2013).

2.5.1 Effects of Nicotine

Nicotine is known to be highly addictive where studies show that it is among the hardest addiction to break as it provides pleasure (Jensen *et al.,* 1991; National Institute on Drug Abuse, 2012; Egesie *et al.,* 2013)**.** On the other hand, the detrimental effects of nicotine towards the smokers are well-recorded. Tobacco smoking is known as the main reason for mortality and morbidity (Peto *et al.,* 1992). Statistically, 50% of smokers from both

gender had increased risk for diabetes mellitus. Smokers were more at risk to suffer type 2 diabetes as they exhibited insulin resistance syndrome including higher serum free fatty acids and triglycerides levels, lower high-density lipoprotein (HDL) cholesterol, and higher number of atherogenic small dense low-density lipoprotein (LDL) particles and fibrinogen levels (Facchini *et al.,* 1992; Eliasson *et al.,* 1997; Eliasson, 2003).

Nicotine was also highlighted to cause free radical generation not only in the rodent species but also in different types of human cells (Campain, 2004). This will then promote an increase in the amount of reactive oxygen species (ROS) resulting in oxidative stress (Yildiz *et al.,* 1998; Bandopadhyay *et al.,* 2008; Sudheer *et al.,* 2008).

Besides, nicotine was suggested to cause reduction in body weight as it also acted as an appetite suppressor (Grunberg *et al.,* 1984; Crisp *et al.,* 1999; Genn *et al.,* 2003). This was in compliment with a finding that withdrawal from consuming nicotine would lead weight gain as a result of a decrease in metabolism and an increase in appetite. Nicotine consumption was also been recorded to have positive effect on the learning and memory. However, this latter finding was only obtained from the animal studies. (Yildiz, 2004).

2.5.2 Nicotine and Infertility

Studies had shown that nicotine caused severe detrimental effect on the function of male reproductive system (Ghaffari *et al.,* 2009; Oyeyipo *et al.,* 2010; Sadeu *et al.,* 2010). Dharwan and Sharma (2002) showed that chronic nicotine consumers grieved due to impotence, loss of libido, premature or delayed ejaculation, infertility and complication with penile erection.

Nicotine was recorded to have effect on the spermatogenesis (Rajpurkar *et al.,* 2002). This was confirmed with findings that showed presence of defects on the sperm including low sperm count, abnormal sperm shape, impaired sperm motility and sperm maturation which suggested an early potential of infertility (Dharwan and Sharma, 2002; Jorsaraei *et al.,* 2008).

The abnormal spermatogenesis is thought to be related to the decrease in serum level of testosterone in alliance with the administration of nicotine (Dharwan and Sharma, 2002; Sarasin *et al.,* 2003; Oyeyipo *et al.,* 2010). It has been suggested that the diminishing level of testosterone would indirectly affect the spermatogenesis process as testosterone was responsible in providing stimulus to initiate spermatogenesis (Ojeda and Urbanski, 1994).

Besides, nicotine administration was also noted to inhibit the release of follicle stimulating hormone (FSH) and luteinising hormone (LH) from the pituitary which would lead to the negative effect on the reproductive system as both hormones are pivotal in ensuring the negative feedback of testosterone secretion regulation (Blake, 1974; 1978).

The nicotine administration would also have detrimental effects on the sex accessory glands as development and maintenance of the glands are influenced by the androgen. Nicotine was shown to alter the androgenic action in the prostatic tissue which led to atrophy of the gland (Meikle *et al.,* 1988; Reddy *et al.,* 1998). However, there was lack of reports on the effects of nicotine on seminal vesicle.

2.6 *NIGELLA SATIVA* (*HABBATUS SAUDA)*

Nigella sativa (NS) is an annual flowering herbaceous plant, belongs to the *Ranunculaceae* family while *Nigella* is its genus (Mozaffarian, 1998; Swamy and Tan, 2001; Dwivedi, 2003). The taxonomy group of NS was first identified and described by Linnaneus in 1753 (Table 2.1) (Kahsai, 2002).

Table 2.1: Taxonomy group of *Nigella sativa*

Kingdom	Plantae
Division	Magnoliophyta
Order	Ranunculales
Family	Ranunculaceae
Genus	Nigella
Spesies	Sativa

Nigella sativa (NS) also known as the Black Cumin, Black Seeds, Black Caraway, 'Blessed Seed' (or Habbatul-ul-Baraka) in Arabic, Syuwainiz' in the Persian language and Black Kammum (or India Kammum) by the Indians (Gray, 2013; Rifqi, 2012). The fruit of these dicotyledons is a capsule that made up of several united follicles containing numerous white trigonal seeds (Schleicher and Saleh, 1998; Goreja, 2003; Warrier *et al.*, 2004). As the capsule is opened when the fruit ripens, the previously white colored seeds turn into black (Figure 2.8) (Schleicher and Saleh, 1998; Gray, 2013).

Figure 2.8: The seeds of *Nigella sativa.* (Adapted from Aftab *et al.,* 2013; Sharma *et al.*, 2009).

2.6.1 Importance of *Nigella sativa*

Dating back to approximately 2000 years ago, *Nigella sativa* (NS) had been extensively consumed traditionally by people around the world as spices, carminative, condiments, natural food addictives and aromatic. It was also consumed with honey and used in cakes, breads, pastries, curries, pickles and seasoning (Gali-Muhtasib *et al.*, 2006; Salem, 2005; Mathur *et al.*, 2011).

Nigella sativa (NS) was also traditionally used as an alternative medicine, as herb or pressed oil for respiratory, stomach, and intestinal health. It is well known for its diuretic, analgesic, anti-inflammatory, anticonvulsant, antidiabetic, anticancer and antioxidant properties and was claimed to improve kidney and liver functions (Anwar, 2005; Sharma *et al.*, 2009; Mathur *et al.*, 2011).

In the five volume text of 'The Canon of Medicine' written by Ibnu Sina or else known as Avicenna in the West, it was stated that NS could stimulate the body energy in recovery from fatigue and dispiritedness (Paarakh, 2010; Rifqi, 2012). *Nigella sativa* was also listed as a drug in the natural drugs categories in a book called 'Al-Tibb Al-Nawawi' (Medicine of the Prophet Muhammad). It was recommended by the Prophet Muhammad (prayers and peace be upon him) as the medicine for every disease except death which explained the extensive used of NS among the moslem as medication for ages (Al-Bukhari, 1976; Ilaiyaraja and Khanum, 2010; Hajra, 2011). In addition, NS was also used as remedy in the Unani Tibb and Indian system of medicine as stimulant and antihelmintic (The Ayurdevic Formulary of India, 1978; Warrier *et al.*, 2004).

Studies also recorded that using the combination of NS seed decoction with some sweet oil forms could also be beneficial in treating skin diseases (Evans, 1996). It could

remove hand and feet swellings when brayed in water. It was also useful in treating leucoderma, alopecia, eczema, freckles and pimples by external usage (Usmanghani *et al.*, 1997).

2.6.2 Biochemical Constituents in *Nigella sativa*

The biochemical constituents of *Nigella sativa* (NS) seed is extremely complex with approximately 20% proteins, 38% fixed oils, 0.5 – 1.6% of volatile oils and about 6.5% trace substances such as amino acids, reducing sugar, alkaloids, saponin, crude fiber, similarly minerals including calcium, iron, sodium, potassium, copper and zinc (Duke, 1992; Al-Gaby, 1998).

The main active constituent in the volatile oil of NS is thymoquinone (TQ, 2-iso-propyl-5-methyl-1, 4-benzoquinone) which had been recorded to exhibit strong antioxidant properties (Mahmood *et al.*, 2004; Salem, 2005; Al-Ali *et al.*, 2008). Besides, it also has other pharmacological effects such as antibacterial, diuretic and antihypertensive (Hailat *et al.*, 1995; Medenica *et al.*, 1997; Swamy and Tan, 2000).

Apart from the TQ, nigellone, is another active component present in NS. It was reported to have an antimicrobial effect whereby it could increase the production of interleukin-3 and 1β which has an impact on macrophages (Hanafi *et al.*, 2005; Zaher *et al.*, 2008).

2.6.3 *Nigella sativa* and Fertility

The high percentage of unsaturated fatty acids contained in NS was thought to be beneficial in reproductive health (Ali and Blunden, 2003; Taskin *et al.*, 2005). Previous study showed that animals fed with oil diet rich in polyunsaturated fatty acids have

positive effect on the reproductive function whereby preventing the reduction of sperm in chicken (Surai *et al.,* 2000). Similar finding was also found in another study using male turkeys which were given rich unsaturated fatty acid diet. It was concluded that the fatty acid could help to sustain the reproductive capacity in older age (Blesbous *et al.,* 2004; Bashandy, 2007).

The fatty acid in NS also has positive effect on the androgen metabolism. The highest level of androgen plasma level was found in the experimental male rats given unsaturated fatty acids diet (Gromadzka-Ostrowska *et al.,* 2002). The fatty acids was claimed to stimulate the activity of 17 β-hydoxysteroid dehydrogenase which is the key enzyme in testosterone synthesis pathway affecting the metabolism and steroid secretion in the testis (Gromadzka-Ostrowska *et al.,* 2002; Al-Sa'aidi *et al.,* 2009). However, this finding of increased level of testosterone was contrary to findings by Datau *et al.* (2010) whom found no significant increase in the free testosterone level after NS consumption.

Many studies showed that rats treated with NS through oral administration had improved their reproductive efficiency, seminal vesicle weight, prostate gland weight, testosterone level, sperm motility and sperm quality (Bashandy, 2007; Al-Sa'aidi *et al.,* 2009; Ghlissi *et al.,* 2012). The ameliorating effects in the testicular weight and size, similarly epididymal caudal sperm parameters had also been recorded with a co-administration of NS and cadmium chloride (Al-Mayali, 2007; Al-Sa'aidi *et al.,* 2009). These were concluded to be due to the presence of proteins, vitamins (Vitamin A, B and C) and vital minerals (zinc, copper and magnesium) in the seed of NS (Al-Okbi *et al.,* 2000; Ahlobom *et al.,* 2001; Kanter *et al.,* 2005).

Nigella sativa (NS) oil and thymoquinone (TQ) were also beneficial in inhibiting membrane lipid peroxidation (Hosseinzadeh *et al.,* 2007; Zaher *et al.,* 2008). The TQ by itself showed a positive effect in inhibiting non-enzymatic lipid peroxidation in liposomes which might have a positive effect on infertility (Houghton *et al.,* 1995). *Nigell sativa* (NS) had been shown as a better antioxidant compared to vitamin C by reducing the level of malionaldialdehyde (MDA) and increased the antioxidant level in tissue (Zaher *et al.,* 2008).

2.7 ANDROGEN RECEPTOR AND FERTILITY

Androgen is a steroid hormone pivotal to ensure normal sexual development, determine gender-specific adult male phenotypes and secondary male traits and maintain the function of the male reproductive system (Brinkmann, 2011; Sampson *et al.*, 2013; Chang *et al.,* 2013). Besides, it is also critical for development, growth and function of the prostate gland and seminal vesicle (Mata, 1995; Bianco *et al.,* 2002; Risbridger *et al.,* 2003).

In men, the biosynthesis of androgen involves a two-step process where testosterone, the major circulating androgen is synthesised largely (95%) by the Leydig cell of the male gonads (testes) (Welsh *et al.,* 2010; Alwyn Dart *et al.,* 2013; Hiort, 2013). The synthesis of testosterone is stimulated by luteinising hormone (LH), while it is controlled by gonadotrophin-releasing hormone (GnRH) of the hypothalamus (Davison and Bell, 2006).

The less potent androgen (5-20%) such as dehydroepiandrosterone (DHEA) which is produced by adrenal gland can also be converted into testosterone. Its secretion from the adrenal gland is controlled by adrenocorticotropic hormone from the anterior

pituitary and regulated by cortocotrophin-releasing factor from hypothalamus (Taplin and Ho, 2001; Davison and Bell, 2006).

Androgen exhibit its effects by binding to androgen receptor (AR). Androgen receptor (AR) is a type of nuclear receptor gene superfamily which acts as ligand-dependent transcription factor with two native ligands, resulting androgen-responsive gene transcription in target cell (Lu *et al.,* 2006; Heemers and Tindall, 2007; Patrao *et al.,* 2009). Although AR is expressed in many tissues, its highest level was observed in the male reproductive organs such as efferent ductules, urogenital sinus, Wolffian ducts, epididymides, ductus deferens, seminal vesicles, coagulating glands, prostate and bulbouretheral glands since embryonic day 13 till postnatal day 10 (Cooke *et al.,* 1991; Mckenna *et al.,* 2009).

Androgen activates its effect through two main paths; i) genomic action and ii) non-genomic mechanism. However, the effects of androgen are mostly activated via the former mechanism which involves the binding of androgen to the AR (Figure 2.9) (Heinlein and Chang, 2002; Foradori *et al.,* 2008). The activation of AR is mediated by dihydrotestosterone (DHT) which is converted from its precursor, testosterone by 5α-reductase enzyme (Taplin and Ho, 2001). Dihydrotestosterone (DHT) which is a 100-fold more potent than its precursor (Mitchell, 2012), binds to the AR rather than testosterone because it has higher affinity towards the AR. It slowly dissociates from the receptor thus protect the receptor from proteolytic degradation (Kicman, 2008; Datta and Tindall, 2013).

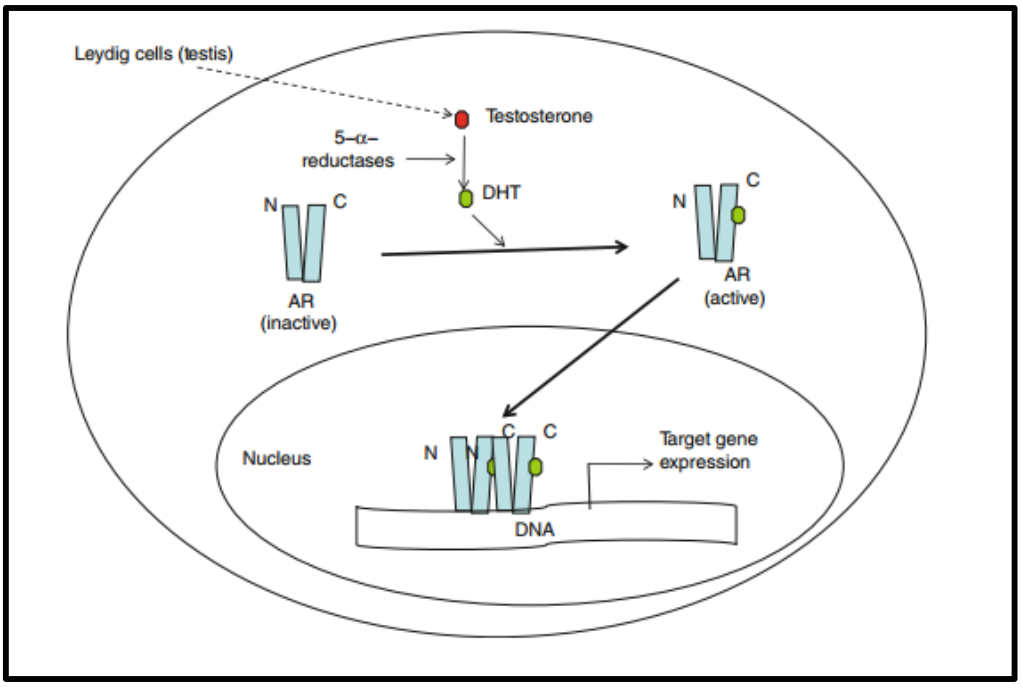

Figure 2.9: Androgen receptor (AR) activation. (Adapted from Datta and Tindall, 2013).

The male gonad, testis, which produces sperm via spermatogenesis is highly dependent on the androgen action (Tsai *et al.*, 2006; Wang *et al.*, 2009; Stanton *et al.*, 2012). Androgen receptor (AR) protein of the mouse testis was expressed in the Leydig, myoid and Sertoli cells (Zhou *et al.*, 2002). Studies showed that deficiency in selective AR knockout of testicular somatic cells rats resulted in spermatogenic defects (Holdcraft and Braun, 2004; Chang *et al.*, 2004; De Gendt *et al.*, 2004).

In addition, study showed that compounds contain high anti-androgenic effect such as bisphenol A (BPA) would compete with DHT in binding to the androgen receptor (AR). Thus, exposure to BPA could reduce the AR response of Sertoli cell towards testosterone signal leading to failure of spermatogenesis (Qiu *et al.*, 2013). Another study had shown that Leydig cell-specific AR knockout mice have a decrease in seminiferous tubules diameter compared to their control group. Similar finding was found in Sertoli cell-specific AR knockout mice that led to infertility (Tsai *et al.*, 2006).

In adult prostate gland, earlier study showed that AR was abundant in the nucleus of epithelial cell and the prostatic stromal cells such as fibroblasts and smooth muscle (Prins *et al.,* 1991; Iwarmura *et al.,* 1994; Shabisgh *et al.,* 1999). Similarly, other studies had also shown that AR was expressed in stromal, smooth muscles and epithelial cells of the seminal vesicle (Takeda *et al.,* 1990; Majdic *et al.,* 1995). Lack of functional AR was found to lead to luminal cells apoptosis in the prostate gland (Evans and Chandler, 1987; Mirosevich *et al.,* 1999; Yeh *et al.,* 2002). In addition, removing the epithelial cells also resulted in apoptotic changes in the seminal vesicles (Mata, 1995).

CHAPTER 3:
MATERIALS AND METHODS

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3.1 ANIMALS

3.1.1 Rearing and Maintenance of Rats

Male *Sprague dawley* rats, 7-9 weeks old with body weight ranging from 150 – 250g were obtained from Animal Experimental Unit, Faculty of Medicine, University Malaya. The rats were reared at Animal House, Centre for Foundation Studies in Science, University of Malaya.

Each rat was housed in a separate cage using sawdust as the bedding. The rats were placed under standard laboratory condition, ±27^{o}C in light and dark cycles with good ventilation. Natural light period was approximately 12 hours (0600 – 1800 hours) per day, while natural dark period was approximately 12 hours (1800 – 0600 hours) per day. Chow pellet and water were given to rats *ad libitum* for 100 days throughout the experimental periods.

3.1.2 Grouping and Treatment of Rats

Sprague dawley male rats (n=30) were randomly divided into five groups, *Nigella sativa* (NS), corn oil (CO), nicotine (N), saline (S) and nicotine-*Nigella sativa* (NNS) with 6 rats for each group. Rats were weighed every three days for exact dosage of *Nigella sativa* corn oil, nicotine and saline to be given to each of the respective treatment groups for 100 days. Experimental procedures were conducted in accordance to the approval of the Institutional Animal Care and Use Committee (IACUC), University Malaya [Ref. Number: ISB/20/04/2012/DSHA(R)].

Nigella sativa (NS) group was force-fed with *Nigella sativa* oil at 6µl/100g body weight. Its control group, corn oil (CO) group was force-fed with corn oil which was used as vehicle for dilution of HS at 0.1ml/100g body weight. Corn oil (Brand: Vecorn) was obtained from local supermarket produced by Yee Lee Edible Oils while *Nigella sativa* (Brand: Dogaci, Turkey) was purchased from local market. Force feeding was done using 20G blunt end feeding needle.

The nicotine (N) group was intramuscularly (i.m.) injected with nicotine at 0.5mg/100g body weight. Nicotine (L-Nicotine, 99+%, CAS RN: 54-11-5) was purchased from Acros Organics. The nicotine control group, saline (S) group was intramuscularly (i.m.) injected with normal saline solution which was used for dilution of nicotine at 0.1ml/100g body weight (Figure 3.1). Normal saline solution, 0.9% NaCl was prepared from sodium chloride (NaCl, SIGMA: S9625-500G) and distilled water (Appendix 1). Intramuscular injection (i.m.) was administered at the thigh muscle of rat hind legs, alternately legs daily. Nicotine and saline was administered using a 26G needle.

The nicotine-*Nigella sativa* (NNS) group was treated with both nicotine, intramuscular injection of 0.5mg/100g body weight and *Nigella sativa*, force-fed with 6µl/100g body weight.

It was recorded that the lethal dose (LD_{50}) values for rats and human are at 50mg/kg and 0.5mg – 1mg/kg of nicotine, respectively (Okamoto *et al.*, 1994). The dosage of nicotine administered to rats in current study were modified based on the previous studies which reported administration of 0.5 – 1.0mg/kg of nicotine had negative effects on the male reproductive system (Jana *et al.*, 2010; Oyeyipo *et al.*, 2010; Sankako *et al.*, 2012).

Administration of 6µl/100g body weight of *Nigella sativa* oil in present study were based on improvisation of preliminary study conducted by Juliyana *et al.* (2011).

Figure 3.1: Rats intramuscularly injected with nicotine (0.5mg/100g of body weight) and saline (0.1ml/100g of body weight) solution.

3.1.3 Harvesting of Selected Tissues

Prior to perfusion, rats in all groups were anesthetised with 1.0ml/100g body weight of 3.5% chloral hydrate (Chloral hydrate, UNILAB: 148-500G) (Appendix 2) via intraperitoneal injection (i.p) at the peritoneal cavity of the rat, specifically at the lower right quadrant of the abdomen as to prevent from hitting other organs such as liver and bladder.

The euthanised rat was placed on a perfusion board and the abdominal and thoracic cavities were opened. Using a peristaltic perfusion pump (Masterflex, Cole-Parmer Instrument Company, Model No: 7553-75) with 23 gauge needles attached to the silicone rubber tube, the needle was inserted via the left ventricle of the heart. Animal was first perfused with normal saline solution in order to clear all the blood from the vasculature system of the animal.

Fixative solution, 10% formalin (Fisher Scientific, CAT: F/1501/PB17) (Appendix 3), was then introduced into the animal vasculature system to fix the tissue. The indication for successful perfusion was observed by discoloration of the internal organs together with the hardening of animal body. From each of the perfused rats, a pair of testes, a pair of seminal vesicles, and a whole structure of prostate glands were harvested soon after the completion of fixation by exposing the pelvic cavity (Figure 3.2). The harvested tissues were further immersed and fixed in 10% formalin solution at room temperature (Figure 3.3).

The rat carcasses were kept in deep freezer at -20^{o}C prior to collection by a licensed bio-disposal company.

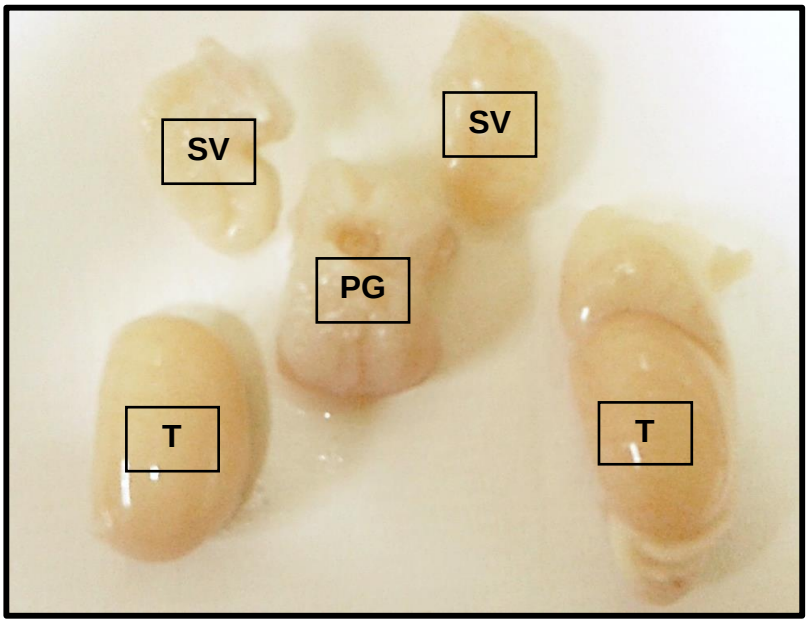

Figure 3.2: The harvested testes, seminal vesicles and prostate gland. T: Testis; SV: Seminal vesicles; PG: Prostate gland.

Figure 3.3: The extracted tissues were fixed in 10% formalin solution.

3.2 MORPHOLOGICAL AND HISTOLOGICAL STUDIES

3.2.1 Tissue Processing

The fixed specimens were rinsed with 0.1M of phosphate buffer solution (PBS) pH 7.4 (PBS, SIGMA: P7059) (Appendix 4) for three times, 30 minutes each. Then, the tissues were dehydrated in increasing graded series of alcohol solution (Appendix 5) for an hour each. The tissues were then immersed for clearing in solution containing an equal part of cedar wood oil (SIGMA: 96090) and absolute alcohol for overnight. Next, the tissues were cleared in pure cedar wood oil for overnight (Figure 3.4).

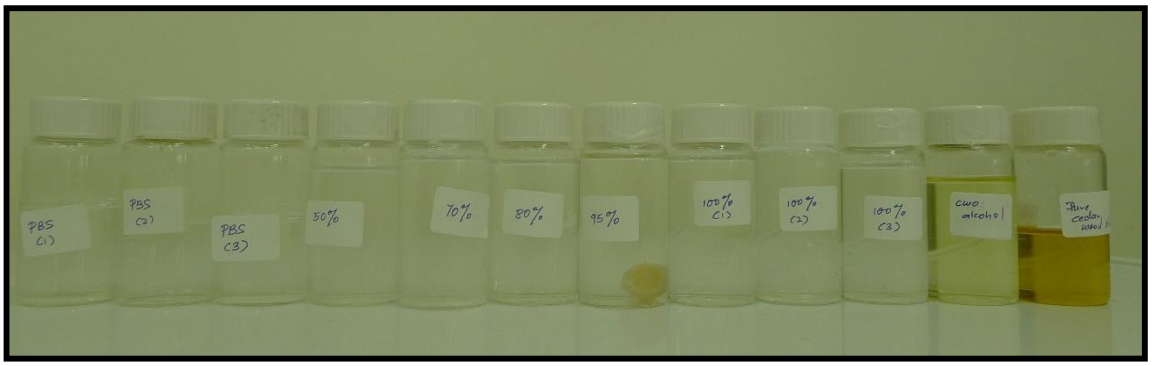

Figure 3.4: Tissues processing for light microscopy.

3.2.2 Tissue Impregnation and Embedding

The processed tissues were firstly dip in benzene (Merck: 1440083 830) for few seconds, prior to immersion in an equal part solution containing paraffin wax (Leica Biosystem: 39601006) and benzene for 1 hour (Appendix 6). The tissues were then immersed in four series of paraffin wax at the melting point of 55 – 60^{o}C for an hour each prior to embedding the specimens in paraffin wax as blocks (Appendix 7).

3.2.3 Tissue Sectioning and Staining

The specimen blocks with embedded tissues were sectioned at 5µm thickness using microtome (JUNG Multicut, Leica: 2045). Sectioned tissues were mounted on labelled glass slide for light microscopy study. Glass slide with sections were dried overnight in oven at 40^{o}C. The slides were stained with Haematoxylin and Eosin (H&E) (Appendix 8) and mounted with mouting medium, Dibutylphthalate Polystyrene Xylene (DPX, LABCHEM: 3197). The slides were covered with coverslip and viewed under the light microscope (Olympus) for histological analysis.

3.2.4 Histological Study

Haematoxylin and Eosin (H&E) stained the harvested tissues to enable visualisation of the tissue morphology. Haematoxylin stained the nucleic acid with deep blue-purple color while the cytoplasm and extracellular matrix were stained pink by eosin (Fisher *et al.,* 2008).

3.3 TESTICULAR CELL COUNT

Quantitative study of spermatogenesis was conducted whereby the number of germ cells of the spermatogenetic cycle, namely spermatogonia, spermatocyte, spermatid and spermatozoa were counted. This included the counts of the Leydig and Sertoli cells in the seminiferous tubules (Figure 3.5).

Nuclei of different germ cells, Leydig and Sertoli cells were counted from 10 rounded seminiferous tubules per rat. Data were presented as number of germ cells. Leydig and Sertoli cells per tubular cross section (Jana *et al.,* 2010). Evaluation was conducted under the light microscope using NIS-Elements Imaging System Software (Nikon) under 20x magnification.

Spermatogonia were located at the basal membrane of the seminiferous tubule and are characterised by large, spheroidal nuclei. The spermatocytes were identified by its large nuclei containing clumps of chromatin granule. In addition, spermatids were characterised by its small nuclei with more condensed chromatin compared to their predecessors. Spermatozoa are found in the lumen of seminiferous tubule. As for Sertoli cell found at the basement membrane and extend to the lumen of the tubule was identified by its the triangular or ovoid nucleus with a very prominent nucleolus. Leydig cells located in the interstitial space of seminiferous tubule were noted by their ovoid shaped cells and eosinophilic cytoplasm. Besides, Leydig cells have large and rounded nuclei (Steven and Lowe, 2005).

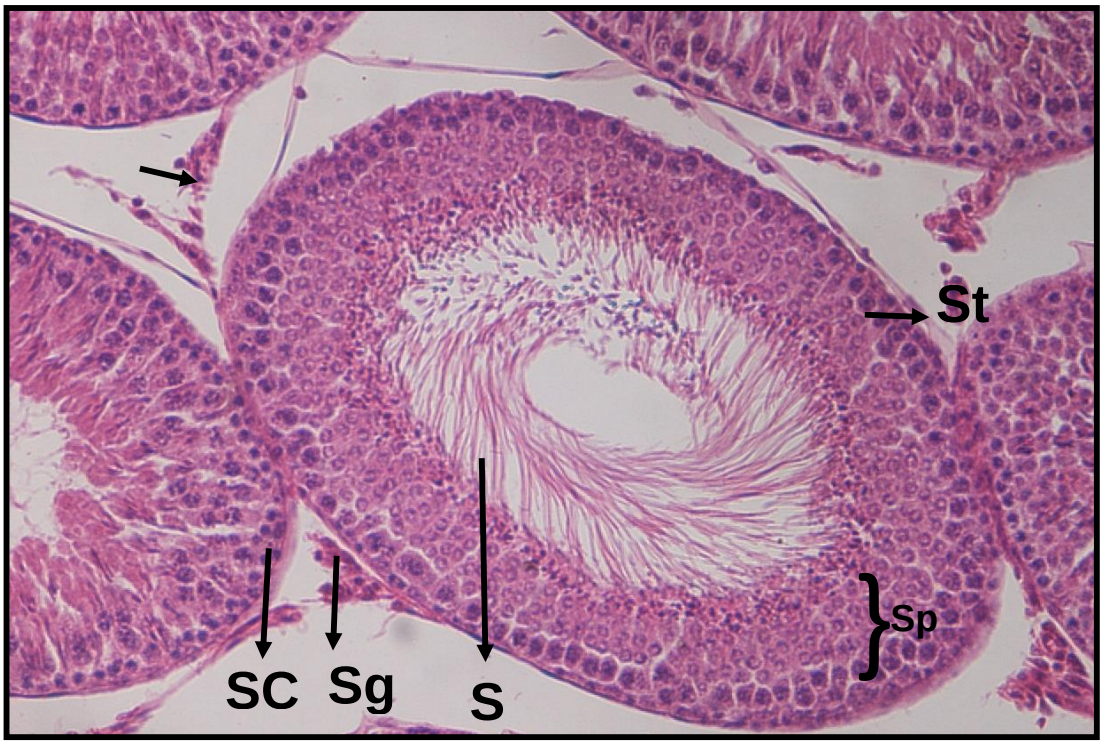

Figure 3.5: H&E stained spermatogenesis at different stages, Leydig and Sertoli cells (40x). Arrow: Leydig cell, SC: Sertoli cell, Sg: spermatogonium, St:spermatocyte, Sp: Spermatid, S: spermatozoa.

3.4 IMMUNOHISTOCHEMISTRY (IHC) STUDY

Specimen blocks were sectioned at 5µm thickness using microtome (JUNG Multicut, Leica: 2045). Sectioned specimens were placed on the Poly-L-Lysine coated slides (Thermo Scientific: J2800AMNZ) for immunohistochemical study. The coated slides were dried overnight in oven at 40^{o}C prior to staining. Immunohistochemical staining procedure was conducted according to the protocol in immunoproxidase secondary detection kit with modification (CHEMICON IHC: DAB 500) (Appendix 9). Sectioned tissues were deparaffinised using xylene for five minutes in four changes. The tissues were then rehydrated in descending series of alcohol graded solution and distilled water for a minute each. Next, the sectioned tissues were introduced to 10mM citrate buffer with pH 6.0 by placing into heated microwave oven at 70^{o}C and 100^{o}C for 6 and 7

minutes, respectively. Citrate buffer solution was used as antigenic retrieval solution (Appendix 10).

The slides were placed in running water to allow cooling prior to quenching with 3% hydrogen peroxide (H_2O_2) (Sigma Aldrich: 88597) for an hour. The slides were then rinsed in tris buffered saline (TBS) three times for 2 minutes each (Appendix 11). Next, the slides were pre-incubated for 2 hours in blocking serum at room temperature, 27^{o}C. Excess of blocking serum on the sectioned slides was washed with TBS solution. All slides were incubated overnight with mouse anti-androgen receptor primary (1^{o}) antibodies (Santa Cruz Biotechnology: AR 441: sc-7305) at room temperature. The negative control slides were incubated in antibody diluent.

On the second day, all treated and negative control slides were incubated in secondary (2^{o}) antibody for 2 hours prior to washing with TBS solution to remove any excess antibody on the slides. The sections were then incubated in streptavidin-horsedish peroxidase (HRP) for 10 minutes and rinsed with TBS solution. Next, the slides were incubated in 3,3' diaminobenzidine (DAB) solution (Appendix 12) for 10 minutes and counterstained with Haematoxylin stain prior to dehydration in increasing alcohol concentration. Finally the slides were cleared in xylene and covered with coverslip. Qualitative assessment of the androgen receptor in the tissue samples of the selected organs were evaluated based on strength of the brown color staining observed indicating the receptor immunoreactivity (Favaro and Cagnon, 2007).

3.5 STATISTICAL ANALYSIS

Testicular cell count data was analysed statistically using one-way analysis of variance (ANOVA) test and Duncan Multiple Range Test (Duncan-MRT) from Statistical Package for the Social Sciences (SPSS) version 21. The data were presented as mean±standard error of means (SE) with significant value $p<0.05$.

3.6 EXPERIMENTAL DESIGNS

Five treatment groups: saline (S), nicotine (N), corn oil (CO), *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) with specific treatment dosages and route of administration (Table 3.1).

Table 3.1: The dosage and number of rats in the treatment groups

Treatment group	Number of rats (n)	Dosage
Saline (S)	6	0.1ml/100g body weight of saline via intramuscular injection (i.m.)
Nicotine (N)	6	0.5mg/100g body weight of nicotine via intramuscular injection (i.m.)
Corn oil (CO)	6	0.1ml/100g body weight of corn oil via force-fed
Nigella sativa (NS)	6	6µl/100g body weight of *Nigella sativa* oil via force-fed
Nicotine-*Nigella sativa* (NNS)	6	Co-administration of 0.5mg/100g body weight of nicotine via intramuscular injection (i.m.) and 6µl/100g body weight of *Nigella sativa* oil via force-fed

3.6.1 Histological Features on the Testes, Prostate Gland and Seminal Vesicle

Testis, seminal vesicle and prostate gland from the treatment groups were harvested after 100 days of treatment. Tissues form the organ were prepared for histological analysis. (Figure 3.6). The histoarchitecture of the selected tissues were observed under 20x magnification.

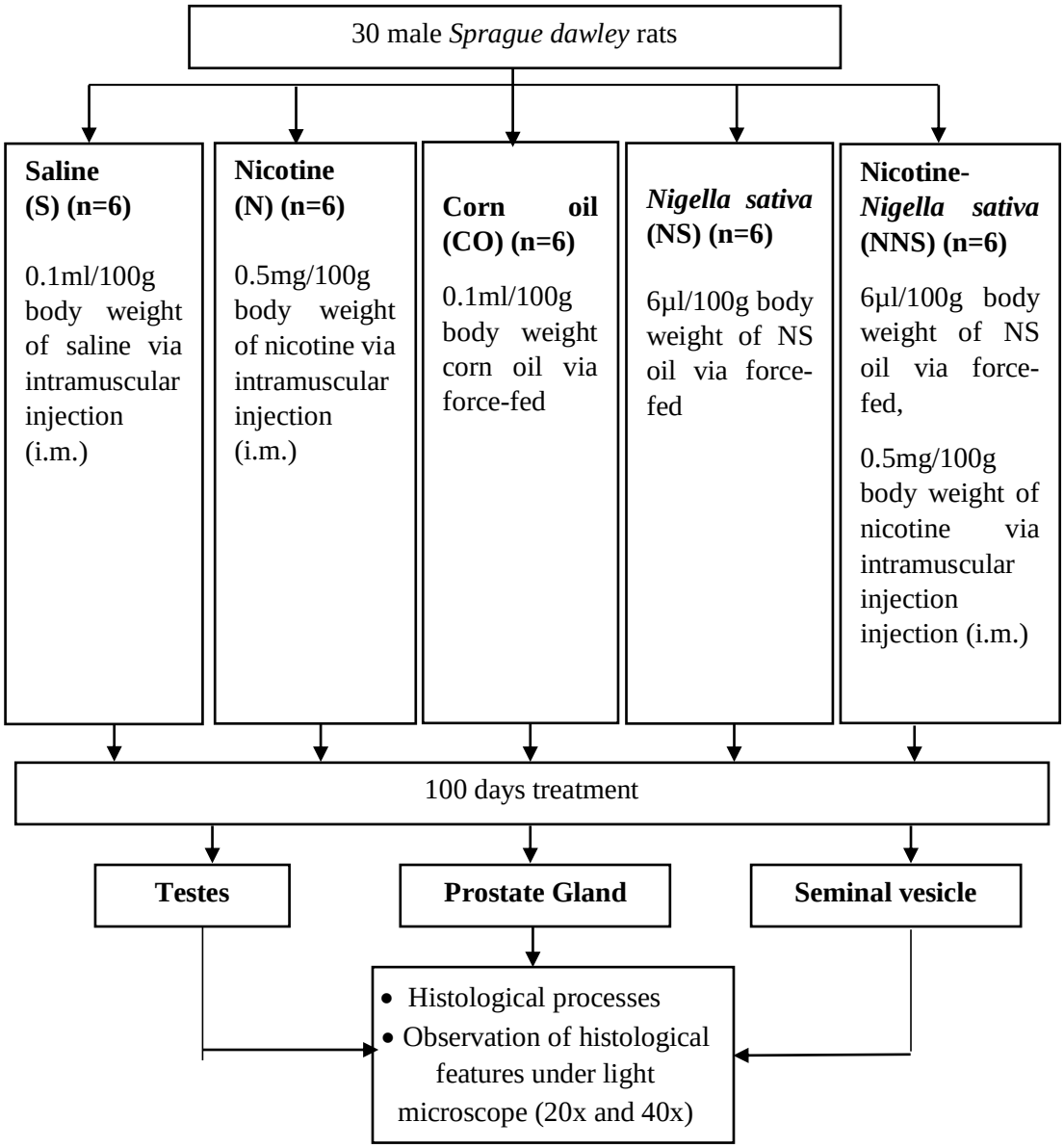

Figure 3.6: Experimental design for histological features of the testes, prostate gland and seminal vesicle.

3.6.2 Testicular Cell Count

Testis from the treatment groups were harvested after 100 days of treatment. The testicular tissue were prepared for cell count analyses under 20x magnification. Raw data collected were analysed using SPSS software version 21 (Figure 3.7).

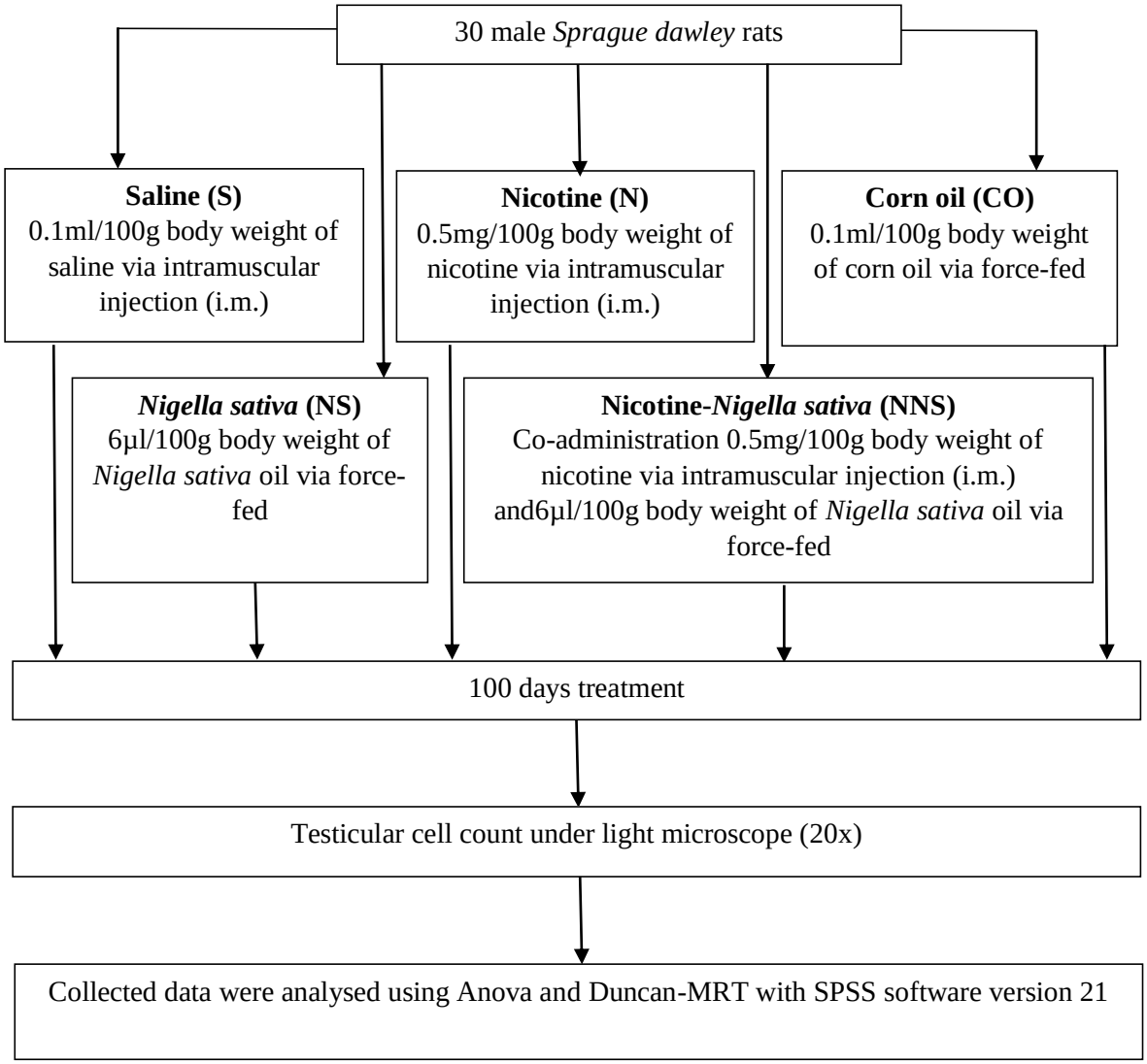

Figure 3.7: Experimental design for testicular cell count.

3.6.3 Immunohistochemistry Studies on the Testes, Prostate Gland and Seminal Vesicle

Immunohistochemistry study were conducted on testis, seminal vesicle and prostate gland from the treatment groups after 100 days of treatment (Figure 3.8). Presence of androgen receptor staining in the tissues were observed under 40x magnification.

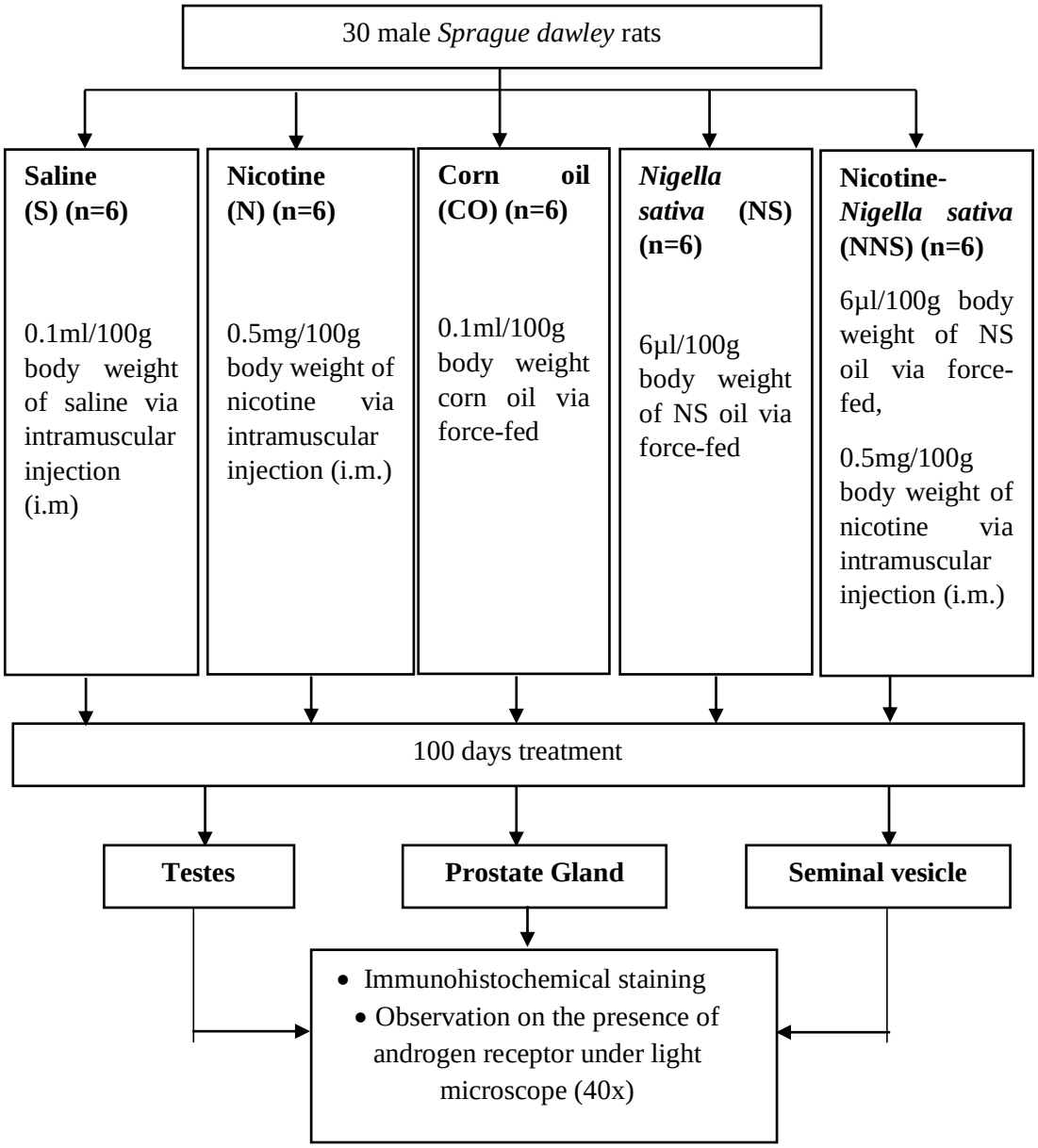

Figure 3.8: Experimental design for immunohistochemistry studies of the testes, prostate gland and seminal vesicle.

CHAPTER 4:
RESULTS

CHAPTER 4: RESULTS

4.1 HISTOLOGICAL STUDIES

Histoarchitecture observation of the harvested tissues was performed on the testes, seminal vesicles and prostate gland of the control and treated groups.

4.1.1 Histology of the Testes

Testis is composed of seminiferous tubules. Each of the seminiferous tubule is surrounded by tunica propria and lined by thick seminiferous epithelium. Both tunica propria and seminiferous epithelium are separated by basal lamina. The seminiferous epithelium consists of two groups of cell, 1) spermatogenic lineage and 2) Sertoli cell (supporting cell). Spaces between the seminiferous tubules, interstitial spaces are filled with fibroblasts and Leydig cell or interstitial cell.

Germ cells of various generations and Sertoli cell are located within the seminiferous tubule. Spermatogonia are found resting at the basal membrane of the tubule followed by spermatocytes which are located in a layer above from the spermatogonia. Spermatid cell are found occupying one or two layers above the spermatocytes whereas spermatozoa are commonly found in the lumen.

The saline (S) and nicotine-*Nigella sativa* (NNS) groups showed a densely packed seminiferous tubules compared to the nicotine (N) group. This normal appearance of tubules were also noted in the saline (S) and nicotine-*Nigella sativa* (NNS) groups. However, shrinkage of the seminiferous tubules were observed in the nicotine (N) group which resulted in increased interstitial space and more loosely arranged seminiferous tubules (Figure 4.1).

More numerous spermatogenic cells were noted in the seminiferous tubules of the saline (S), corn oil (CO), *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups compared to that of in the nicotine (N) group. Number of Leydig cells were also noted to be less in the nicotine group compared to the *Nigella sativa* (NS), nicotine-*Nigella sativa* (NNS), saline (S) and corn oil (CO) groups (Figure 4.1).

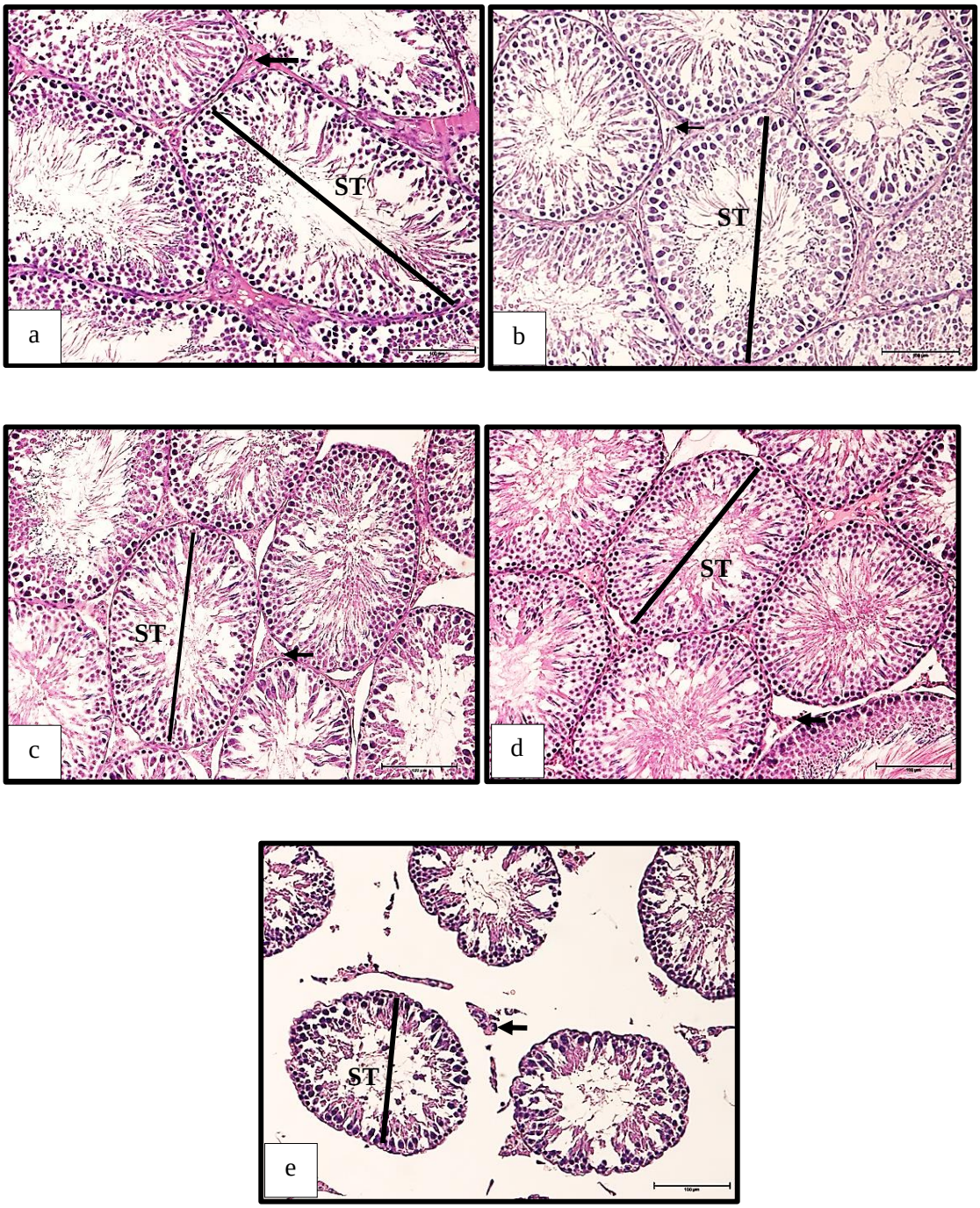

Figure 4.1: Photomicrograph of transverse histological section of seminiferous tubules for the (a) saline, (b) corn oil, (c) *Nigella sativa*, (d) nicotine-*Nigella sativa*, and (e) nicotine groups stained with H&E staining (20x).
ST: seminiferous tubules, Arrow: Leydig cell.
Bar = 100µm.

4.1.2 Histology of the Seminal Vesicle

Seminal vesicles are highly coiled structures and unbranched tubular diverticulum of the vas deferens. Its wall is lined by an 1) inner mucosa layer which is supported by smooth muscle layer and 2) outer stroma cell layer. The lumen of seminal vesicle is highly irregular and recessed forming a honeycomb appearance.

From light microscopy study of the seminal vesicles, it was observed that in the saline (S) group, honeycomb-like appearance from the anastomosing epithelial of the mucosa folds was visible with dense acidophilic secretion substance in the lumen. Similar observation was also seen in the corn oil (CO), *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) group (Figure 4.2).

However, the honeycomb-like appearance was absent in the seminal vesicles of the nicotine (N) group and there was minimal acidophilic secretion seen in the lumen as compared to the *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups (Figure 4.2).

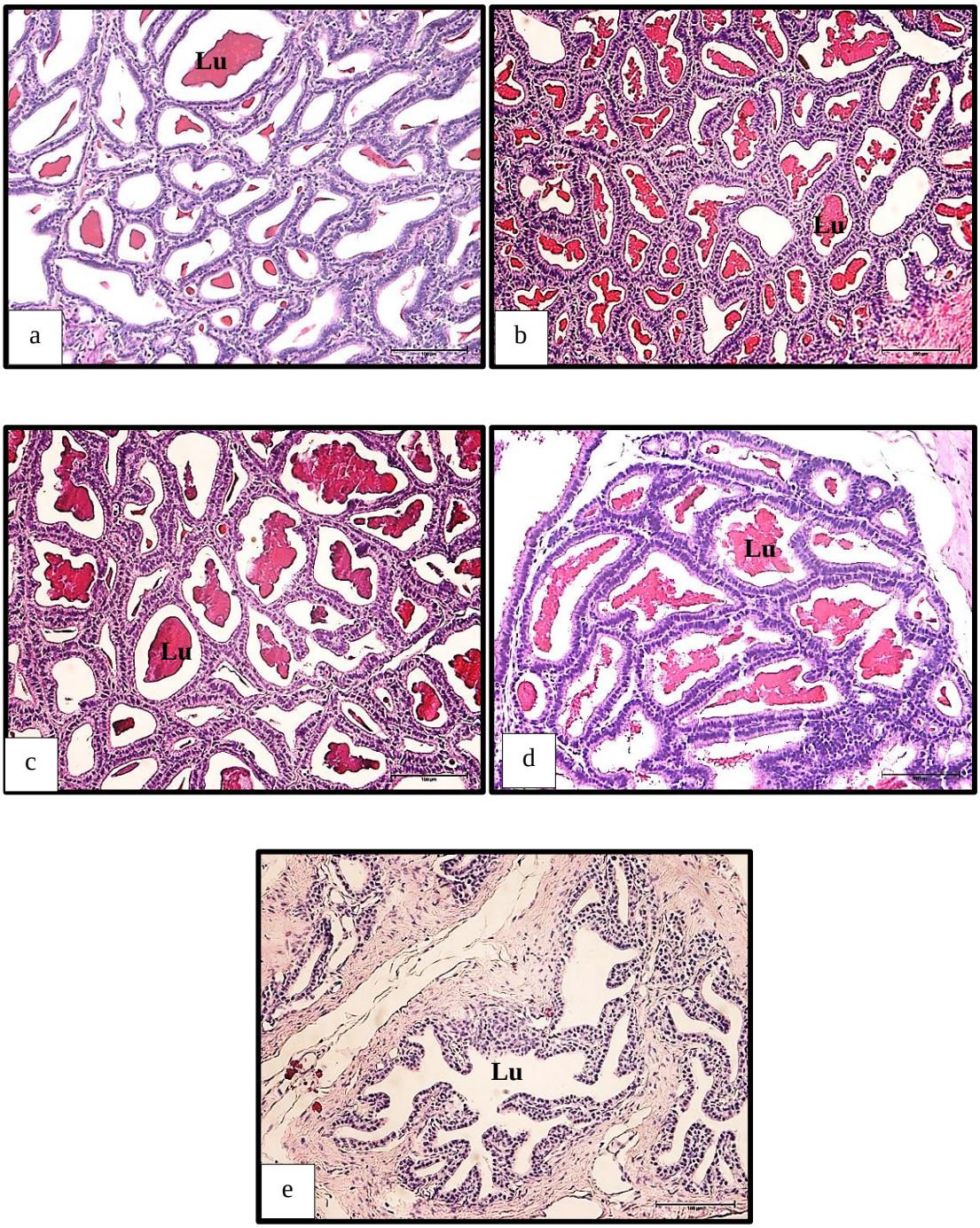

Figure 4.2: Photomicrograph of seminal vesicle for the (a) saline, (b) corn oil, (c) *Nigella sativa*, (d) nicotine-*Nigella sativa*, and (e) nicotine groups stained with H&E staining (20x). Lu: Lumen. Bar = 100µm.

4.1.3 Histology of the Prostate Gland

Prostate glands are accessory sex gland of male reproductive organ where in rodents it consists of three pairs of lobes. The mucosa layer of prostate gland is lined with tall epithelium cells. Its lumen contained acidophilic secretion.

In the control groups, saline (S) and corn oil (CO), the lining epithelial of the prostate was surrounded with columnar cells with relatively minimal spaces among prostatic acini. Presence of acidophilic secretion was also seen in the lumen of prostate gland. In addition, presence of mucosa glandular infoldings was also noted. Similar findings were noted on the *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups with more abundant mucosa glandular infoldings in the *Nigella sativa* (NS) group (Figure 4.3).

However, in the nicotine (N) group, the epithelium lining surrounding the gland was flattened as compared to the saline (S), corn oil (CO), *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups. Absence of acidophilic secretion were also seen in the lumen of the nicotine group compared to the *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups. Besides, the increase spaces between the prostatic acini were also noted in the nicotine (N) group with minimal mucosa glandular infoldings (Figure 4.3).

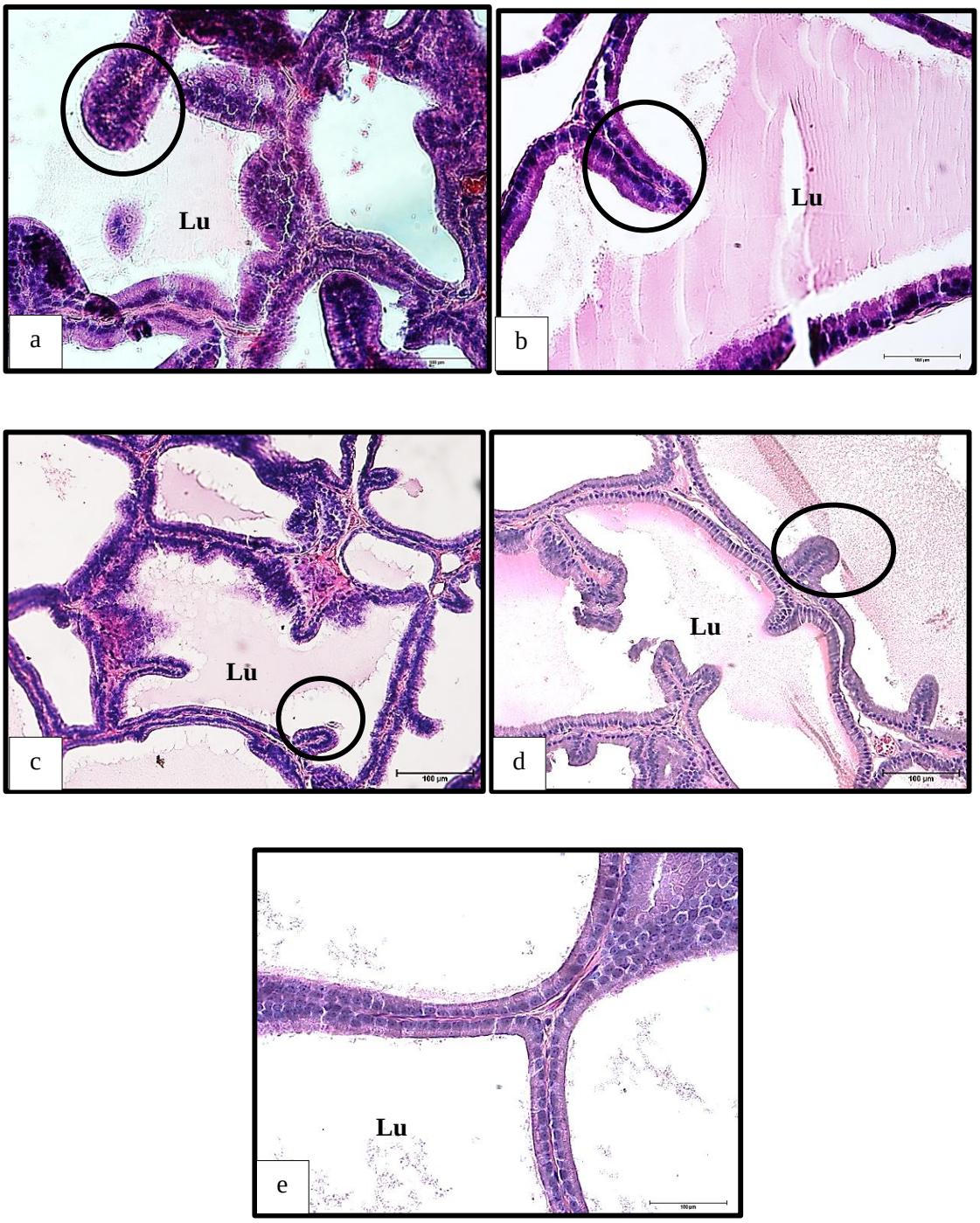

Figure 4.3: Photomicrograph of prostate gland for the (a) saline, (b) corn oil, (c) *Nigella sativa*, (d) nicotine-*Nigella sativa*, and (e) nicotine groups stained with H&E staining (40x).

Lu: Lumen. Circle: Mucosa infoldings. Bar = 100µm.

4.2 TESTICULAR CELL COUNT

Spermatogenic cell count of different stages in the seminiferous tubules were counted and the analysis of variance indicated that treatments had significant ($p<0.05$) effect on the spermatogenic cell count of rats testis (Table 4.1). In general, the nicotine group showed significantly lower spermatogenic cell count as compared to the other groups. In contrary, spermatogenic cell count in the *Nigella sativa* group was significantly higher than the saline, nicotine, corn oil and nicotine-*Nigella sativa* groups.

4.2.1 Spermatogenic Cell Count

The nicotine-*Nigella sativa* (30.35±1.93) and saline (35.62±1.72) groups showed a significantly higher ($p<0.05$) number of spermatogonia cells compared to the nicotine (25.05±1.60) group. The number of spermatocytes in the *Nigella sativa* (57.77±1.76) group showed a significantly higher ($p<0.05$) number than the nicotine (49.47±1.54), nicotine-*Nigella sativa* (51.58±1.62) and corn oil (52.68±1.48) groups (Table 4.2).

In addition, a significantly higher ($p<0.05$) number of spermatid was obtained in the saline (161.60±4.35) and nicotine-*Nigella sativa* (139.30±4.78) as compared to the nicotine (124.60±5.25) group. Similarly, the number of spermatozoa cells were also observed to be significantly higher ($p<0.05$) in both the saline (63.13±2.88) and nicotine-*Nigella sativa* (60.22±3.34) groups than the nicotine (46.22±2.68) group (Table 4.2).

Table 4.1: Mean square analyses of variance for spermatogenic cells of saline, nicotine, corn oil, *Nigella sativa* and nicotine-*Nigella sativa* groups

Source of variation	d.f.	Mean square			
		Cell count			
		Spermatogonia	Spermatocytes	Spermatid	Spermatozoa
Treatment	4	1115.45*	584.86*	23842.51*	4264.09*
Error	295	195.44	143.41	1442.70	504.22
Total	299				

*$p<0.05$

Table 4.2: Least square means from analyses of variance for spermatogenic cells per seminiferous tubule of saline, nicotine, corn oil, *Nigella sativa* and nicotine-*Nigella sativa* groups

Parameter / Treatment	Mean±SE			
	Spermatogonia	Spermatocyte	Spermatid	Spermatozoa
Saline (S) n=10	35.62±1.72^{c}	54.35±1.29^{bc}	161.60±4.35^{c}	63.13±2.88^{bc}
Nicotine (N) n=10	25.05±1.60^{a}	49.47±1.54^{a}	124.60±5.25^{a}	46.22±2.68^{a}
Corn oil (CO) n=10	29.78±1.59^{ab}	52.68±1.48^{ab}	153.57±4.97^{c}	60.15±2.52^{b}
Nigella sativa (NS) n=10	34.98±2.12^{bc}	57.77±1.76^{c}	176.23±5.12^{d}	69.20±3.02^{c}
Nicotine-*Nigella sativa* (NNS) n=10	30.35±1.93^{bc}	51.58±1.62^{ab}	139.30±4.78^{b}	60.22±3.34^{b}

^{abcd} different superscripts within the same column show significant differences ($p<0.05$)

4.2.2 Sertoli and Leydig Cell Count

The analysis of variance showed that treatments had significant ($p<0.05$) effects on the Sertoli and Leydig cell count of rats testes (Table 4.3). The Sertoli cell count showed that the *Nigella sativa* (20.83±0.87) and saline (21.55±0.87) groups had a significantly higher ($p<0.05$) number of cells as compared to the nicotine-*Nigella sativa* (17.68±0.81) group (Table 4.4). Similarly, the number of Leydig cells in the *Nigella sativa* (42.77±1.77), nicotine-*Nigella sativa* (33.08±1.91) and saline ((33.08±1.91) groups were significantly higher ($p<0.05$) than that of in the nicotine (26.55±1.64) group (Table 4.4).

Table 4.3: Mean square analysis of variance for Sertoli and Leydig cells of saline, nicotine, corn oil, *Nigella sativa* and nicotine-*Nigella sativa* groups

Source of variation	d.f.	Mean square	
		Cell count	
		Sertoli cell	Leydig cell
Treatment	4	126.98*	4953.97*
Error	295	40.78	205.60
Total	299		

*$p<0.05$

Table 4.4: Least square means from analysis of variance for Sertoli and Leydig cells per seminiferous tubule of saline, nicotine, corn oil, *Nigella sativa* and nicotine-*Nigella sativa* groups

Parameter / Treatment	Mean±SE	
	Sertoli	Leydig
Saline (S) n=10	21.55±0.87[b]	50.15±2.25[d]
Nicotine (N) n=10	20.03±0.74[ab]	26.55±1.64[a]
Corn oil (CO) n=10	20.08±0.83[ab]	40.57±1.62[c]
Nigella sativa (NS) n=10	20.83±0.87[b]	42.77±1.77[c]
Nicotine-*Nigella sativa* (NNS) n=10	17.68±0.81[a]	33.08±1.91[b]

[abcd] different superscripts within the same column show significant differences ($p<0.05$)

4.3 IMMUNOHISTOCHEMISTRY STUDIES

Immunohistochemistry detection for the androgen receptor (AR) using mouse monoclonal anti-androgen antibody was performed on the testes, seminal vesicles and prostate gland of the control and treated groups.

4.3.1 Immunohistochemistry Study of the Testes

In the testis of the saline (S) and corn oil (CO) groups, the androgen receptor was demonstrated with strong immunoreactivity of brown staining in the Sertoli and Leydig cells (Figure 4.4). Similarly, the *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups also showed presence of strong immunoreactivity androgen receptor in the Sertoli and Leydig cells of the testis. However, weak immunoreactivity of brown staining was observed in the Sertoli and Leydig cells of the nicotine (N) group (Figure 4.4).

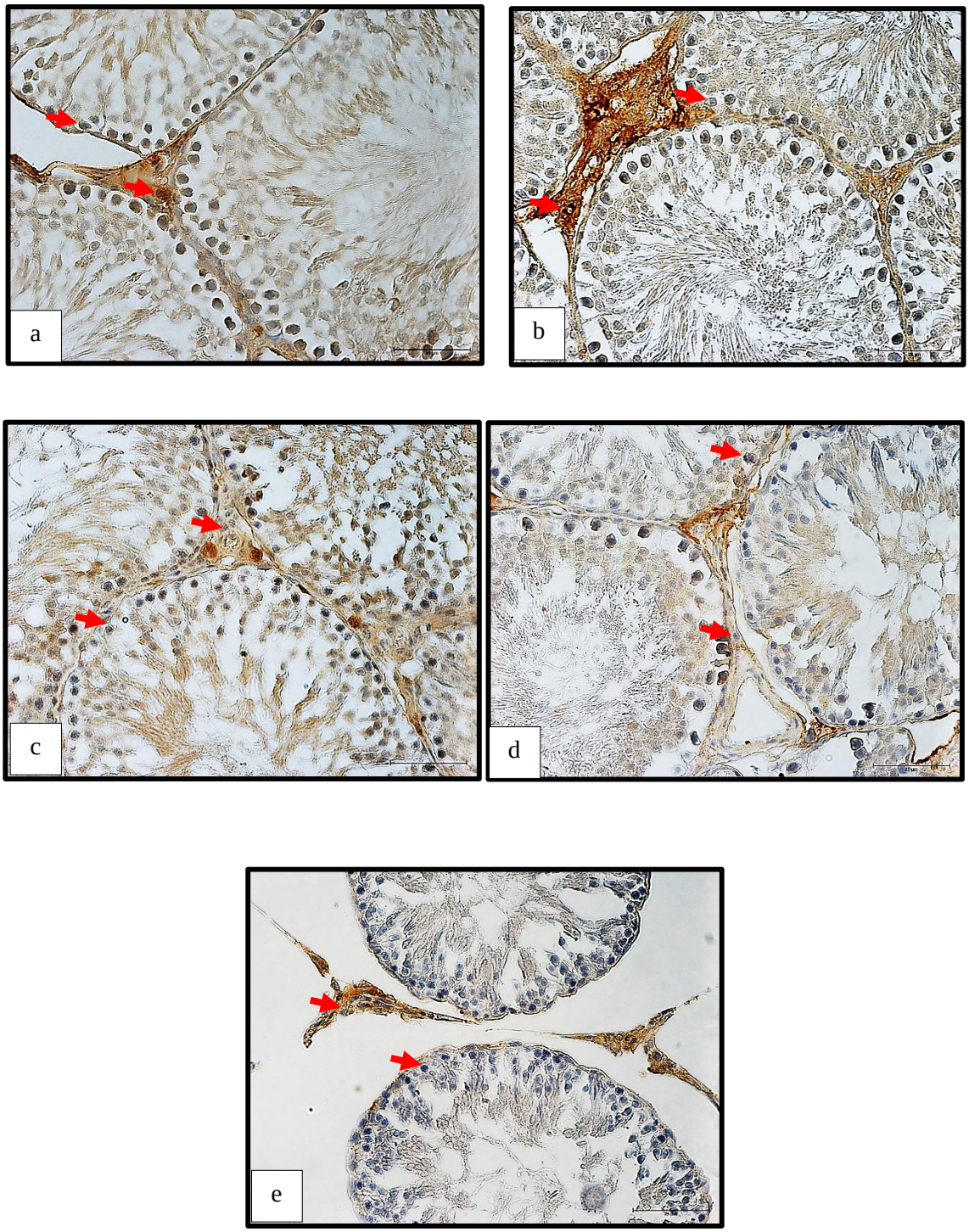

Figure 4.4: Photomicrograph of seminiferous tubules for the (a) saline, (b) corn oil, (c) *Nigella sativa*, (d) nicotine-*Nigella sativa*, and (e) nicotine groups stained with anti-androgen antibody (40x). AR staining (Arrow).
Bar = 100µm.

4.3.2 Immunohistochemistry Study of the Seminal Vesicle

In the seminal vesicle, the expression of androgen receptors was demonstrated with strong immunoreactivity of brown staining in the glandular epithelial cells of the gland (Figure 4.5). Both the *Nigella sativa* (NS) and nicotine-*Nigella sativa* (NNS) groups showed the presence of strong immunoreactivity of brown staining. In contrast, the androgen receptor staining was demonstrated to be less intense in the nicotine (N) group (Figure 4.5).

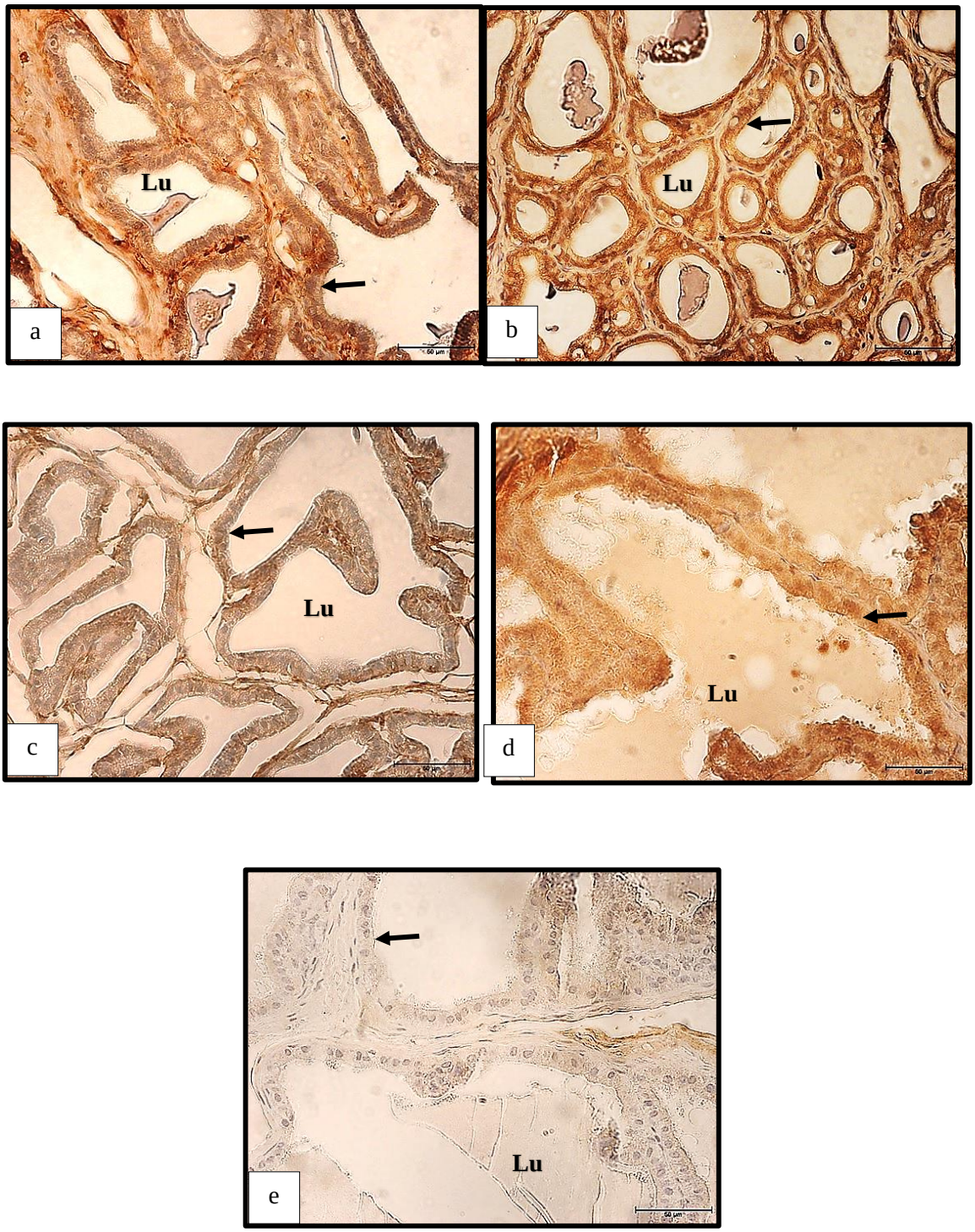

Figure 4.5: Photomicrograph of seminal vesicle for the (a) saline, (b) corn oil, (c) *Nigella sativa*, (d) nicotine-*Nigella sativa*, and (e) nicotine groups stained with anti-androgen antibody (40x). Lu: lumen, AR staining (arrow). Bar = 50µm.

4.3.3 Immunohistochemistry Study of the Prostate Gland

Strong immunoreactivity of brown staining was observed in the epithelial cells of prostate glands in saline (S) and corn oil (CO) groups (Figure 4.6). The *Nigella sativa* (NS) and nicotine- *Nigella sativa* (NNS) groups exhibited strong immunoreactivity of immunostaining in the epithelial cells of prostate glands as compared to the nicotine (N) group. The nicotine (N) group showed absence of androgen immunostaining in the epithelial cells of prostate glands, thus exhibited weak immunoreactivity of brown staining in the epithelial cells (Figure 4.6).

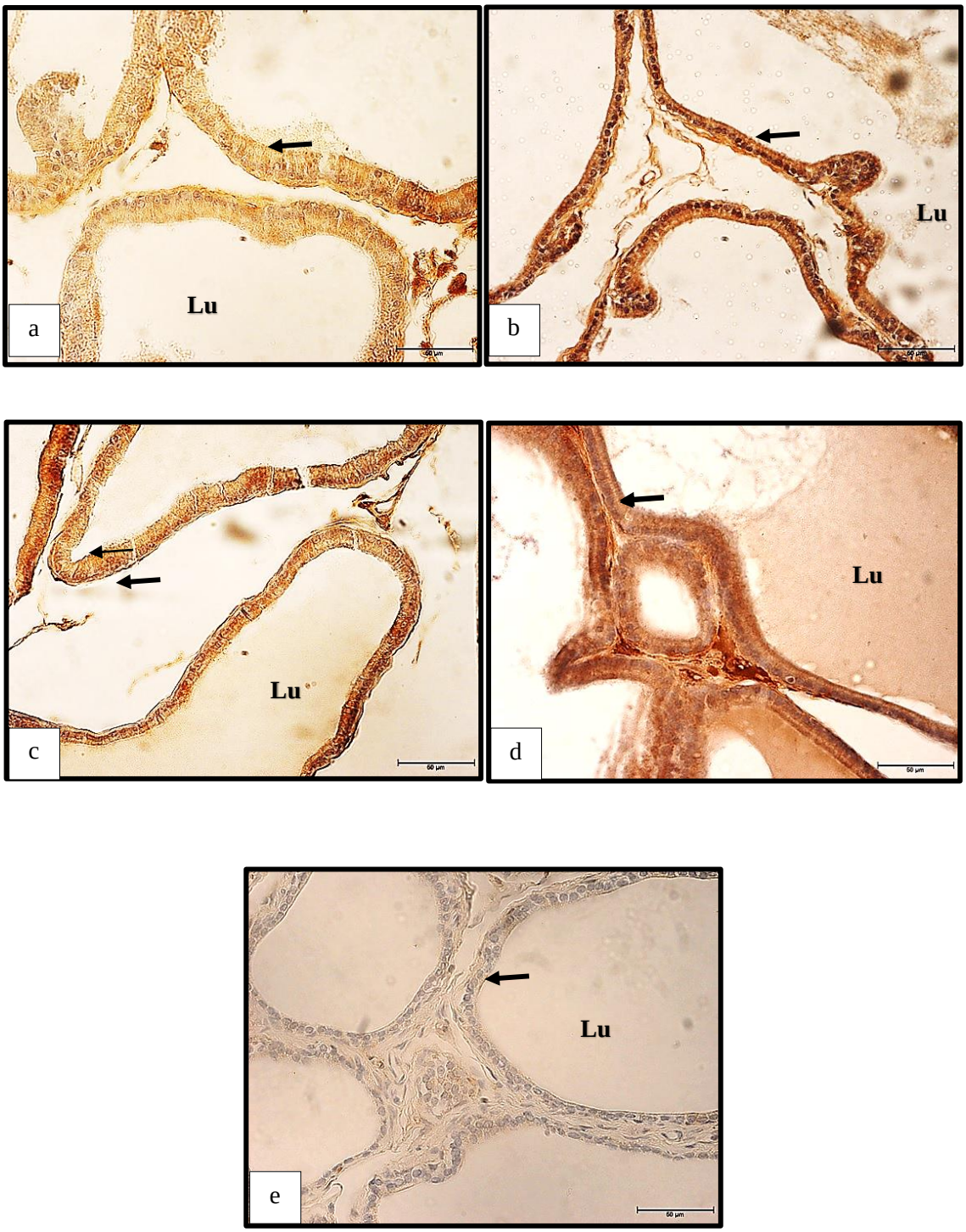

Figure 4.6: Photomicrograph of prostate gland for the (a) saline, (b) corn oil, (c) *Nigella sativa*, (d) nicotine-*Nigella sativa*, and (e) nicotine groups stained with anti-androgen antibody (40x). Lu: lumen, AR staining (arrow). Bar = 50µm.

CHAPTER 5:
DISCUSSION

CHAPTER 5: DISCUSSION

5.1 HISTOLOGICAL FEATURES OF MALE REPRODUCTIVE ORGANS

Normal histological features of the testes can be used as a parameter to indicate its normal function. A study had shown that intact epithelial lining of the seminiferous tubule resulted in a normal cyclic pattern of cell renewal and development within the testes (Standring *et al.,* 2004).

In this present study, histological features of the testes in the nicotine group were noted to be loosely packed with seminiferous tubules as compared to densely packed seminiferous tubules in the other four groups. Previous study reported that exposure to 1.5 hour of cigarette smoke for 10 weeks would lead to impairment in the animal testicular histology with a reduction in the diameter of seminiferous tubules indicating disruption in the spermatogenesis (Ahmadnia *et al.,* 2007). This was supported by a study which showed atrophy and epithelium degeneration of the seminiferous tubules in rats treated with 1mg/kg of nicotine for 2 months (Rachid *et al.,* 2011). This might have led to the decline in the size of the tubules and hence loose arrangement of the tubules within the testes.

Apart from causing reduction on the seminiferous tubule size, nicotine was also found to cause degeneration and disorganisation of germ cells in the seminiferous tubules of the nicotine group (Kushwaha and Jena, 2014; Jalili *et al.,* 2014). In the present study, thinner layer of spermatogenic cells was observed lining the inner wall of the seminiferous tubule of the testes belonging to rats in the nicotine group compared to that of testes from the *Nigella sativa* and nicotine-*Nigella sativa* groups. This might be due to the inadequate testosterone level which affect the thickness of the spermatogenic cell

layer. Sufficient amount of testosterone presence in the testes is critical for maintenance of the structural morphology and physiology of the seminiferous tubule (Cremades *et al.,* 2004). The hormone is also essential to maintain the spermatogenesis process (Zhang *et al.,* 2003). This was in agreement with previous study that nicotine administration resulted in reduction of spermatogenic cell mass which was found to be associated with a significant low testosterone level after 4 weeks of 100µg/ml of nicotine administration (Kushwaha and Jena, 2014). Another study reported a poor development of germ cell in the seminiferous tubule due to a low testosterone level in rats treated with a higher dose (0.6mg/100g) of nicotine as compared to a lower dose (0.2mg/100g) (Egesie *et al.,* 2013). Therefore, thinner spermatogenic cell layer in the nicotine group in the present study indicated an interruption of spermatogenesis which was most likely resulted from a low testosterone level.

Nicotine exposure also caused overproduction of reactive oxygen species (ROS) which gave unfavourable effect on the seminiferous tubule (Mosbah *et al.,* 2015). Excessive production of ROS consequently led to lipid peroxidation that can directly cause structural damage and dysfunction of the testes (Saalu, 2010). This was manifested in a previous study where testicular damage was associated with the alleviated lipid peroxidation levels (Uygur *et al.,* 2014; Mohamed and Mohamed, 2015). Thus, this might explain the abnormal histoarchitecture of seminiferous tubule in the nicotine group of present study.

The detrimental effect of nicotine on the testes histological features could be overcome with administration of medicinal plant such as *Nigella sativa.* It was observed in the present study that co-administration of *Nigella sativa* oil would give similar histological features of the testes as observed in the control group. Few other studies had

also reported the beneficial effects of *Nigella sativa* oil. It was reported to be able to ameliorate the damaging effects of hydrocortisone by the presence of numerous germ cells lining the seminiferous tubules which surrounded by healthy basal lamina (El-Hakem *et al.,* 2013). In addition, different study on the testes histological features of *Habbatus sauda* treated rats showed a smaller lumen with thick germinal cell layer lining the seminiferous tubule (Ng *et al.,* 2014). Another study showed that co-administration of colchicine and aqueous suspension of powdered *Habbatus sauda* seed had led to a recovery in the germ cells linings of seminiferous tubules including the basement membrane and the Leydig cell in the interstitial space (Shebab *et al.,* 2014). A pretreatment with 250mg/kg of *Habbatus sauda,* linseed and celery oils to male rats with testicular injury caused by sodium valproate for 4 weeks had also demonstrated an improvement to the histological features of the seminiferous tubules which also suggesting the protective effect of *Nigella sativa* oil (Hala, 2011).

The protective effect exhibited by *Nigella sativa* oil against testicular toxicity is possibly due to the antioxidant properties of *Nigella sativa*. Researchers reported that an active compound in *Nigella sativa*, the thymoquinone exhibited antioxidant pharmacological activities would protect organs against the oxidative damage (Chandra *et al.,* 2009; Ragheb *et al.,* 2009; Saleem *et al.,* 2012). This was supported by previous study that co-administration of cadmium chloride and thymoquinone would improve the testes histology to normal histological appearance (Sayed *et al.,* 2014). The presence of thymoquinone in the *Nigella sativa* oil would maintain the structure and functional integrity of the spermatogenic epithelium thereafter explained the ameliorated seminiferous tubules that were surrounded by basal lamina and lined by epithelium layer of normal thickness (Richardson *et al.,* 1998; El-Hakem *et al.,* 2013). This was in

agreement with numerous studies that antioxidant could attenuate testicular oxidative stress induced by nicotine (Li *et al.,* 2014).

Nigella sativa also contains unsaturated fatty acids, linoleic and oleic acid which may help to protect cell damage caused by nicotine. Study reported that linoleic acid would increase membrane fluidity of spermatozoa which was pivotal in fertilisation (Lovejoy, 2002; Elzanaty *et al.,* 2004). This was supported by a study that mice treated with oat oil which is rich with unsaturated fatty acids had a normal cellular arrangement within the seminiferous tubules of their testes (Halima *et al.,* 2014). In addition, presence of amino acid, specifically arginine in *Nigella sativa* may also have beneficial effect on the testicular structure. It had been reported that arginine was able to protect testicular germ cell specifically spermatozoa against lipid peroxidation (Srivastava *et al.,* 2000).

In the present study, the histological features of seminal vesicle in the nicotine group demonstrated minimal presence of acidophilic secretion in its lumen as compared to the other groups. It was reported that any disruption in the structure of seminal vesicle could lead to a decrease in its secretion (Gupta *et al.,* 2007). This was supported by a study that the reduction in the secretion was accompanied with the atrophy of the glandular tissue (Mathur and Malarvizhi, 1995). Previous researchers hypothesised that nicotine probably caused the impairment of seminal vesicle function by either defect in the mitochondrial genome or mitochondrial enzymatic activities (Sunanda *et al.,* 2014). The abnormal histoarchitecture of seminal vesicles thus could distrupt normal function of the seminal vesicle secretion for activation, motility and nourishment of spermatozoa (Gonzales, 1989; Bhatia *et al.,* 2010; Ambika and Selvasabhanayakam, 2012) which is important in ensuring male fertility (Pang *et al.,* 1979; Pakrashi and Chatterjee, 1995; Zhang and Jin, 2007).

Similarly, absence of acidophilic secretion in the lumen of prostate gland was observed in the rats exposed to the nicotine. This could probably be due to the decrease of testosterone level as found in the previous study by Khaled *et al.* (2014). In addition, previous study reported that acute depletion of testosterone might lead to the apoptotic cell death of prostate gland, thereafter caused reduction in the secretory content (Shappell *et al.*, 2004). Thus, depletion in the testosterone level would affect the quantity of acidophilic secretion presence in the lumen.

Besides, low testosterone level also affected the epithelial cell height of the accessory glands. In the present study, the histological features of seminal vesicle in the nicotine group showed a flattened epithelial cell that lined the seminal vesicle as compared to the tall epithelial cell in the other groups. This was supported by a previous study that rat intraperitoneally treated with nicotine exhibited lower epithelial cell height in the seminal vesicle compared to the control group (Londonkar *et al.*, 2000). This atrophy of epithelial cells was thought to be due to a decrease in the level of testosterone as what had been demonstrated in the seminal vesicles of castrated rats (Farooq and Hayfaa, 2011).

Similar finding was observed in the prostate glands of rats in the nicotine group that exhibited a flattened epithelial cells lining the mucosa of the gland. This was in accordance with previous report that the prostate gland of rats exposed to cigarette smoke had decreased epithelial height with severe interstitial oedema (Carvalho *et al.*, 2006; Mohamed *et al.*, 2012). Reduction in testosterone level subsequently altered the prostate gland histology such as decrease in epithelial cells height as manifested in previous studies (Yamashita *et al.*, 1996; Vilamaior *et al.*, 2000). This was also supported by other study that nicotine significantly reduced the circulating testosterone level suggesting that

prolonged nicotine intake might affect fertility (Hekmat, 2010; Carvalho *et al.,* 2012; Galam *et al.,* 2013).

Nicotine is recognised as an important agent involved in the damaging effect of biological molecules such as increased free radical production (Wetscher *et al.,* 1995; Yildiz *et al.,* 1999). In the past, studies had shown that nicotine induced intracellular oxidative stress both *in vivo* and *in vitro* which affected male reproductive system leading to infertility (Wetscher *et al.,* 1995; Chen and Greene, 2004; Micinski *et al.,* 2011). A study reported that chlopyrifos, an insecticide, which was known to cause oxidative stress, had damaging effects on the seminal vesicle. Fat infiltration and fibrosis were observed at the basement membrane of the seminal vesicle of the treated group but not in the control group (Olorunshola *et al.,* 2011). Therefore, reduction in the epithelial height of seminal vesicle and prostate gland in nicotine treated rats of the current study could also be due to the oxidative stress.

On contrary, antioxidant was reported to influence the integrity and minimised the harmful effects of oxidative damage in the reproductive tissues of laboratory animals (Altan *et al.,* 2003; Tauler *et al.,* 2003; Dehghani *et al.,* 2012). In present study, it was shown that the normal histology features of seminal vesicle and prostate gland were restored in the nicotine-*Nigella sativa* group. Similar finding was reported with co-administration of chlopyrifos and a common antioxidant, ascorbic acid, whereby presence of secretion in the lumen and normal epithelial cells were noted (Olorunshola *et al.,* 2011). Similarly, another study which used selenium as antioxidant supplementation also showed an increase in the epithelial cell height of prostate gland (Sakr *et al.,* 2012). In addition, another study using a typical antioxidant, vitamin E, also showed a normal histology of the seminal vesicle. Vitamin E was reported to be able to improve the

reproductive function by protecting the glands against lipid peroxidation (Naji and Fakhir, 2013). Moreover, onion was also reported to exhibit antioxidant which would maintain the histology structure of seminal vesicle (Slimestad *et al.,* 2007; Odumosu *et al.,* 2013).

The protective effect of *Nigella sativa* oil was also demonstrated in the prostate gland tissue. In the present study, it was observed that administration of *Nigella sativa* oil exhibited tall columnar epithelial cells with relatively packed prostatic acini in the *Nigella sativa* and nicotine-*Nigella sativa* groups. Probably, *Habbatus sauda* oil exhibited its antioxidant effect that would protect the glands. Previous study by Mohamed *et al.* (2010) using honey which is well known to have antioxidant pharmacological affect gave similar result. A restoration of height in the epithelial cell lining the mucosa of prostate gland was observed in rats exposed to cigarette smoke and supplemented with honey (Mohamed *et al.,* 2012). Other researchers revealed similar findings that spaces between acini were reduced with administration of antioxidant such as zinc, vitamin C and vitamin E (Sayed Gabry *et al.,* 2014). In addition, a study using supplementation of *Allium sativa* from the same *sativa* family as *Nigella sativa* also demonstrated absence of histopathological lesions, minimal acinar distortion and atrophy of the glands (Isaac *et al.,* 2014). Thus, ameliorated histological noted structures in the accessory glands of the current study might also be due to the antioxidant pharmacological effect of the *Nigella sativa* oil.

Numerous studies also reported that *Nigella sativa* oil also increased the testosterone level in male (Mansi, 2005; Khaled *et al.,* 2014; Farooq and Hayfaa, 2011). Testosterone is critical in differentiation and maintenance of the seminal vesicle (Ojeda and Urbanski, 1994). A Previous study had reported that castrated rats treated with *Nigella sativa* oil showed an improvement in the histological features of their seminal vesicle which was associated with high concentration of testosterone (Farooq and Hayfaa,

2011). In present study, the *Nigella sativa* oil might have counteracted the damage caused by nicotine by increasing the level of testosterone and thereafter maintained the normal histological features of the seminal vesicle.

In addition, increase level of testosterone would also affect the histoarchitecture of prostate gland as demonstrated in the *Nigella sativa* and nicotine-*Nigella sativa* groups. Interestingly, it was shown that in the non-treated castrated rats, the histological features of prostate gland had showed atrophy of secretary cells with presence of vacuolation. On the other hand, castrated rats treated with *Nigella sativa* oil showed more secretion in lumen of the gland with highest secretory cells height (Faroq and Hayfaa, 2011). This finding was in agreement with present study which suggesting the ameliorating effect of *Nigella sativa* oil on the histological features of the prostate gland, seminal vesicle and testes of nicotine treated rats.

5.2 TESTICULAR CELL COUNT

Nicotine that present in cigarette smoke has mutagenic consequences towards the germ cell production and maturation as well as the reproductive organ (Yamamoto *et al.,* 1998). These effects might be manifested by sperm count, sperm motility grade and sperm morphology features in histology of reproductive organs (Mahanem *et al.,* 2006). Previous researchers had stated that nicotine served as an independent predator in male infertility whereby it adversely affects testicular spermatogenesis (Aydos *et al.,* 2001; Nessiem *et al.,* 2011). The histomorphometric analysis of testicular cell count showed that nicotine treated group had a deteriorated spermatogenesis which was demonstrated by a reduction in number of the spermatogenic cells. In the present study, it was observed that the number of spermatogonia was significantly lower in the nicotine group compared

to the other groups. The finding was also in agreement with most reports from previous studies (Zhang *et al.,* 2009; Gambo *et al.,* 2013; Kushwaha and Jena, 2014).

Paniagua *et al.* (1991) also reported that germ cells namely spermatocyte and spermatids were susceptible to toxic materials. It is because exposure to cytotoxic agents such as nicotine, cigarette smoke and/or polycyclic aromatic hydrocarbons lead to testicular atrophy, abnormal morphology of the germ cell and disruption in spermatogenesis. Furthermore, study also reported that any disruption in the latter could cause lethal damage to the differentiated spermatocytes (Keating *et al.,* 1997). It was supported by previous finding which reported that nicotine administration resulted in decline number of spermatocytes and spermatids cells in mice treated with graded doses of nicotine for 15 days (Reddy *et al.,* 1998). In addition, the accumulation of electron-densed deposits in the spermatids observed in previous study corroborated the sensitivity of germ cells towards nicotine (Aydos *et al.,* 2001). Similar finding was also observed in the study done by Aslan *et al.,* (2015). Therefore, presence of harmful material such as nicotine might interfere spermatogenesis which lead to significantly lower number of spermatogenic cells as exhibited in the nicotine group of present study.

The presence of free radical in the testes might also lead to germ cell apoptosis; subsequently reduced the number of spermatozoa cell (Ozen *et al.,* 2002; Fujii *et al.,* 2003; Zhou *et al.,* 2006). It was hypothesised that nicotine was able to provide free radicals either by releasing accumulated lipid hydroperoxides from sperm membranes or by direct generation of oxygen derivatives (Arabi, 2004). It served as a powerful fuel for lipid peroxidation cascade in attacking the polyunsaturated fatty acids (PUFA) and caused oxidative damage (Girotti, 1998; Ayala *et al.,* 2014). Spermatozoa are extremely sensitive to free radical due to high PUFA content in their cells membrane, thus making them

particularly vulnerable to oxidative damage (Alvarez and Storey, 1995). Furthermore, the oxidation of the membrane fatty acid also lead to loss of membrane fluidity, subsequently decreased the activity of enzymes and ion channels in spermatozoa cells (Rao *et al.,* 1989). This will eventually lead to cell death that explains the reduce number of spermatogenic cells in the nicotine treated group of present study.

Moreover, presence of oxidative stress might also affect the number of spermatozoa in the nicotine group as observed in present study. The stress occurred due to imbalance in the reactive oxygen species (ROS) and antioxidant level (Arabi, 2006). Numerous studies had showed the positive association between cigarette smoking and increased level of oxidative stress, which adversely affected the spermatozoa (Ramadan *et al.,* 2002; Erat *et al.,* 2007; Jana *et al.,* 2010). This was concurrent with another study which recorded a significant decrease in the spermatozoa concentration and motility of nicotine treated rats (Gambo *et al.,* 2013).

Notably, oxidative stress that occurs among the Leydig cells may also lead to reduce spermatogenic cell count. A previous finding reported that the oxidative stress which was triggered by streptozotocin could alter the normal spermatogenesis (Ricci *et al.,* 2009). Oxidative stress due to cigarette smoking was reported to cause a marked reduction in the number of Leydig cells, thereafter leading to deprivation of pituitary gonadotrophin hormone which is vital for spermatogenesis and steroidogenesis processes (Reddy *et al.,* 1998; La Maestra *et al.,* 2015). In an *in vitro* study, researchers reported that the production of sexual hormones would be indirectly altered by apoptosis of Leydig cell due to nicotine exposure (Kim *et al.,* 2005). Reduction in Leydig cells count as seen in the present study, may cause disruption in the spermatogenesis and results in male infertility.

Follicle stimulating hormone (FSH) is another gonadotrophin hormone that also influence the testicular cell count since the conversions of testicular germ cells are highly dependent on FSH (Reddy *et al.*, 1998). It has an important role to ensure the initiation and maintenance of spermatogenesis (Hall, 1994). This is because upon stimulation of both the FSH and also luteinising hormone (LH), testosterone will be produced by the Leydig cells for normal spermatogenesis (Razi *et al.,* 2012). This was supported by an *in vivo* study that reported a low number of spermatogenic cells was associated with a significant decline in the testosterone level after an exposure to cigarette smoke for 8 weeks (Zhang *et al.,* 2009). Moreover, FSH helps to enhance Sertoli cell in the synthesis of androgen binding protein which helps in sustaining a high concentration of testosterone level (Shan *et al.,* 1995; Koksal *et al.,* 2003; Akkoyunlu *et al.,* 2007). Thus, diminished number of spermatogenic cell count in current study may indicate an inhibition of pituitary FSH release caused by nicotine.

In addition, nicotine might also inhibit the LH metabolism by either affecting the intracellular calcium content or blocking the effects of calcium on steroidogenesis of the Leydig cells in mouse (Patterson *et al.,* 1990). This was supported by previous study that cigarette smoking caused secretary dysfunction of Leydig cells (Parazzini *et al.,* 1993; Yamamoto *et al.,* 1998). Another scientific study recorded the presence of high accumulation of positive Sudan Black B indicating lack of LH in the testis interstitial and seminiferous tubule of high dose nicotine-treated group (Reddy *et al.,* 1998). The result was supported by another *in vivo* study which showed a significant reduction in LH level measured by radio-immunity method after 8 weeks of passive smoking exposure in rat models (Zhang *et al.,* 2009). Therefore, deprivation of hormones could cause degeneration of the Leydig cell demonstrated by the low cells count in present study.

Interestingly, the present study showed that co-administration of 6µl/100g *Nigella sativa* oil and nicotine in rats was able to inhibit the harmful effects induced by nicotine on the testicular cells count. This was demonstrated by an improvement in the germ cell count in the *Nigella sativa* and nicotine-*Nigella sativa* groups suggesting an undisrupted spermatogenesis. Numerous earlier studies recorded the favourable effects of *Habbatus sauda* oil not only in spermatogenesis, but also in the overall reproductive parameters in male rats and chickens (Al-Sa'aidi *et al.,* 2009; Mukhallad *et al.,* 2009; Abdulkarim and Al-Sardary, 2009). In a study done on a group of infertile men also demonstrated an improvement in sperm count, morphology and motility that was treated with *Habbatus sauda* oil (Kolahdooz *et al.,* 2014).

In addition, another study showed that male mice administered with aqueous suspension of *Nigella sativa* (*Habbatus* sauda), 1000mg/kg of body weight had a normal spermatogenesis as in the control group (Al-Nailey, 2010). In a different study, minimal suppression of spermatogenesis was also observed in the mice given *Nigella sativa* prior to cimetidine, a drug to reduce gastric acid secretion, compared to their control group (Al-Nailey, 2010). This showed that *Nigella sativa* oil reduced the testicular toxicity of cimetidine which was clinically reported to have anti-androgen activity at high dose (Al-Nailey, 2010; Okon and Okon, 2014).

It had also been recorded that treatment with aqueous extract of *Nigella sativa* would restore spermatogenesis by increasing the primary spermatocytes, secondary spermatocytes cells and spermatids cell counts (Al-Sa'aidi *et al.*, 2009; Mukhallad *et al.*, 2009). Previous study showed that *Nigella sativa* seed could act on the coenzymes in metabolic pathways of steroid hormone production which would stimulate the Leydig cells function in the testes (Al-Zamely, 2008). Thus, the increase of luteinising hormone

level by *Nigella sativa* oil could explain the important role of luteinising hormone on stimulating Leydig cells to produce testosterone. Testosterone is critical in order to stimulate growth and secretary activity of the reproductive organs. The increase in the testosterone level would also indirectly stimulate a normal, uninterrupted spermatogenesis (Bhasin *et al.,* 1988; O'donnel *et al.,* 1994; Singh *et al.,* 1995). Therefore, a significant increase in testosterone which improved testicular cell count could possibly due to either direct action of *Nigella sativa* or testosterone indirect action on the seminiferous tubules which had thereafter elevated the number of testicular somatic and germinal cells in present study.

Besides, *Nigella sativa* is known to exhibit antioxidant pharmacological activities. The antioxidant properties of *Habbatus sauda* oil may be involved in protecting the reproductive system although the underlying mechanism of it is still unclear (Al-Ali *et al.,* 2008). To date, antioxidants are known to have the ability to reduce the oxidative stress by breaking the oxidative chain reaction (Chen *et al.,* 2006; Bilaspuri and Bansal, 2008; Elbetieha *et al.,* 2011). Under normal conditions, the reactive oxygen species (ROS) is neutralised by antioxidants found in the ejaculatory fluid (Lamirande and Gagnon, 1999). However, at times of insufficient antioxidant activity or increase production of ROS level, an increase in the oxidative stress level may lead to cell damage (Aitken and Krausz, 2001; Schulte *et al.,* 2010).

In support with the antioxidant activity, previous study showed that *Nigella sativa* oil had an excellent ability to scavenge free radicals (Burits and Bucar, 2000). Moreover, the antioxidant effect of the oil was also proven in another study which demonstrated a significant protection against aflatoxicosis in rats given with *Nigella sativa* diet (Abdel-Wahhab and Ali, 2005). The protective effect of *Nigella sativa* oil on

the rat testes was manifested by an increase in the weight of testes, improve sperm count and quality, increase serum testosterone level, and decrease lipid peroxidation in the testes (Hala, 2011).

The main active compound in the *Nigella sativa* oil, thymoquinone was recorded to exhibit strong antioxidant properties and could reduce the reactive oxygen species level in semen (Butt and Sultan, 2010; Al-Wafai, 2013; Tembhurne *et al.,* 2014). Numerous studies indicated protective effect of thymoquinone on mice testicular parameters with reduced malondialdehyde, a marker for oxidative stress level (Al-Ali *et al.,* 2008; Gokce *et al.,* 2010a).

In light of a previous study, the restoration in the germ cells count might be due to the presence of thymoquinone (Gokce *et al.,* 2010b). Administration of thymoquinone was shown to preserve the spermatogenesis. This finding was indicated by a significant increase in the Johnsen scoring which was used to access spermatogenesis in testicular biopsies (Gokce *et al.,* 2010b; Fouad and Jresat, 2014). Presence of this antioxidant compound in the *Nigella sativa* oil might also explain the improvement in the testicular cell count of the *Nigella sativa* and nicotine-*Nigella sativa* treated rats in the current study.

5.3 IMMUNOHISTOCHEMISTRY STUDIES OF MALE REPRODUCTIVE ORGANS

In the testes, androgen receptor (AR) is detected in peritubular of seminiferous tubules, Leydig and Sertoli cells (Walker, 2011). The localisation of AR in germ cells however was controversial. Some studies found AR staining while others showed absence of AR staining in the germ cells (Sar *et al.,* 1990; Bremner *et al.,* 1994; Wang *et al.,* 2009). Androgen receptor (AR) in the accessory glands, seminal vesicle and prostate gland are

strongly characterised in their epithelium and stromal cells (Cooke *et al.,* 1991; Pelletier *et al.,* 2000).

In the present study, weak AR staining was noted in the testes, seminal vesicle and prostate gland of the nicotine group. The minimal AR protein staining might be attributed to the oxidative stress caused by nicotine. This was proven by an increase in the level of malondialdehyde (MDA) which was a marker for oxidative stress and reactive oxygen species (ROS) level in rats exposed to nicotine (Kiziler *et al.,* 2007; Al-Malki and Moselhy, 2013). The damage was observed when oxidative stress affect the genetic material causing lipid peroxidation in cell membranes as well as inactivated the membrane-bound enzymes (Savarino *et al.,* 2001). This was supported by latest literature study whereby the prostate glands of aged rats showed a negative immunoreactivity in the epithelial and stromal cells of the tissue due to oxidative stress (Banerjee *et al.,* 2001; Sayed Gabry *et al.,* 2014). The disrupted AR protein receptor site caused by oxidative stress would lead to weak AR staining as observed in current study.

In addition, nicotine was also found to interfere the production of steroid hormones (Rannikk *et al.,* 1995; Krishnamoorthy *et al.,* 2013; Tinti and Soory, 2014). Previous study suggested that reduction in serum testosterone level would lead to minimal expression of AR in the nicotine treated rats (Nair and Rajamohan, 2014). Testosterone, the main androgenic steroid hormone is synthesised by Leydig cells and regulated by luteinising hormone (LH) (De Gendt *et al.,* 2004). It was reported that testosterone was an important hormone responsible for initiation and maintenance of spermatogenesis as well as male fertility (Sharpe, 1994; Weinbauer and Neschlag; 1998; McLachlan *et al*., 2002). Apoptosis of Leydig cell would interrupt the normal function of Leydig cell subsequently suppress the testosterone secretion in male rats (Yeh *et al.,* 1989; Chen *et*

al., 2001; Walker, 2011). This was confirmed where weak AR staining was observed in the seminal vesicle and prostate gland of orchietomised rats (Nishino *et al.,* 2004). Low testosterone could affect the staining of AR in the testes of nicotine group since AR was highly regulated by steroid hormone.

Low testosterone level which lead to weak AR staining might also due to aromatisation of testosterone to oestrogen (Klaiber and Brovermann, 1988; Pasqualotto *et al.,* 2004). Researchers reported that tobacco intake would increase the norepinephrine level which was able to further enhance the aromatisation of testosterone (Pasqualotto *et al.,* 2004). This would lead to a disturbance in androgen/ oestrogen ratio causing hormonal imbalance (Pasqualotto *et al.,* 2004; Sunanda *et al.,* 2014). Increase in oestrogen level could subsequently activate the oestrogen receptor (ER) signalling pathway while the fluctuated testosterone level could minimise the optimum regulation of the AR (Rannikk *et al.,* 1995; O'donnell *et al.,* 2001). This was also proved by Candido *et al*., (2012) that imbalance of testosterone/ oestrogen ratio in the prostate gland could lead to weak AR expression. Thus, the obstruction in the balance of androgen/ oestrogen level would lead to minimal AR staining as noted in all male reproductive tissues of the nicotine group in the present study.

On the other hand, it was showed in the present study that co-administration of nicotine and *Nigella sativa* (*Habbatus sauda*) oil improved the quality of AR staining. This might be due to the presence of thymoquinone in the *Nigella sativa* seeds and oil that exhibited antioxidant properties (Mansour *et al.,* 2002; Ince *et al.,* 2013). Antioxidant is pivotal to inhibit oxidation of membrane fat polyunsatured acids and DNA from oxidative stress (Savarino *et al.,* 2001). Thymoquinone also increased the antioxidant enzymes activity and provided protection to the reproductive organs. Although lack of

studies was conducted on the antioxidant properties of thymoquinone on the AR of male reproductive tissues, exemplary findings had demonstrated the importance of antioxidants to protect the tissues against oxidative stress (Hatch *et al.,* 1987; Kolleck *et al.,* 2002). As for the prostate gland, administration of antioxidants such as zinc, vitamin E and vitamin C showed presence of AR immunoreactivity in the nuclear cell and stroma with the intensity differed from moderate to intense AR immunoreactivity (Sayed Gabry *et al.,* 2014). Therefore, high intensity of AR staining in the *Nigella sativa* and nicotine-*Nigella sativa* groups of presence study might be due to the direct effect of the antioxidant properties in *Nigella sativa* oil on the prostate gland, seminal vesicle and testes.

It was also been reported in a previous study that administration of *Nigella sativa* oil would increase testosterone, LH and FSH levels (Mosbah *et al.,* 2014). It was hypothesised that *Nigella sativa* oil would give androgenic effects by stimulation of Leydig cells in the testes. This would then trigger the action of coenzymes in the metabolic pathways and led to an increase in production of steroid hormones (Mukhallad *et al.,* 2009; Al-Nailey, 2010). Furthermore, *Nigella sativa* increased the testosterone level by converting the cholesterol molecule into testosterone via stimulating the activity of 17β-hydroxysteroid dehydrogenase which was an important key enzyme in the testosterone synthesis pathway (Gromadzka-Ostrowska *et al.,* 2002; Al-Zamely, 2008). The increase in testosterone level would affect the expression of AR staining since it was positively correlated with androgen level (Zalata *et al.,* 2013). This was demonstrated by a previous study that administration of testosterone propionate to orchiectomied rats could enhance the intensity of AR immunoreactivity in the seminal vesicle and prostate gland (Nishino *et al.,* 2004). High intensity of AR staining demonstrated in rats treated with *Nigella sativa* oil could be related to the elevated serum testosterone level which associated with administration of *Nigella sativa*.

CHAPTER 6:
CONCLUSION

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This research was conducted to investigate the protective effects of *Nigella sativa* and nicotine administration on male rat reproductive system. The observations involved testicular cell count, histological features and presence of androgen receptor in the testes, seminal vesicle and prostate gland.

It was observed that administration of 0.5mg/100g body weight of nicotine for 100 days had a damaging effect on the histological features of rat testes, seminal vesicle and prostate gland. Present results indicated that administration of *Nigella sativa* could increase the testicular cells count and the co-administration of nicotine and *Nigella sativa* could reduce the damaging effect caused by nicotine on the testicular cells. In addition, he protective effects of *Nigella sativa* were manifested by the strong immunoreactivity of androgen receptor observed in the testes, seminal vesicle and prostate gland of nicotine-*Nigella sativa* treated rats..

The co-administration of *Nigella sativa* with nicotine had resulted in a reduction of damaging effects on the male reproductive organs exposed to nicotine. This protective effect of *Nigella sativa* could be due to the present of antioxidant properties that scavenged free radicals released by nicotine.

The limitation of presence study would mostly be on lacking of a quantitative evaluation on the histological findings. In addition, quantitative analysis was also needed for the intensity evaluation of androgen receptor in the selected tissues using immunofluorescent technique.

Therefore, it would be interesting to conduct a further study on the mechanism of how *Nigella sativa* helps to promote the protective effects on the damaged histological features and androgen receptor of the male reproductive organs exposed to oxidative stress presence in the nicotine. Moreover, antioxidant study could also be performed in order to determine the capability of *Nigella* sativa to scavenged free radicals. This could can be conducted by evaluating the effects of *Nigella sativa* active compound such as thymoquinone on the male reproductive system. The role of *Nigella sativa* on the expression of androgen receptor protein and mRNA can also be performed in the future. Lastly, the effects of *Nigella sativa* at the cellular level could be carried out by evaluating the ultrastructural changes on the tissues using transmission electron microscope (TEM).

It is essential to understand the mechanism in order to develop new strategies in the assisted reproductive technologies as well as to maintain a good male reproductive health, particularly among the cigarette smokers.

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APPENDICES

Appendix 1

Preparation of normal saline solution

1. Measure 9g of sodium chloride
2. Add 1000ml of distilled water
3. Stir well until all dissolved

Appendix 2

Preparation of 3.5% chloral hydrate solution

1. Measure 100 ml of distilled water into a measuring cylinder
2. Weight 3.5 g of chloral hydrate
3. Add in the measured chloral hydrate into the distilled water
4. Stir well and stored

Appendix 3

Preparation of 10% formalin solution

1. Measure 100ml of 40% formaldehyde and empty it into a conical flask
2. Add distilled water up to 1000 ml and stir until dissolved

Appendix 4

Preparation of 0.1M phosphate buffer solution (PBS) pH 7.4

3. Measure 200 ml of distilled water and empty into a conical flask.
4. Add in a tablet of PBS into the distilled water
5. Stir well until all dissolved
6. Store in refrigerator

Appendix 5

Procedure of specimen processing for light microscopy study

1.	Wash specimen with 0.1M PBS pH 7.4 (3x)	30 minutes/each
2.	Dehydrate specimen in series of ascending alcohol	
	50% alcohol	1 hour
	70% alcohol	1 hour
	80% alcohol	1 hour
	95% alcohol	1 hour
	Absolute alcohol (3x)	1 hour/each
3.	Immerse specimen in solution containing an equal part of clearing agent (cedar wood oil) and absolute alcohol	Overnight
4.	Clear in pure cedar wood oil	Overnight

Appendix 6

Procedure for wax impregnation

1. Dip specimen in benzene for few seconds
2. Immerse in an equal part solution containing paraffin wax and benzene for 2 hours
3. Immerse in a series of 4 paraffin wax jars at melting point of 55 – 60 ^{o}C (1 hour each)

Appendix 7

Procedure for tissue embedding

1. Mould is filled with clean paraffin wax at melting point of 50 – 60 ^{o}C.
2. Specimen is placed inside the mould using forcep
3. The filled in mould is placed on a cold plate to allow it to solidify
4. A labelled embedding ring is placed on top of the mould
5. Wax is added until the embedding ring is filled
6. The solidified block of embedded specimen is kept in a cool dry place

Appendix 8

Procedure for Haematoxylin and Eosin (H&E) staining

1.	Deparaffinise sections in xylene (2x)	3 minutes/each
2.	Rehydrate sections in series of descending alcohol	
	Absolute alcohol (2x)	2 minutes/each
	90% alcohol	2 minutes/each
	80% alcohol	2 minutes/each
	70% alcohol	2 minutes/each
	50% alcohol	2 minutes/each
3.	Immerse sections in distilled water	3 minutes
4.	Stain with filtered Haematoxylin (Appendix 13)	20 minutes
5.	Differentiate with acid alcohol (Appendix 14)	Few seconds
6.	Blueing under running tap water	5 minutes
7.	Stain with Eosin (Appendix 15)	5 minutes
8.	Dehydrate sections in series of ascending alcohol	
	95% alcohol (2x)	2 minutes/each
	Absolute alcohol (3x)	2 minutes/each
9.	Clearing with xylene (2x)	3 minutes/each

Appendix 9

Procedure for immunohistochemical staining

1^o antibody = mouse anti-androgen receptor (AR) (Santa Cruz) with dilution of 1:100

2^o antibody = goat anti-mouse (Chemicon)

Normal serum = normal goat serum

1.	Deparaffinise sections in xylene (4x)	5 minutes/each
2.	Rehydrate sections in series of descending alcohol	
	Absolute alcohol (2x)	1 minutes/each
	90% alcohol	1 minutes/each
	80% alcohol	1 minutes/each
	70% alcohol	1 minutes/each
	50% alcohol	1 minutes/each
3.	Immerse sections in distilled water	1 minutes
4.	Perform antigen retrieval treatment using citrate buffer solution (Appendix 10). Microwave is heated at:	
	70^oC	6 minutes
	100^0C	7 minutes
5.	Cool sections in ice water	
6.	Quench sections with 3% hydrogen peroxide (H_2O_2)	1 hour
7.	Wash with tris buffered saline (TBS) (3x) (Appendix 11)	2 minutes/each
8.	Treat sections with normal serum	2 hours
9.	Wash with TBS (3x)	2 minutes/each
10.	Treat with primary antibody	overnight
11.	Wash with TBS (3x)	2 minutes/each

12.	Treat with secondary antibody	2 hours
13.	Wash with TBS (3x)	2 minutes/each
14.	Incubate sections with streptavidin-horseradish peroxidase (HRP) solution	10 minutes
15.	Wash with TBS (3x)	2 minutes/each
16.	Incubate sections with chromogen reagent 3,3' diaminobenzidine (DAB) solution (Appendix 12)	10 minutes
17.	Wash with TBS (3x)	2 minutes/each
18.	Counterstain with Haematoxylin	45 seconds
19.	Wash with TBS (3x)	2 minutes/each
20.	Dehydrate with a series of ascending alcohol	
	95% alcohol	1 minute
	Absolute alcohol	1 minute
21.	Clear sections with xylene	5 minutes
22.	Mounting	

Appendix 10

Preparation of 10mM citrate buffer pH 6.0 for antigen retrieval

1. Measure 1000ml of distilled water
2. Add in 2.94g of tri-sodium citrate (dehydrate)
3. Stir until dissolved
4. Slowly add in hydrochloric acid to adjust to pH 6.0 using pH meter

Appendix 11

Preparation of tris buffered saline (TBS)

1. Measure 10ml of 20x TBS
2. Add distilled water up to 200ml
3. Add 200µl of Tween® 20
4. Stir well before use

Appendix 12

Preparation of chromogen reagent 3,3' diaminobenzidine (DAB) solution

[Preparation for one test]

DAB chromogen A	25µl
DAB chromogen B	0.625ml

1. Add DAB chromogen A and DAB chromogen B in a 1:25 ratio using separate pipette tips for each chromogen to avoid contamination.

Appendix 13

Procedure for Harris Haematoxylin

Mercuric oxide	1g

Solution A

Haematoxylin	2g
Absolute alcohol	20ml

Solution B

Distilled water	400ml
Aluminium potassium sulphate	40g

1. Dissolve Haematoxylin in the absolute alcohol (**Solution A**)
2. Boil aluminium potassium sulphate in distilled water (**Solution B**)
3. Add in **Solution A** into **Solution B**
4. Stir and heat again until simmer
5. Add mercuric oxide into the beaker. The solution will turn dark purple
6. Cool the beaker in the ice water
7. Filter before use

Appendix 14

Procedure of acid alcohol

1. Measure 140ml of absolute alcohol
2. Add 60ml of distilled water into the absolute alcohol
3. Add in 1ml of hydrochloric acid in the solution and stir well

Appendix 15

Procedure of Eosin

1. Measure 160ml of absolute alcohol
2. Add 40ml of distilled water into the absolute alcohol
3. Add in 2g of eosin
4. Stir until all eosin dissolved
5. Add in 1ml of acetic acid
6. Stir

Appendix 16

List of proceedings and publication

2014

Lina, S., Hashida, N.H., and Eliza, H. (2014). Role of *Habbatus sauda* towards the histological features of nicotine treated male rats seminal vesicle and prostate gland. *Biomedical Research.* 25 (1): 11-18. (*ISI-Cited Publication).*

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Lina, S., Hashida, N.H., and Eliza, H. Role of *Habbatus sauda* towards the histological features of nicotine treated male rats seminal vesicle and prostate gland. INTERNATIONAL CONFERENCE ON NATURAL PRODUCTS 2012 (ICNP 2012). Shah Alam Convention Centre, Malaysia. 4^{th} – 6^{th} March, 2013. *(International Proceedings).*

Lina, S., Hashida, N.H., and Eliza, H. Effect of *Habbatus sauda* on the spermatogenic cell of nicotine treated male rats. 5^{th} GLOBAL SUMMIT ON MEDICINAL AND AROMATIC PLANTS 2013 (GOSMAP 2013). Miri, Sarawak, Malaysia. 8^{th} – 12^{th} December 2013. *(International Proceedings).*