AMELIORATIVE POTENTIAL OF THYMOQUINONE IN NICOTINE-TREATED RATS: SPERM CHARACTERISTICS AND EXPRESSION LEVEL OF *PRM1* AND *TNP2* GENES

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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AMELIORATIVE POTENTIAL OF THYMOQUINONE IN NICOTINE-TREATED RATS: SPERM CHARACTERISTICS AND EXPRESSION LEVEL OF *PRM1* AND *TNP2* GENES

ABSTRACT

Thymoquinone (TQ), the main constituent of the volatile oil derived from *Nigella sativa* has been extensively studied for its various therapeutic properties. This study was conducted to assess the effects of TQ in ameliorating the injurious state of sperm in infertility-induced rats caused by nicotine, the active component in cigarettes. Experiments were conducted on adult male Sprague Dawley rats which were divided into four groups: 1) control group, received normal saline orally for 60 days; 2) nicotine group, was subcutaneously injected with 5 mg/kg/day nicotine for 30 days and then given normal saline for next 30 days; 3) TQ group, was given normal saline for 30 days and followed by TQ 5 mg/kg/day for another 30 days; 4) nicotine-TQ group, received 5 mg/kg/day nicotine for 30 days and followed by TQ 5mg/kg/day for 30 days. The sperm count, motility, membrane integrity, mitochondrial function and DNA integrity in sperm were evaluated. Also, the expression level of genes responsible for chromatin condensation, *PRM1* and *TNP2* were analyzed. Results showed significantly lower number of sperm count (26.72 ± 1.64 10^6/ml) and sperm motility (66.24 ± 1.01 %) in nicotine group but higher number in nicotine-TQ group (30.97 ± 0.88 10^6/ml and 85.02 ± 2.24 %, respectively; $p<0.05$). Results of sperm membrane integrity test and the number of MitoTracker positive sperm also showed a significantly lower percentage in nicotine group (47.34 ± 0.69 % and 75.68 ± 0.90 %, respectively) but a notable improvement in nicotine-TQ group was observed (52.58 ± 1.14 % and 79.08 ± 0.74 %, respectively). Moreover, TQ significantly decreased DNA fragmentation in sperm of nicotine treated rats in Comet assay (2.40 %; $p<0.05$). Sperm mitochondrial function showed a significant correlation with DNA damage which was inversely proportional to

each other (r = -0.480; p<0.01). By RT-qPCR analysis, the expression level of *PRM1* and *TNP2* genes were evaluated to ascertain if DNA damage occurring in this study as a result of dysregulation of chromatin protein gene. There were no changes in expression level of *PRM1* in nicotine, TQ and nicotine-TQ group, whilst *TNP2* was downregulated in nicotine group (0.047 ± 0.009) and slightly but significantly upregulated in nicotine-TQ group (0.111; p<0.05). This suggests that the DNA damage observed in this study may not be induced by abnormal chromatin packaging. Nevertheless, further investigations on the effects of nicotine and TQ on *TNP2* need to be explored in the future. In conclusion, the present study demonstrates the potential benefits of TQ in improving the sperm quality of nicotine-induced damage.

Keywords: thymoquinone, nicotine, infertility, sperm quality, chromatin condensation genes

POTENSI AMELIORATIF TIMOKUINON DALAM TIKUS DIRAWAT NIKOTIN: CIRI-CIRI SPERMA DAN ARAS EKSPRESI GEN *PRM1* DAN *TNP2*

ABSTRAK

Timokuinon (TQ), konstituen utama minyak mudah ruap daripada *Nigella sativa* telah dikaji secara meluas kerana sifat terapinya yang pelbagai. Kajian ini telah dijalankan untuk menilai kesan amelioratif TQ terhadap kemudaratan sperma dalam tikus teraruh kemandulan yang disebabkan oleh nikotin, komponen aktif di dalam rokok. Penyelidikan telah dijalankan ke atas tikus matang 'Sprague Dawley' yang telah dibahagikan kepada empat kumpulan: 1) kumpulan kawalan, menerima salin normal secara oral selama 60 hari; 2) kumpulan nikotin, telah disuntik subkutaneus dengan 5 mg/kg/hari nikotin selama 30 hari dan kemudian diberi salin normal selama 30 hari berikutnya; 3) kumpulan TQ, telah diberi salin normal selama 30 hari dan diikuti 30 hari TQ 5 mg/kg/hari; 4) kumpulan nikotin-TQ, menerima 5 mg/kg/hari nikotin selama 30 hari dan diikuti TQ 5 mg/kg/hari selama 30 hari. Bilangan, motiliti, integriti membran, fungsian mitokondria dan integriti DNA sperma telah dinilai. Aras ekspresi gen, *PRM1* dan *TNP2* yang bertanggungjawab dalam kondensasi kromatin juga dianalisa. Keputusan menunjukkan penurunan bilangan sperma ($26.72 \pm 1.64\ 10^6$/ml) dan motiliti sperma (66.24 ± 1.01 %) yang signifikan pada kumpulan nikotin tetapi peningkatan bilangan pada kumpulan nikotin-TQ (masing-masing, $30.97 \pm 0.88\ 10^6$/ml dan 85.02 ± 2.24 %; $p<0.05$). Keputusan ujian integriti membran sperma dan bilangan sperma positif MitoTracker juga menunjukkan peratusan rendah yang signifikan pada kumpulan nikotin (masing-masing, 47.34 ± 0.69 % dan 75.68 ± 0.90 %) tetapi peningkatan yang jelas diperhatikan pada kumpulan nikotin-TQ (masing-masing, 52.58 ± 1.14 % dan 79.08 ± 0.74 %). Tambahan pula, TQ mengurangkan fragmentasi DNA pada sperma tikus terawat nikotin secara signifikan dalam ujian Comet (2.40 %; $p<0.05$). Fungsi mitokondria sperma mempamerkan korelasi signifikan dengan kerosakan DNA dimana

ianya berkadar songsang terhadap satu sama lain ($r = -0.480$; $p<0.01$). Melalui analisis RT-qPCR, aras ekspresi gen *PRM1* dan *TNP2* telah dinilai untuk memastikan samada kerosakan DNA berlaku di dalam kajian ini adalah disebabkan oleh gangguan regulasi gen protin kromatin. Tiada perubahan pada ekspresi gen *PRM1* pada kumpulan nikotin, TQ dan nikotin-TQ, manakala *TNP2* menunjukkan penurunan aras ekspresi gen pada kumpulan nikotin (0.047 ± 0.009) dan peningkatan regulasi yang signifikan pada kumpulan nikotin-TQ (0.111; $p<0.05$). Ini mencadangkan bahawa, kerosakan DNA di dalam kajian ini mungkin tidak dicetuskan oleh pengemasan kromatin yang abnormal. Walau bagaimanapun, penyelidikan lanjutan terhadap kesan nikotin dan TQ pada *TNP2* perlu dikaji pada masa hadapan. Kesimpulannya, kajian ini memperlihatkan potensi manfaat TQ dalam memulihkan kualiti sperma terhadap kerosakan teraruh nikotin.

Kata kunci: timokuinon, nikotin, kemandulan, kualiti sperma, gen kondensasi kromatin

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LIST OF SYMBOLS AND ABBREVIATIONS

2n	:	Diploid
ABP	:	Androgen binding protein
ACTB	:	Beta actin
ACTH	:	Adrenocorticotropic hormone
ANOVA	:	Analysis of variance
ATP	:	Adenosine triphosphate
b.w.	:	Body weight
BBB	:	Blood brain barrier
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
CAT	:	Catalase
CREM	:	cAMP response element modulator
cDNA	:	Complementary DNA
c-GT	:	c-glutamyl transpeptidase
CRH	:	Corticosteroid Releasing Hormone
CSE	:	Cigarette smoke extract
CT	:	Cycle threshold
DAPI	:	4',6-Diamidino-2-Phenylindole
DEPC	:	Diethyl carbopyronate
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleotide triphosphates
em	:	Emission wavelength

ESR1	:	Estrogen receptor 1
ESR2	:	Estrogen receptor 2
ex	:	Excitation wavelength
FISH	:	Fluorescence in situ hybridization
FSHB	:	Follicle-stimulating hormone beta
FSHR	:	Follicle-stimulating hormone receptor
FSH	:	Follicle-stimulating hormone
G-6-PDH	:	Glucose-6-phosphate dehydrogenase
GATS	:	Global Adult Tobacco Survey
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
gDNA	:	genomic DNA
GnRH	:	Gonadotropin-releasing hormone
GPx	:	Glutathione peroxidase
GR	:	Glutathione reductase
GSH	:	Gluthathione
HOST	:	Hypo-osmolality test
IACUC	:	Institutional Animal Care and Use Committee
ICR	:	Institute of Cancer Research
i.p.	:	Intraperitoneal
IUI	:	Intrauterine insemination
i.v	:	Intravenous
IVF	:	In Vitro Fertilization
LD_{50}	:	Lethal death 50%
LH	:	Luteinizing hormone
LM	:	Low melting agar
LPO	:	Lipid peroxidation

MDA	:	Malondialdehyde
MMP	:	Mitochondria membrane potential
mRNA	:	Messenger ribonucleic acid
n	:	Haploid
nAChRs	:	Nicotinic cholinergic receptors
NCBI	:	National Center for Biotechnology Information
NMA	:	Normal melting agar
NTC	:	No-template control
NS	:	*Nigella sativa*
OTM	:	Olive tail moment
p.o.	:	Per oral
PUFA	:	Polyunsaturated fatty acids
PRM	:	Protamines
PRM1	:	Protamine 1
PRM2	:	Protamine 2
qPCR	:	Quantitative Polymerase Chain Reaction
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
S.E	:	Standard error
SCSA	:	Sperm chromatin structure assay
SPSS	:	Statistical package for social science
s.c.	:	Subcutaneous
TL	:	Tail length
TM	:	Tail moment
TBARS	:	Thiobarbituric acid reactive substances
TBE	:	Tris Boric EDTA

TCM	:	Traditional Chinese Medicine
TUNEL	:	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay
TNP	:	Transition nuclear proteins
TNP1	:	Transition nuclear protein 1
TNP2	:	Transition nuclear protein 2
TNP4	:	Transition nuclear protein 4
TYH	:	Toyoda–Yokoyama–Hosi
TQ	:	Thymoquinone
UV	:	Ultra violet
WHO	:	World Health Organization
WPRO	:	World Health Organization Regional Office for the Western Pacific

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Infertility is a very important worldwide health issue recognized by the World Health Organization (WHO) due to the declining fertility rate displayed globally. According to Vital Statistics Malaysia 2018 report (Department of Statistics Malaysia, 2018), the total national fertility rate in 2017 was 1.9 babies per women aged 15 to 49 which is a decrease from 2.0 babies in 2015. The total fertility rate has been declining for the past 38 years and this is the lowest ever recorded since the formation of Malaysia in 1963. The fertility rate in Malaysia has been below the replacement level of 2.1 babies that is the average number of babies born per woman throughout her reproductive life that is sufficient to replace herself and her spouse. In an interview with Bernama, Dr. Azirawaty Mohd Tadzri, a local gynaecologist believed that Malaysians' wide perception of the inability to conceive being mainly due to female infertility is no longer applicable as many studies have shown that male infertility was on the rise due to declining sperm quality (Bernama.com, 2017). Among numerous factors causing infertility of couples, almost half of the cases are caused by male (Sharlip et al., 2002).

Cigarette smoking have been recognized as one of the risk factors for many fatal diseases and is reported to have damaging effects on the male reproductive function which could lead to infertility. The cigarette smoke has detrimental effects on sperm functions and sperm chromatin condensation (Ali Josaraei et al., 2008; Calogero et al., 2009; Mostafa et al., 2018).

The adverse effects of cigarettes on fertility could be the result of its notorious contents with nicotine being its primary psychoactive component (Colagar et al., 2007; Harlev et al., 2015). Nicotine is revealed to have unfavourable results on gametogenesis

mainly the sperm (Lodonkar et al., 1998; Ali Jorsaraei et al., 2008; Oyeyipo et al., 2011). Nicotine has negative impact towards sperm membrane and DNA integrity (Arabi, 2004). The component is well-documented to be able to raise oxidative stress by producing free radicals that is damaging at cellular level (Yildiz, 2004). The plasma membrane of sperm is particularly vulnerable to oxidative stress because of its structure (Agarwal et al., 2014). Impairment to the structure is associated with decline sperm motility which consequently affects the fertilization process. Damage of sperm by oxidative stress will cause defective sperm functions which account for a high fraction of infertility cases (Sharma & Agarwal, 1996). As an oxidant agent, nicotine could also induce cellular oxidative injury that results in DNA breakage (Mosadegh et al., 2017).

Damage of sperm DNA may also be the result of abnormal chromatin packaging during chromatin remodelling (Hekmatdoost et al., 2009). The sperm chromatin is extremely compact and is organized in a specific manner to provide a safe and secure transfer of the paternal DNA without being damaged or mutated (Oliva, 2006). During the late stage of spermatogenesis, histone proteins are replaced by transition nuclear proteins before being replaced by protamines (Shirley et al., 2004; Carrell et al., 2007). These proteins are important in promoting chromatin condensation and its dysregulation could risk DNA to become vulnerable towards damaging agents (Lewis et al., 2008).

Moreover, significant increase of free radicals has demonstrated to affect mitochondrial functions (Agarwal & Prabakaran, 2005) and since mitochondria of the sperm play a major role in supplying energy for their motility, disruption in the function of this vital organelle will ultimately lower the chances of fertilization.

The alarming infertility rate has forced scientist to further look into the matter. Natural products have been researched upon in tackling the problem as they are generally considered to be safe and comparable to the modern medicines. This is proven as 25% to 30% of modern medicines prescriptions have active ingredients originated

from plants (Kumar et al., 2012). Among many medicinal plants, *Nigella sativa* (NS) which is also known as Habbatus sauda is a prominent herb which various scientists have researched on for its pharmacological benefits and its very long historical and religious connections.

A study by Kolahdooz et al. (2014) showed that the quality of sperm and semen parameters of infertile patients were improved significantly after treatment with NS oil for 2 months. In addition, a recent study revealed the protective role of NS against reproductive toxicity (Mosbah et al., 2018).

Most of the pharmacological activities of NS are attributed to the presence of thymoquinone (TQ) as an active component and main constituent of the volatile oil derived from NS. Thymoquinone (TQ) has received particular consideration and has been extensively studied for its healing properties. A review paper on the therapeutic potentials of TQ showed that it has beneficial medicinal effects encompassing various areas such as antibacterial, anti-inflammatory, anxiety modulatory, and anticancer activities (Shoieb et al., 2003; Hannan et al., 2008; Nehar & Kumari 2012; Sayeed et al., 2014). Thymoquinone demonstrated strong antioxidant properties and oral administration of TQ was capable of protecting several organs against oxidative injuries (Nagi & Mansour, 2000; Mansour et al., 2002; Salem, 2005). In the male reproductive system in mice, TQ has been great protective and healing properties against heat stress and morphine treatment (Al-Zahrani et al., 2012; Salahshoor et al., 2018).

However, the role of TQ against infertility caused by nicotine, through sperm characteristic and DNA integrity assessment has not been studied so far. Therefore, the present study aims to investigate the potential ameliorative role of TQ on nicotine-induced sperm damage and its effects on genes responsible for sperm chromatin

condensation. Additionally, this study also provides some additional data on the adverse effects of nicotine on sperm quality and sperm chromatin condensation.

1.2 Objectives of Study

1. To elucidate sperm concentration and motility of nicotine and thymoquinone treated rats.
2. To analyse the sperm membrane integrity, mitochondrial function and DNA integrity of nicotine and thymoquinone treated rats.
3. To compare the expression level of *protamine 1* (*PRM1*) and *transition nuclear protein 2* (*TNP2*) genes on nicotine and thymoquinone treated rat testis.

CHAPTER 2: LITERATURE REVIEW

2.1 The Worldwide Scenario of Male Infertility

Infertility is commonly defined as the inability to conceive after 12 months or more of regular unprotected sex (Practice Committee of the American Society for Reproductive Medicine, 2013). The World Health Organization (WHO) recognized infertility is a major worldwide public health issue that transcends culture and society. It has become apparent that developed countries are experiencing rapid decline in fertility rates.

Infertility affects 10% to 15% of couples trying to conceive and male factor infertility accounts for almost half of the cases (Sharlip et al., 2002). Male infertility is any condition which adversely influences the chances of initiating pregnancy with female partner. Most commonly, those problems arise when the man is unable to produce or deliver fully-functioning sperm. The etiology of male infertility is multifactorial with largely remained idiopathic (Agarwal & Prabakaran, 2005). Causal of male infertility is often associated with an array of environmental, behavioural and genetic factors that may affect spermatogenesis at different stages (Toshimori et al., 2004). The negative changes usually result in reduced sperm count, abnormal sperm quality (e.g., reduced motility and altered morphology), or altered levels of sex hormones (e.g., reduced testosterone) which will eventually hinder the fertilization process (Agarwal et al., 2014).

2.2 Smoking and Male Infertility

World Health Organization (WHO) reported that 30% of men aged 15 years and older are smokers (Saleh et al., 2002). Previously, Trummer et al. (2002) reported that, men aged between 20 to 39 years who are of reproductive age form approximately 46% of the smokers. Cigarette smoking remains a global phenomenon despite its well-known damaging effects on health. It has been recognized as one of the important risk factors for many notable diseases such as cancer, hyperlipidemia and hypertension that could lead to heart attack and stroke (Celermajer et al., 1993; Ambrose & Barua, 2004).

In addition, a number of studies have reported the damaging effect of cigarette smoke on the male reproductive system which constitutes it as a risk factor of infertility. Several reports have argued that smokers displayed lower sperm count, sperm motility, abnormal morphology and may also result in DNA fragmentation compared to non-smokers (Ali Josaraei et al., 2008; Calogero et al., 2009).

Cigarette smoke comprises of 400 compounds and the major constituents which affect health are nicotine, particulate phase tar and gaseous phase carbon monoxide. Nicotine, the active and main component of cigarette has revealed to have adverse effects on gametogenesis mainly the spermatozoa. Nicotine is reported to have direct effects on sperm concentration and sperm motility characteristics (Lodonkar et al., 1998; Ali Jorsaraei et al., 2008). This finding is supported by Oyeyipo et al. (2011) who showed that sperm count and sperm motility of rats decline after treatment with nicotine. Nicotine also showed the ability to elevate oxidative stress by free radicals production that is harmful at the cellular level (Arabi, 2004; Yildiz, 2004). Sperm damage by oxidative stress may impair sperm functions (Makker et al., 2009).

Oxidative stress is stated to be the main cause of male infertility. Studies showed low level and adequate level of ROS (reactive oxygen species) played an important role in sperm physiology processes such as capacitation, hyper-activation, acrosome reaction and signaling process to ensure a complete fertilization. Conversely, ROS at a high level causes oxidative stress which consequently promotes male infertility through peroxidative damage of the sperm plasma membrane, DNA breakage and apoptosis (Tafuri et al., 2015).

2.3 Smoking Prevalence in Malaysia

In Malaysia, 10% to 12% causes of death are due to smoking habit which contributes to more than 10,000 deaths per year. In 2011, the fraction distribution of death attributed to smoking in government hospitals is 11,056 in total (Lim et al., 2013). A report by the World Health Organization Regional Office for the Western Pacific (WPRO) in 2012 estimated that the prevalence of smoking among adults in 2008 aged more than 15 years old is 15% to 74% in males and 2% to 62% in females. This report also estimated that the prevalence of male smokers in Malaysia is 43% while it is only 3% in females (Lim et al., 2013).

Meanwhile, according to Tan and Yen (2016), a survey by GATS (Global Adult Tobacco Survey) performed in Malaysia in 2011 found that 4.7 million adults (23.1%) which comprised of 43.9% male and 1.0% female were smokers. This study also demonstrated that 4.3 million adults (20.9%) in Malaysia smoked daily, comprising of 39.9% adult male and 0.7% adult female. The result also revealed that as much as 2.3 million adults (4 out of 10) is passively exposed to cigarette smoke in the work place,

7.6 million adults (4 out 10) is exposed to it at home and 8.6 million adults (7 out 10) is exposed to cigarette smoke in the restaurants each day (Tan & Yen, 2016).

2.4 Scenario of Smoking Related Male Infertility in Malaysia

Despite cigarette smoking being widely known as a health threat and a main cause of premature deaths worldwide (Practice Committee of the American Society for Reproductive Medicine, 2004), alarmingly the habit continues to grow. Mathers and Loncar (2006) reported an estimated increase of smokers from 1.3 billion to 1.6 billion globally by 2025 and they also predicted that the number of mortality due to smoking-related diseases would presumably reach 8.3 million by the year 2030. According to National Health and Morbidity Survey 2015, smoking-related diseases account for 15% of hospitalizations and 35% of inpatient hospital deaths in Malaysia. Smoking-related diseases have been a major cause of mortality in Malaysia with 20,000 deaths reported yearly (Institute for Public Health, 2015).

As was pointed out before, male infertility is very much on the rise and a contributing factor to declining fertility globally. Additionally, smoking causes destructive effects on the male reproductive system that may lead to infertility. There are rather scarce reports on the prevalence of smoking related male infertility in Malaysia. Nevertheless, with the number of male smoker and male infertility increasing in parallel over the years, it is safe to presume that Malaysia is not excluded from the rest of the world in regards to smoking related male infertility occurrence.

2.5 Male Reproductive System in Mammalian

In general, the organs of the male reproductive system consist of testes, duct system, accessory sex glands and several supporting structures, including scrotum and the penis. The testes produce sperm (male gamete) and secrete hormones. The system of ducts (comprising of epididymis, ductus deferens, ejaculatory ducts, and urethra) is responsible in transporting and storing sperm, provide a maturation site and conveys them to the exterior (Figure 2.1). The accessory sex glands provide secretions that complement sperm in the semen. The scrotum supports the testes outside of the abdomen which is necessary for its function and the penis deposits sperm into the female reproductive tract. Penis of rodents such as rat is enclosed in a sheath, called the prepus. Prior to mating, the contraction of muscles moves bacula, a type of bone, into the penis to stiffen it for copulation (Tortora & Derrickson, 2006; Costabile, 2013).

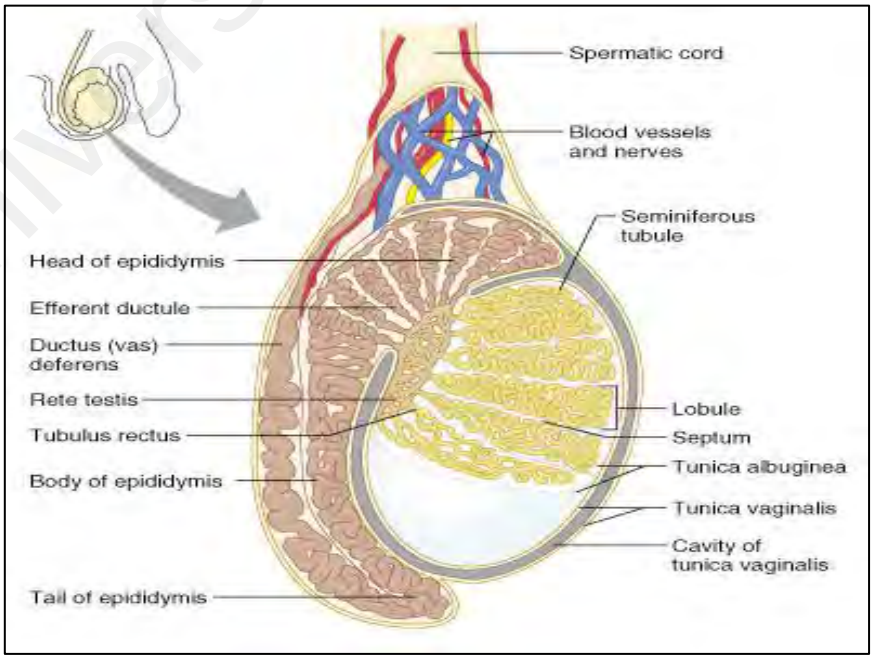


Figure 2.1: Male reproductive duct system (Marieb, 2016 - Reprinted with permission)

The male reproductive system is maintained by the hypothalamic-pituitary-testis axis through coordinated release of hormones. Gonadotropin-releasing hormone (GnRH) is released by hypothalamic neurosecretory cells and the secretion increases at puberty. This hormone will in turn stimulate gonadotrophs in the anterior pituitary to synthesize and secrete two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones enter the bloodstream and reach the testis. The LH stimulates testosterone (the most prevalent androgen) production by Leydig cells which are located in the interstitium between seminiferous tubules. Meanwhile, FSH indirectly supports spermatogenesis by acting synergistically with testosterone on Sertoli cells. Through a negative feedback mechanism, testosterone suppresses the secretion of GnRH by hypothalamus and LH by the anterior pituitary. In addition, when a point of spermatogenesis has been reached, Sertoli cells release inhibin, a glycoprotein hormone that suppresses FSH secretion by the anterior pituitary (Tortora & Derrickson, 2006; Costabile, 2013).

2.6 Spermatogenesis

Spermatogenesis is a process in which spermatogonia, the most immature germ cell develops into a mature spermatozoa or sperm. The spermatogenic cycle takes place along the length of the seminiferous tubule in a cyclic manner over time progressing through a number of stages. The spermatogonia contains diploid (2n) number of chromosomes and reside in the basement membrane of seminiferous tubules. Spermatogonia differentiates by mitosis into primary spermatocytes as they lose contact with the basement membrane and squeeze through the tight junctions of blood-testis barrier. The primary spermatocytes will undergo meiosis I that yields secondary

spermatocytes with a haploid (n) number of chromosomes that subsequently divides into spermatid in meiosis II. Once the process of meiosis is completed, no more cell division occurs as spermiogenesis begins (Figure 2.2).

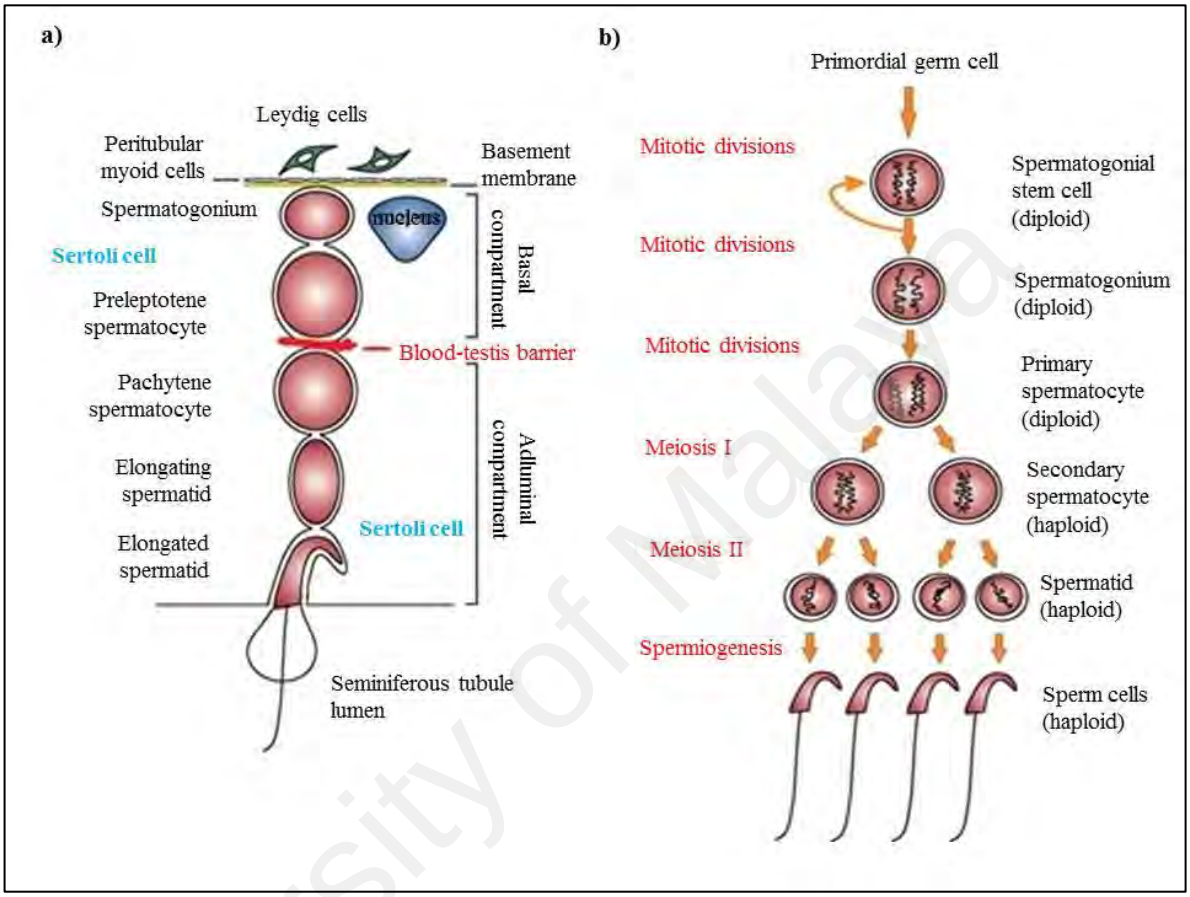


Figure 2.2: Spermatogenesis in the rat. (a) Drawing illustrating the morphological features of different cells during development from the basement membrane to the release in the lumen of seminiferous tubules. (b) Schematic drawing depicting the process that occurs in male germ cells during spermatogenesis (modified from Cheng & Mruk, 2010)

This final stage of spermatogenesis involves the development of each round spermatid into elongated sperm capable of motility. The metamorphosis occurring in this stage includes the development of acrosome, condensation of chromatin, formation of the flagellum, and migration of cytoplasmic organelles (Kretser et al., 1981). Sperm then enter the seminiferous tubule lumen as fluid secreted by Sertoli cells drives the sperm towards the ducts of testes for maturation, storage and transport (Tortora &

Derrickson, 2006; Varghese et al., 2010; Costabile, 2013). The numbers of stages differ among species, in the rat there are 14 stages (Aleem et al., 2005). A complete spermatogenic cycle for rats is about 56 days (Russell, 1990; Figure 2.3) and human spermatogenesis takes 65-75 days (Tortora & Derrickson, 2006).

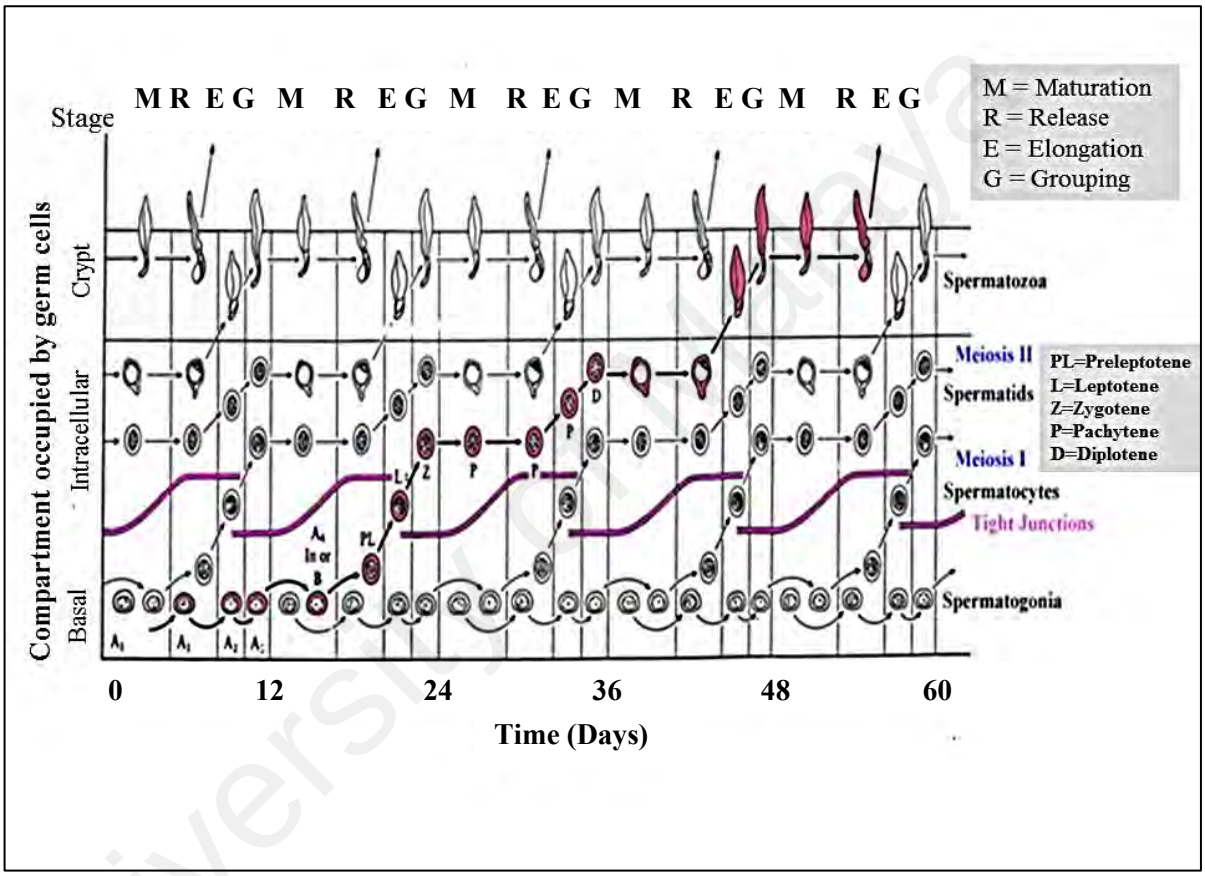


Figure 2.3: Stages of spermatogenesis in rat (modified from Austin & Short, 1972)

2.7 Sperm

Sperm is a male reproductive cell or a male gamete that contributes almost half of genetic information which fuses with female gamete to form offspring. Sperm or spermatozoon comprises of sperm head and sperm flagellum (tail). The head and tail

structures are covered by sperm plasma membrane. The human sperm heads are spatula-shaped (spatulate) and rodent sperm head is hook-shaped (falciform). The main contrast between human and rodent sperm is the complete lack of sperm centrosome and centrioles in the rodents compared to the reduced form of centrosome with a single proximal centriole in human. Apart from that, all eutharian mammals generally share similar features of sperm structure (Eddy & O'Brien, 2006; Sutovsky & Manandhar, 2006).

2.7.1 Sperm Head

The sperm head is composed of nucleus in which genetic material deoxyribonucleic acid (DNA) resides. The DNA of immature sperm contains histone linker protein that is later partially replaced by protamines during spermiogenesis (Zalenskaya et al., 2000). This positively charged protein ensures that the sperm nucleus is extremely compact and organized in a specific manner to protect it from damage and secure transfer of genome to the ovum for fertilization (Oliva, 2006; Gill-Sharma et al., 2011). Covering the anterior of nucleus with a less dense tip is acrosome, a cap like structure filled with enzymes that help a sperm to penetrate an oocyte (Tortora & Derrickson, 2006).

2.7.1.1 Sperm Chromatin Structure

During spermiogenesis, the process spermatids develop into mature sperm, haploid sperm chromatin undergoes an important change where histones are replaced by transition nuclear proteins (TNP) which are then replaced with protamines (Meistrich et

al., 2003; Shirley et al., 2004; Carrell et al., 2007; Figure 2.4). The organization of sperm chromatin maintains secure transfer of the very tightly packed paternal genetic information to the oocyte. The chromatin DNA of sperm is condensed with specific basic proteins and is at least six times more condensed than in somatic cells (Talebi, 2011). Nevertheless, damage to the sperm DNA can still occur in certain conditions such as due to free radical attacks and heightened apoptosis (Singh et al., 2003).

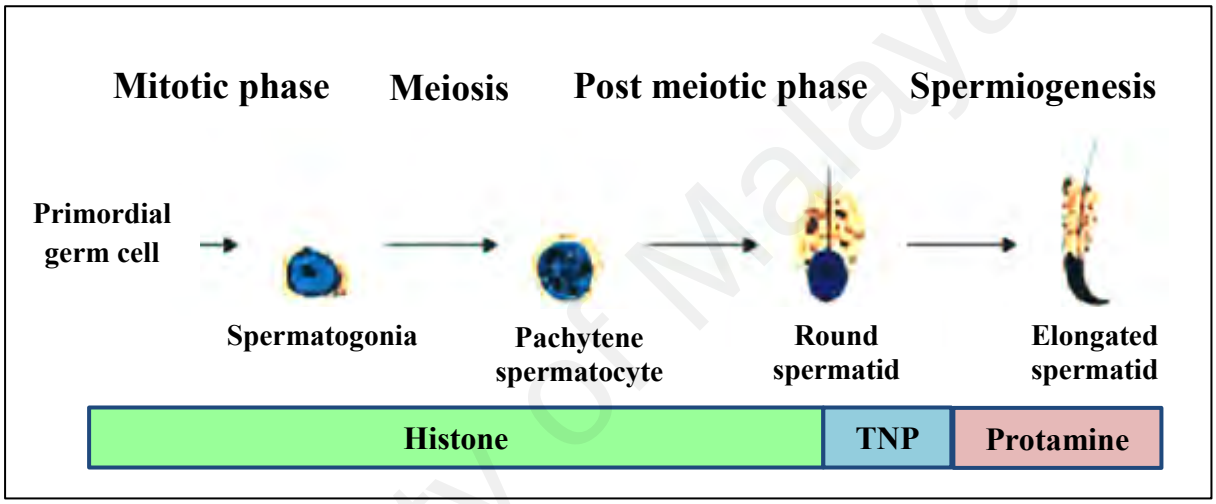


Figure 2.4: Chromatin remodeling during spermatogenesis which shows the transition from histones to protamines (modified from Pradeepa & Rao, 2007)

2.7.1.2 Transition proteins

In the final stage of sperm differentiation during spermiogenesis, sperm DNA is subjected to a major reorganization to facilitate a tighter, less vulnerable packaging. This is achieved by replacing most of histones with more basic proteins, protamines with the involvement of transition proteins (TP) or transition nuclear proteins (TNP) (Barone et al., 1994). Transition nuclear proteins are the intermediate proteins of sperm chromatin condensation which is the period of transition from histone to protamine

associated DNA. There are TNP1 to TNP4 variants with only TNP1 and TNP2 are well characterized and considered most important transition proteins. Both single-copy genes, *TNP1* and *TNP2* encodes TNP1 and TNP2 proteins, respectively (Meistrich et al., 2003). The three important events that occur during the TNP phase of spermiogenesis are 1) transformation of nucleosomal type chromatin into a smooth chromatin fiber, 2) beginning of chromatin condensation, and 3) cessation of transcription. The TNPs are believed to be involved in at least one or more of these processes (Kundu & Rao, 1996).

TNP2 is closely linked to two *protamine* genes (Engel et al., 1992), suggesting that they have an evolutionary relationship and have common functions. In contrast, *TNP1* is positioned on a separate chromosome (Heidarana et al., 1989). Disruption in *TNP* expression and binding may impair sperm DNA integrity (Venkatesh et al., 2011). The TNP1 protein is proposed to be actively involved in the displacement of histones from DNA as it relaxes the DNA, reducing the interaction of DNA with the nucleosome core (Dadoune, 2003). Caron et al. (2001) demonstrated that TNP1 also appears to facilitate DNA strand break repair by neutralizing the phosphodiester backbone of DNA and bringing nick-ends into close proximity. The TNP2 appears to be involved in chromatin condensation better than TNP1 and contribute in the beginning of chromatin packaging prior to the expression of protamines (Kundu & Rao, 1996; Lévesque et al., 1998).

2.7.1.3 Protamines

Protamines (PRM) are approximately half the size of a typical histone (5-8 kDa) and the strong DNA binding affinity is owing to the presence of arginines from 55% to 79% of the amino acid residues (Fuentes-Mascorro et al., 2000). Moreover, protamines have

increased number of positively charged residues in evolution which led to the formation of a highly condensed structure with the strong negatively charged, paternal genomic DNA (Oliva & Dixon 1990; Talebi, 2011). Additionally, the next stage of chromatin organization occurs during sperm epididymal maturation in which protamine cross-linking by disulphide bond formation takes place.

Protamine variants differ from species to species with two protamines found in mammals, protamine 1 (PRM1) and protamine 2 (PRM2) are the most widely studied. Protamine 1 is present in sperm of all mammals, whereas protamine 2 can be found only in some species including, hamster, mouse, stallion and man (Corzett et al., 2002). In rats, the experimental subject of the present study, have only one form of protamine which is PRM1 due to limited transcription and translation of *PRM2* as a result of inefficient promoter in addition to altered processing of the mRNA transcript (Balhorn, 1982; Bunick et al., 1990). Protamine is a crucial factor for proper chromatin condensation and irregular protamination increases the susceptibility of sperm to DNA injury (Simon et al., 2011).

Protamines are presently understood to be required for 1) the condensation of paternal genome to produce a more compact and hydrodynamic nucleus as sperm with a more hydrodynamic nucleus have the ability to move faster and hence have higher potential to fertilize the oocyte, 2) protecting the genetic information carried by the sperm from nucleases, mutagens or damage from reactive oxygen species or other toxic agents, 3) epigenetic remodeling during the process of spermiogenesis and 4) removal of transcription factors and proteins from spermatids to help reorganize the imprinting code in the oocyte (Oliva & Dixon, 1991; Oliva, 2006). Alteration of sperm protamine content can disturb any of the above mentioned normal sperm functions.

2.7.2 Sperm Flagellum

The sperm tail or flagellum which contains a long axial filament can provide a motile force for the sperm, is subdivided into connecting piece (neck), middle piece (midpiece), principle piece, and end piece. The neck is the constricted region behind the head which contains centriole for most mammals. Sperm initially contain two centrioles which are proximal and distal. At fertilization, only a single (proximal) is present, which in most mammals is considered to reconstitute the zygotic centrosome except for rodents where both centrioles are lost and only a maternal centrosomal inheritance occurs.

The midpiece contains mitochondria arranged in form of a helix, which generate energy for sperm motility taking them to the site of fertilization and for sperm metabolism. The principle piece is the longest portion and functions as sperm locomotion machinery (Kruger et al., 1986). This structure is similar to the midpiece except it is not covered by mitochondria sheath. The end piece is the tapering portion of the tail (Phadke, 2007). Crucial parts of the sperm for fertilization include the head, midpiece and sperm tail (Figure 2.5). Impairment of any of these structures could interfere with the fertilization process (Robinson et al., 2012).

2.8 Nicotine

Nicotine is a naturally occurring alkaloid found predominantly in the members of Solanaceae family, some of which are tobacco, potato, tomato, green pepper, and eggplant (Doolittle et al., 1995; Brčić Karačonji, 2005). It is a natural component in tobacco leaves from the plant *Nicotiana tabacum*, where it acts as a botanical insecticide

(Soloway, 1976; Tomizawa & Casida, 2003). It was first isolated as the major constituent of tobacco in 1828 (Schievelbein, 1982). *Nicotiana tabacum,* named after Jean Nicot de Villemain, the first person to import these plants from America to Europe in 1560 (Sierro et al., 2014) and now has become one of the most extensively cultivated non-food crops worldwide (Peedin, 2011). The term *Nicotiana* was initially used by Adam Lonitzer to describe the tobacco plants in 1630 and in 1788 by Carl von Linne´ (Linnaeus) to designate the entire genus (Sierro et al., 2014). In commercial tobaccos, the major alkaloid is nicotine, accounting for about 1.5% by weight and 95% of the total alkaloid content (Schmeltz & Hoffmann, 1977).

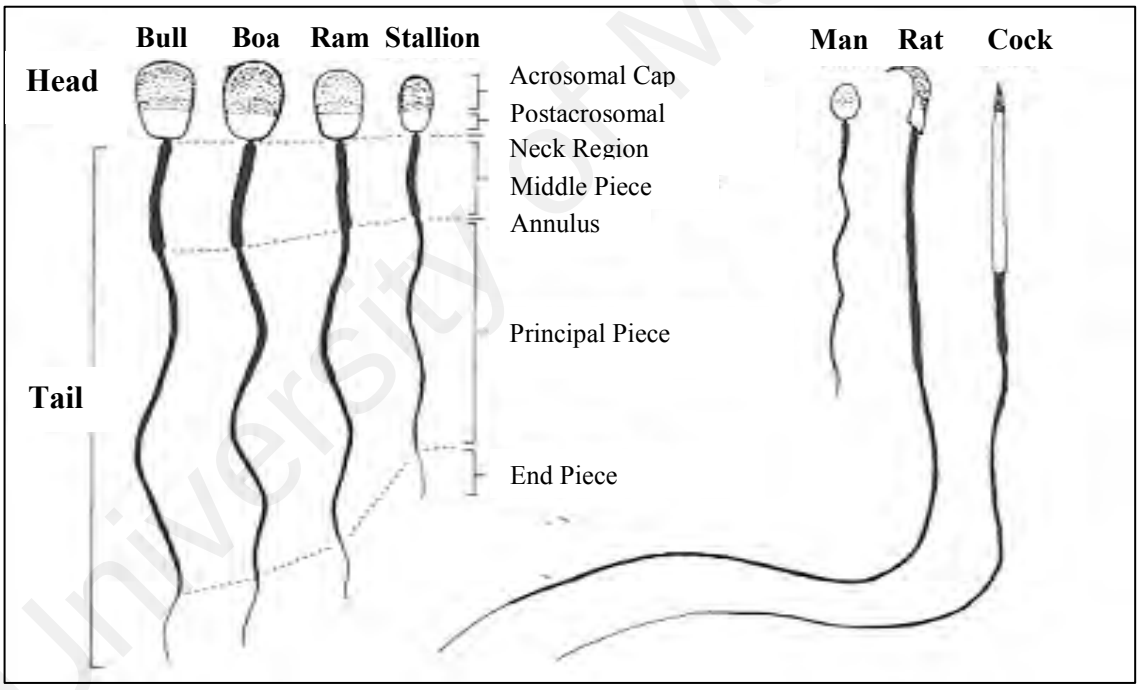


Figure 2.5: Comparison of the spermatozoa of various vertebrates (Frandson, Wilke & Fails, 2009 - Reprinted with permission)

2.8.1 Chemical Properties of Nicotine

The chemical structure of nicotine resembles neurotransmitter acetylcholine which acts on stereospecific nicotinic cholinergic receptors (nAChRs) in the brain and other organs (Benowitz et al., 2009; Figure 2.6). Nicotine has an active center and occurs as stereo isomers (Barlow & Hamilton, 1965). The structure was suggested by researchers in 1892 and was confirmed by synthesis in 1895 (Pictet & Crepieux, 1895). Pure nicotine is a clear liquid with a distinct odor but it turns brown on exposure to air. It is a strong base and has a boiling point of 274.5 °C at 760 Torr (Schievelbein, 1962). In the free base form, nicotine is less ionized, soluble in water and lipid as it can penetrate membranes more easily in alkaline solutions and consequently is readily absorbed via respiratory tissue, skin, and the gastrointestinal tract (Benowitz, 1988).

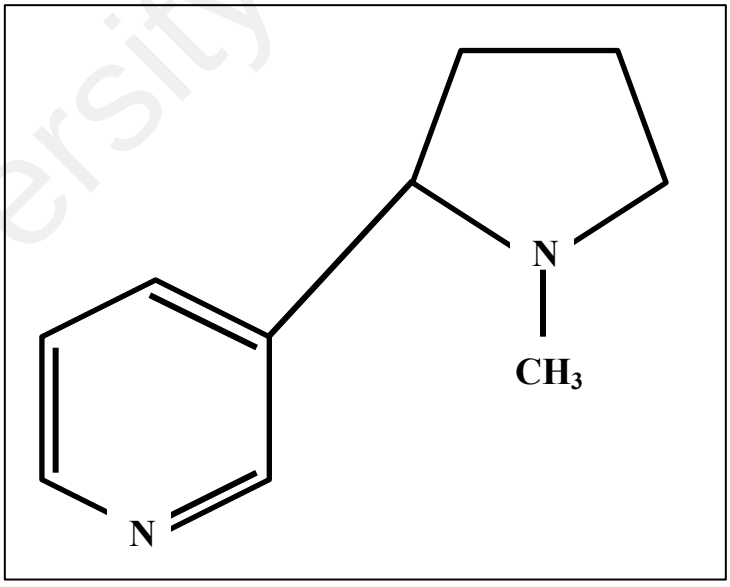


Figure 2.6: Chemical structure of nicotine

2.8.2 Pharmacokinetics of Nicotine

Nicotine absorption can occur through the oral cavity, skin, lung, urinary bladder and gastrointestinal tract (Schievelbein et al., 1973). The rate of nicotine absorption through the biological membranes depends on the pH (Schevelbein et al., 1973; Yildiz 2004). In its ionized state, such as in acidic environments, nicotine does not rapidly cross membranes. Nicotine is less absorbed via the buccal mucosa as the pH of cigarette tobacco is about 5.5 when it is highly positively charged. The principal route of nicotine absorption in smokers is through the alveoli of lungs attributed to its huge surface area and because of dissolution of nicotine at the physiological pH (approximately 7.4), which facilitates its transfer across cell membranes (Armitage, 1974; Benowitz et al., 2009). Due to the acidity of the gastric fluid, nicotine is poorly absorbed from the stomach. However, it is well absorbed in the small intestine which has a more alkaline pH and a large surface area (Yildiz, 2004). Nicotine base is absorbed well through the skin as there have been cases of poisoning after skin contact with nicotine contained pesticides and nicotine toxicity among tobacco harvesters (Saxena & Scheman, 1985; Benowitz et al., 1987; McBride et al., 1998). That is also the basis for transdermal delivery technology.

The time course of nicotine accumulation in the brain and in other body organs and the subsequent pharmacologic effects are greatly dependent on the route and dose. Smoking a cigarette delivers nicotine rapidly to the pulmonary venous circulation, where it moves quickly to the left ventricle of the heart and to the systemic arterial circulation and the brain. The time interval between a puff of a cigarette and nicotine reaching the brain is 10 to 20 seconds (Benowitz et al., 2009). Nicotine enters the bloodstream after absorption where, at pH 7.4, 69% is ionized and 31% is non-ionized with less than 5% binding to plasma proteins (Benowitz et al., 1982). The drug is

distributed extensively to body tissues with a steady-state volume of distribution averaging 2.6 body weight (Benowitz & Jacob, 1994). Human autopsy samples from smokers demonstrated the highest affinity of nicotine for the liver, kidney, spleen, and lung, and the lowest affinity for the adipose tissue (Urakawa et al., 1994). In skeletal muscle the concentrations of nicotine and cotinine are close to that of the whole blood. Nicotine has high affinity binding towards brain tissues, and the receptor binding capacity is increased in smokers compared to non-smokers (Breese et al., 1997; Perry et al., 1999). It can rapidly cross biological membranes and the blood brain barrier (BBB), thus reaching and consequently damaging neurons very easily (Hawkins et al., 2004; Table 2.1).

Nicotine metabolism in living organism is complicated where it is extensively metabolized to a number of metabolites by the liver. Six primary metabolites have been identified with the major metabolite in mammalian species being cotinine (Benowitz et al., 2009). In human, 70% to 80% of nicotine on average is metabolized to cotinine by C-oxidation (Brčić Karačonji, 2005). The most important enzyme in that process is CYP2A6, formerly known as coumarin 7-hydroxylase (Nakajima et al., 1996; Messina et al., 1997). Although most of the nicotine is metabolized through the cotinine pathway, only 10% to 15% of nicotine absorbed by smokers appears in the urine as unchanged cotinine (Benowitz & Jacob, 1994). Cotinine also passes the BBB and thus participates in the central effects of nicotine administration (Patel et al., 2010). Another primary metabolite of nicotine is Nicotine N‟-oxide even though only about 4% to 7% of nicotine absorbed by smokers is metabolized via this passage (Benowitz et al., 1994). Nicotine N‟-oxide shows no further metabolization to any significant extent, except by reduction back to nicotine in the intestines, which may lead to recycling of nicotine in the body (Benowitz et al., 2009) (Figure 2.7). The effects of nicotine have been widely

investigated in animals and in a variety of cell systems. It displayed various effects on multicellular organisms as well as individual cells (Table 2.2).

Table 2.1: Summary of nicotine pharmacokinetics

Absorption	• 10%–50% of available nicotine is absorbed during puffing; 80% during deep inhalation. • Readily and completely absorbed via mucous membranes and skin.
Metabolism	• 80% is metabolized in the liver to cotinine by enzyme CYP2A6 (and to a lesser extent by CYP2B6 and CYP2E1). • The rest is metabolized in the lungs and kidneys.
Distribution	• Readily distributed in all body tissues. • Reaches brain within 10 seconds. • Acute spike in arterial nicotine level occurs. • Steady state volume of distribution is approximately 2.6 times the body weight in kg.
Elimination	• Average elimination half-life is 2 hour. • Average elimination half-life of nicotine metabolite is 16 hour. • Cotinine is not detectable in the urine after complete abstinence for a week. • Unaltered nicotine and its metabolites excreted by kidneys.

(modified from Patel et al., 2010)

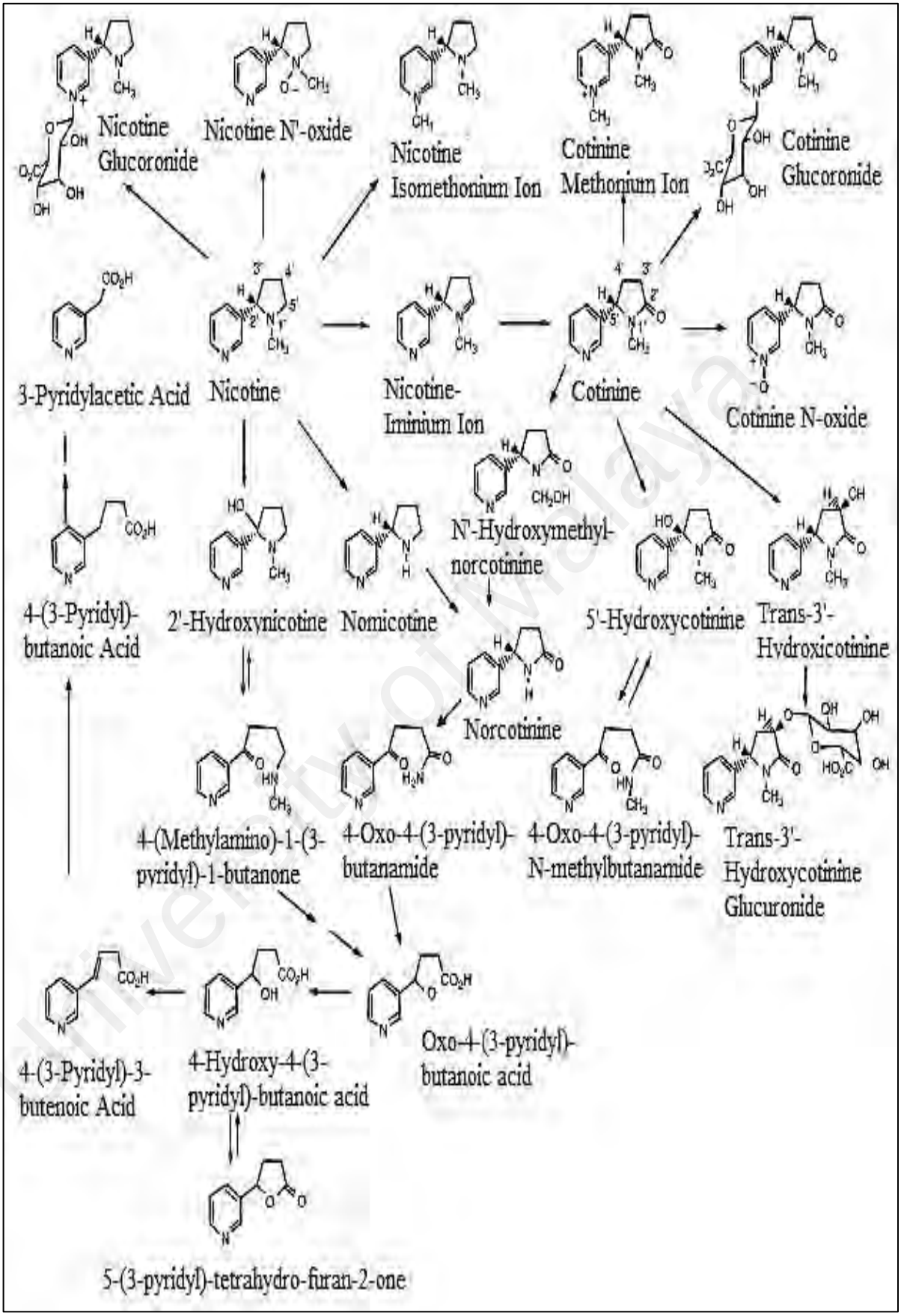


Figure 2.7: Pathways of nicotine metabolism (modified from Hukkanen et al., 2005)

Table 2.2: The general effects of nicotine

The effects at cellular level	The effects in whole organism
• Increased synthesis and release of hormones • Activation of thyrosine hydroxylase enzyme • Activation of several transcription factors • Induction of heat shock proteins • Induction of oxidative stress • Effects on apoptosis • Induction of chromosome aberrations • Induction of sister chromatids exchange	• Cardiac contractility • Increased blood pressure • Mobilization of blood sugar • Increase in free fatty acids in the blood • Increased catecholamine levels in the blood • Arousal or relaxation • Decreased skin temperature • Increased heart rate

(modified from Yildiz, 2004)

2.8.3 Adverse Effect of Nicotine on the Male Reproductive System

Cigarette smoking has been reported to have damaging effects on the male reproductive function that could eventually lead to infertility. Smoking showed its negative impact by exerting injurious effects on sperm count, kinetic parameters, morphology and DNA/chromatin integrity (Ali Jorsaraei et al., 2008; Calogero et al., 2009). The adverse effects of cigarette on fertility could be attributed to its content which includes several toxic chemicals, mutagens and carcinogens with the primary psychoactive component being nicotine. Nicotine represents 90% of total alkaloids in cigarettes (Colagar et al., 2007; Harlev et al., 2015) and mimics most of the deleterious effects of cigarette smoking (Kavitharaj & Vijayammal, 1999). Nicotine is revealed to provide an unfavorable environment for gametogenesis and have toxic effects on male gonadal functions (Table 2.3). It managed to reduce reproductive capacity and has mutagenic

consequences towards the germ cell production and maturation along with the reproductive organ itself (Yamamoto et al., 1998) and the accessory reproductive organs (Patil et al., 1999). Nicotine is reported to have direct effects on sperm counts (Londonkar et al., 1998) and sperm kinetic parameters (Ali Jorsaraei et al., 2008). As mentioned earlier, a study by Oyeyipo et al. (2011) showed that the number of sperm count and motility of male rats decreased after treatment with nicotine and Yildiz (2004) has proven that nicotine is able to cause cellular damage by producing free radicals.

Furthermore, owing to its unsaturated fatty acid structure, plasma membrane of sperm is particularly susceptible to free radicals (Agarwal et al., 2014). Injured membrane will consequently affect sperm motility and hamper successful fertilization. Nicotine has also been associated with DNA breakage resulting from free radical build up that induces cellular oxidative damage. Study by Mosadegh et al. (2017) determined that nicotine causes negative impacts by damaging the DNA through apoptosis activation. Sperm with damaged DNA lose their ability to fertilize an oocyte (Agarwal & Prabakaran, 2005) as its integrity is an important factor for embryo development and offspring production (Silva & Gadella, 2006).

Table 2.3: Chronology of the effect of nicotine on male reproductive system

Year	Authors	Findings
1988	Riesenfeld & Oliva	The reproductive capacity of the male rat is greatly reduced when injected with nicotine. Inflammatory processes, determined by an increased number of lymphocytes and/or polymorphonuclear leukocytes, were responsible for the decrease in fertility.
1995	Reddy et al.	Nicotine at concentrations of $\geqslant$1 mM significantly decreased sperm motion characteristics after different periods of incubation.

Table 2.3, continued

Year	Authors	Findings
1998	Londonkar et al.	Nicotine caused a reduction in the weight of epididymis and vas deferens. The total cholesterol content is increased while protein, DNA and RNA contents and the epididymal sperm count were decreased.
2000	Londonkar et al.	Delay of puberty in male rats treated with nicotine indicated by the absence of spermatozoa in the cauda epididymis and reduction in the activities of accessory sex organs.
2001	Aydos et al.	Ultrastructural alterations of testis in rats exposed to nicotine.
2004	Arabi	Decreased sperm membrane intactness and increased double-stranded DNA breaks of sperm nuclei in nicotine treated rats.
2006	Mahanem et al.	Administration of nicotine reduced the epididymal sperm count, grade of motility, percentage of normal sperm morphology and showed sign of testis degeneration.
2008	Ghaffari et al.	Inhibition of creatine kinase activity for normal sperm energy metabolism by nicotine.
2008	Ali Jorsaraei et al.	Nicotine and cotinine have negative effects on the sperm parameters.
2008	Kim et al.	Nicotine has toxic influences on sperm count and motility in adult ICR mouse.
2010	Jana et al.	Nicotine caused testicular toxicity by germ cell degeneration, inhibition of StAR gene expression and androgen production
2011	Nesseim et al.	Nicotine adversely affects testicular spermatogenesis in a dose- and time-dependent manner in adult albino rats.
2011	Lagunov et al.	Maternal nicotine-exposure during pregnancy and lactation induced transient structural changes in the testis and epididymis of male rats‟ offspring.
2013	Egesie et al.	i. Nicotine caused a significant reduction in sperm count, serum testosterone concentration and testicular weight. ii. Histopathology revealed a marked degeneration of germ cell layers in the seminiferous tubule and disruption of interstitial cells of the testis.

Table 2.3, continued

Year	Authors	Findings
2013	Condorelli et al.	Reduced percentage of viable spermatozoa and increased number of sperm in late apoptosis, with altered chromatin compactness, or DNA fragmentation in sperm exposed to nicotine.
2014	Oyeyipo et al.	Nicotine administration in rats caused decreased testicular antioxidants and increased testicular lipid peroxidation.
2015	Mosbah et al.	i. Nicotine induced oxidative damage in male rats as indicated by a significant reduction in the activities of antioxidant enzymes and an elevation in TBARS levels. ii. Decline in testosterone levels, weights of reproductive organs and sperm characteristics. iii. Thee histological examination of testes revealed atrophy, degenerative alterations and perturbation of spermatogenesis in several seminiferous tubules, together with increased interstitial spaces and reduced number of Leydig cells.
2018	Azad et al.	Nicotine treatment in male mice showed significant decrease in sperm motility, viability and fertilization rates along with poor blastocyst formation and increased sperm DNA damage, MDA levels and chromatin abnormality incidences.

2.9 Alternative Treatment of Male Infertility

Until today, there is no specific treatment that is available for improving fertile potential in a sterile male. Assisted reproductive techniques such as artificial insemination (AI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have helped these men to contribute in conceiving (Zini et al., 2004). Apart from that, assortments of medications have been developed in an effort to improve male fertility potential

(Kamischke & Nieschlag, 1999; Siddiq & Sigman, 2002; Comhaire & Mahmoud, 2003).

Naturally, therapies should be given directly focusing on the effects of the underlying causes. However, this has proven to be an insurmountable challenge as the cause of male infertility is multifactorial and for the most part remains idiopathic. Consequently, this has led to extensive use of alternative or complementary treatments that branch out to traditional treatments and medicine, including natural products supplements which has become a popular choice among patients (National Institute of Health, 1997). Traditional Chinese Medicine (TCM) has also shown to be one of the preferable treatments of choice. Their treatment includes the use of acupuncture, massages and herbal medicine (Crimmel et al., 2001).

In a study set out to review the use of medicinal plants for the improvement of spermatogenesis, Khojasteh et al. (2016) has listed out *Alpinia galanga, Danae racemose, Aloe vera, Phoenix dactylifera. L.* and many more which have been proven to be beneficial. Amidst these listed plants, *Nigella sativa* or Habbatus sauda is expectedly included.

2.10 The Importance of Medicinal Plant

The World Health Organization (WHO) stated that 60% to 80% of the world‟s population mainly in developing countries, depends on herbal remedies or traditional medicine for their primary health care and treatment (World Health Organization, 2002). Appreciating the importance of traditional and complementary medicine, the WHO reappraised „WHO Traditional Medicine Strategy 2002–2005‟ and developed „The WHO Traditional Medicine Strategy 2014-2023‟ to further strengthen the mission

to enhance the application of traditional medicine in the next decade (World Health Organization, 2013). Currently, the use of medicinal herbs or plants in the treatment of various diseases has been gaining worldwide interest due to effectiveness, fewer side effects, availability and being economical. Researchers suggest that two-thirds of the world‟s plant species have medicinal values and many of them have great antioxidant potentials comparable to the synthetic antioxidant (Krishnaiah et al., 2011).

Among many medicinal plants, *Nigella sativa* (NS) is a medicinally promising herb which has been researched upon and well-known for its pharmacological benefits.

2.11 *Nigella sativa*

Nigella sativa, a member of the botanical family of Ranunculaceae is an annual flowering plant. The fruit of the plant is large and its inflated capsule contains 3-7 united follicles with numerous seeds in each. The black colored seeds are 0.2 cm in length and 0.1 cm wide which are flattened, oblong, angular and funnel shaped (Ali & Blunden, 2003; Goreja, 2003). It is also called as black cumin, black seed, or Habbatul Barakah and is native to the south and southwest Asia, and cultivated in several countries of the Mediterranean region, South Europe, Syria, Turkey, and Saudi Arabia (Tariq, 2008; Shrivastava et al., 2011).

The plant has paramount historical and religious background and its seeds and oil have been commonly used as a folk therapy to cure various health conditions for more than 2000 years (Goreja, 2003). The extensive use of black seed by millions of Muslims all over the world is due to its spiritual and religious impact, as the prophet Muhammad (PBUH) advised “Hold onto use the black cumin, because it can heal every disease except death” (Goreja, 2003). It is also known as the curative black cumin in the Holy Bible and is described as the Melanthion of Hippocrates and Discroides and as the

Gith of Pliny (ne mann & Luetjohann, 1998). The seeds, which are the rich source of active ingredients of plant, have been used for a wide range of pathological conditions (Salem, 2005).

It is used in ethnomedicine to treat ailments and symptoms including, asthma, bronchitis, inflammation, eczema, fever, influenza, hypertension, cough, headache, dizziness, diabetes, kidney and liver dysfunctions, nervous disorders, rheumatism, cancer and related inflammatory diseases, gastrointestinal problems, and overall for general well-being (Khan, 1999; Ali & Blunden, 2003; Salem, 2005).

Researchers have attributed the medicinal effects of the black seed to its active constituents and high nutritional content. The seeds are composed of 28% to 36% fixed oils, proteins, alkaloids and saponins, and 0.4% to 2.5% essential oils. Many pharmacologically active compounds have been isolated from black seeds, however, most reported active compounds are thymoquinone (TQ), dithymoquinone, thymol, and thymohydroquinone (Tariq, 2008; Shrivastava et al., 2011; Al-Attas, 2016). Nevertheless, most of the known actions have been attributed mainly to TQ. Illustration of the *Nigella sativa* plant, seeds of plant and the chemical structure of TQ are presented in Figure 2.8.

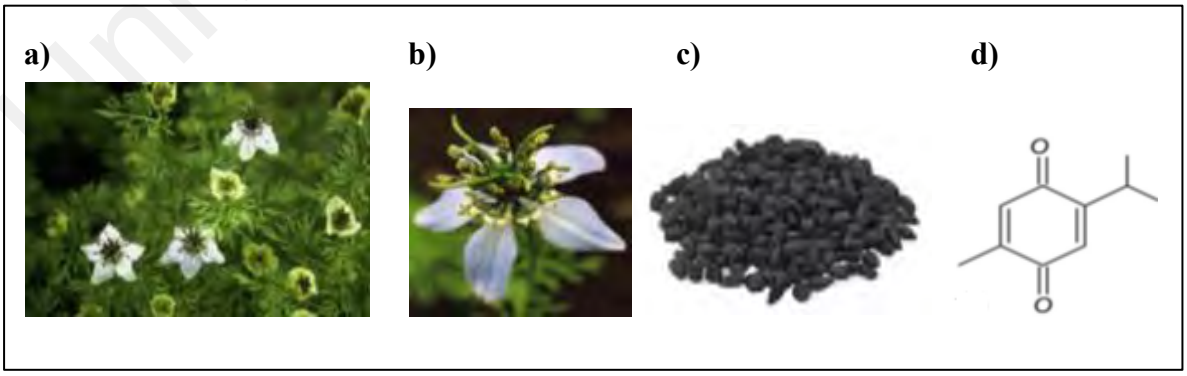


Figure 2.8: (a) The *Nigella sativa* plant (b) its flower (c) black seeds and (d) the chemical structure of bioactive component of seeds, thymoquinone (TQ) (modified from Darakhshan et al., 2015)

2.12 Thymoquinone (TQ)

Thymoquinone, as a naturally derived agent, has lately received particular consideration and has been extensively studied for its therapeutic properties. Thymoquinone (2-isopropyl-5-methylbenzo-1, 4-quinone) is the biologically active and most abundant compound of seeds and the volatile oil (18.4–24%) (Ali & Blunden, 2003). It was first extracted by El-Dakhakhny (1963) using thin layer chromatography on silica gel (Table 2.4).

Table 2.4: The properties of thymoquinone (TQ)

Molecular formula	$C_{10}H_{12}O_2$
Systematic/IUPAC name	2-Isopropyl-5-methylbenzo-1,4-quinone
Molar mass	164.20 g mol^{-1}
Appearance	Crystalline and dark yellow
PubChem CID	10281
CAS number	490-91-5

(modified from Darakhshan et al., 2015)

Thymoquinone is a bioactive component found in many medicinal plants other than Ranunculaceae. It is found in several genera of the Lamiaceae family such as Monarda, and the Cupressaceae families such as Juniperus (Taborsky et al., 2012). Different routes and modes administration of TQ includes intravenous (i.v.) (El Tahir et al., 1993), intraperitoneal (i.p.) (Abdel-Fattah et al., 2000; El-Gazzar et al., 2006), and oral sub-acute and sub-chronic administrations (Abdel-Fattah et al., 2000; Hosseinzadeh & Pavardeh, 2004). Oral administration leads to a biotransformation by the metabolizing activity of liver enzymes such as DT-diaphorase (a quininereductase) that catalyzes the reduction of TQ into a hydroquinone (Nagi & Almakki, 2009).

2.12.1 Therapeutic Effect of Thymoquinone on the Male Reproductive System

Several authors have extensively reviewed TQ‟s therapeutic potentials and showed that TQ has broad beneficial medicinal effects on various diseases such as antibacterial (Hannan et al., 2008), anti-inflammatory (Nehar & Kumari, 2012), anxiety modulatory (Sayeed et al., 2014), anticancer (Shoieb et al., 2003), analgesic, histamine release inhibitor, antihypertensive, hypoglycemic, antioxidant and hepatoprotective activities (Tariq, 2008; Shrivastava et al., 2011). Thymoquinone has been reported to protect vital organs from oxidative damage through its strong antioxidant properties (Nagi & Mansour, 2000; Mansour et al., 2002; Salem, 2005).

In the reproductive studies, this component has shown great protective and healing effects as TQ induced significant improvement of semen quality and reproductive characteristics against heat stress in mice (Al-Zahrani, 2012). Other than that, Salahshoor et al. (2018) demonstrated that TQ boosted sperm quality and improved morphine-induced adverse effects on reproductive parameters in male mice. In addition, a study by Tawfeek (2006) reported protective and antioxidant effects of TQ on testicular and accessory sex glands functions against oxidative stress induced by hydrogen peroxide. Moreover, TQ was shown to re-establish spermatogenesis after testicular injury by chronic toluene exposure (Kanter, 2011) and ameliorated lead-induced testicular function impairment in adult rats (Mabrouk & Ben Cheikh, 2016) (Table 2.5).

While there are vast studies on the effect of *Nigella sativa* on the male reproductive system, studies on the action of TQ on the particular system is rather scarce in comparison. This study was conducted to assess the effects of TQ in ameliorating the injurious state of sperm in infertility induced rats caused by nicotine.

Table 2.5: Chronology of the effect of *Nigella sativa* and thymoquinone on male reproductive system

Year	Authors	Findings
2006	Tawfeek	*Nigella sativa* oil counteracted the impairment in the epididymal sperm characters caused by H_2O_2 treatment through antioxidative actions.
2007	Bashandy	Administration of *Nigella sativa* oil to hyperlipidemic rats improved their reproductive efficiency and produced additional protection in hyperlipidemia-induced infertility.
2009	Al-Sa'aidi et al.	Alcoholic extract of *Nigella sativa* treatment in male rats caused significant increase in body weight, reproductive parameters and hormones.
2009	Mohammad et al.	The aqueous extracts of *Nigella sativa* increased spermatogenesis of male albino rats.
2010	Gökçe et al.	Thymoquinone treatment had a protective effect on testicular ischemia-reperfusion injury in mice through antioxidant activities.
2011	Gökçe et al.	Thymoquinone significantly reversed the histological changes of methotrexate destructive effects on testicular tissue.
2011	Hala	i. Pretreatment with *Nigella sativa* oil in sodium valproate-intoxicated rats induced significant increase in the weight of testis, sperm count and motility. ii. Increased serum testosterone levels and a decreased testicular lipid peroxides (MDA) and testicular degenerative lesions.
2011	Kanter	Thymoquinone treated rats showed an improved histological appearance of testis after testicular injury caused by chronic toluene exposure.
2012	Awadalla	Administration of *Nigella sativa* oil restored the change of MDA and also histopathological changes of the rat testes caused by the toxic effects of cisplatin.
2012	Parandin et al.	Alcoholic extract of *Nigella sativa* seed especially in higher doses increased the fertility potential, LH and testosterone concentrations in male rats.

Table 2.5, continued

Year	Authors	Findings
2012	Ghlissi et al.	i. *Nigella sativa* seeds treatment on male diabetic rats improved semen quantity and mobility, and testosterone levels and testis. ii. Decreased blood glucose and lipid peroxidation product level (LPO) and improved antioxidant enzyme activities.
2012	Al-Zahrani et al.	Supplementation with TQ completely restored the free radicals levels, semen quality and histopahological changes that were induced by heat stress.
2013	Kamarzaman et al.	Administration of TQ in mice caused significant reduction in overall toxicity caused by cyclophosphamide. The integrity of the seminiferous tubule was well preserved and the numbers of total DNA damaged cells were reduced from 49% to 4% in 32 days.
2014	Kolahdooz et al.	In a study conducted on infertile men, the sperm count, motility and morphology and semen volume, pH and round cells were improved significantly in *Nigella sativa* oil treated group compared with placebo group after 2 months.
2014	Ng et al.	*Nigella sativa* oil increased sperm quality and improved testis histological features against the damaging effects of nicotine in male rats.
2014	Sayed et al.	Thymoquinone increased gluthathione (GSH), superoxide dismustase (SOD), catalase (CAT) and testosterone, and reduced lipid peroxidation activity in cadmium-induced reproductive toxicity in rats.
2014	Fouad et al.	i. Thymoquinone significantly attenuated arsenic-induced decreases of serum testosterone, and testicular reduced glutathione level, and significantly decreased the elevations of testicular MDA and nitric oxide levels resulting from arsenic administration. ii. Thymoquinone ameliorated the arsenic-induced testicular tissue injury and decreased the arsenic-induced expression of inducible nitric oxide synthase and caspase-3 in testicular tissue.
2015	Fouad & Jresat	Thymoquinone, through its antioxidant and anti-inflammatory activities, protected testes against the toxic effects of cadmium.

Table 2.5, continued

Year	Authors	Findings
2016	Aithal et al.	The testosterone levels and LH were normalized significantly when diabetic groups were treated with *Nigella sativa* seed powder and TQ.
2017	Assi et al.	Oral *Nigella sativa* administration alleviated the detrimental effects of lead acetate on spermatogenesis and antioxidant defenses in Sprague Dawley rats.
2018	Mabrouk	Thymoquinone showed protective effects against lead-induced testicular histopathological lesions in rat.
2018	Attari et al.	Administration of TQ for 14 days improved sperm quality and biochemical parameters, while reducing apoptotic cells of the testes in a mouse model of aging.
2018	Lina et al.	Administration of *Nigella sativa* resulted in ameliorating effects on both the prostate gland and seminal vesicle structures and functions of the nicotine-treated rats.

2.13 Conventional and Molecular Approaches in Male Infertility Evaluation

2.13.1 Conventional or Traditional Approach

In current fertility clinics and laboratories across the world, the conventional evaluation of infertility in males includes physical examination and semen analyses. A range of standardized semen analyses are considered as the initial step to investigate semen quality. The evaluation relies on the descriptive analysis of semen pH, sperm concentration, motility and morphology with reference values for human that must be exceeded to be considered as functional or fertile sperm (Khatun et al., 2018). The lower reference limits for semen parameters have been adjusted several times (year 1987, 1992, 1999, 2010) in the WHO manual to provide accuracy for predicting fertility status of a man. In animal studies, sperm evaluation is often performed by comparing

the parameters to a control or fertile group. Conventional semen analysis has been considered as the preliminary choice for fertility assessment. However, this method cannot always provide valid information on specific defects of sperm physiology (Khatun et al., 2018). Therefore it is commonly complemented by more intensive sperm function tests such as molecular approach to determine the reason for non-pregnancy in infertile couples.

2.13.2 Molecular Approach

Extensive evaluation techniques in male infertility are often required to assist in the diagnosis process especially involving molecular and genetic defects. A number of methods have been proposed in recent years to assess sperm quality and function through molecular assessments which involve mitochondrial function, DNA fragmentation, protamination, DNA packaging, chromosome aneuploidy (detectable by karyotype analysis) and gene mutations (detectable by molecular diagnostic techniques). Even though the practicality of these methods in the evaluation of male infertility and as prognostic markers are somewhat inconclusive, partly because standardization has not been reached for some of these methods, nevertheless, they provide valuable and important information as additional test to standard the semen analyses (Pizzol et al., 2014).

Mitochondrial status is an important feature of sperm physiology as they generate a major part of the ATP required for sperm metabolism, membrane function and motility. Mitochondrial function could be determined by the evaluation of the electrochemical gradient established during the process of oxidative phosphorylation when the protons are pumped from inside of the mitochondria to the outside (Agarwal & Prabakaran, 2005). The loss of the mitochondria membrane potential (MMP) is one

of the earliest apoptosis mechanisms in cell systems (Peña et al., 2009). This inner mitochondrial membrane potential is explored by assays such as MitoTracker and JC-1 dye assay to determine sperm function (Agarwal & Prabakaran, 2005). MitoTracker probes are cell permeant, mitochondrion-specific vital dyes that contain a mildly reactive chloromethyl moiety. This enables it to react with the thiol groups of mitochondria utilizing its very large membrane potential making the accumulation irreversible. As the dye preferentially target mitochondria due to its membrane potential, the probe is used to assess sperm mitochondrial function. When sperm are dying, the mitochondrial membrane potential is no more persistent. Change in accumulation produces changes in the fluorescence signal indicating changes in membrane potential (Chazotte, 2011).

The integrity of sperm DNA is vital for normal sperm function and embryonic development (Omran et al., 2013). It is also increasingly evident that damaged DNA in sperm that manages to fertilize an oocyte can cause failed implantation, spontaneous miscarriages and might also have a negative impact on fetal development (Virro et al., 2004; Aitken & Baker, 2006; Fernández-Gonzalez et al., 2008). Other alterations that are often associated with sperm DNA damage are defective sperm chromatin packaging, apoptosis, oxidative stress, gene mutations and aneuploidy. As a consequence, several techniques for the assessment of sperm DNA integrity have been described, including the acridine orange staining assay, sperm chromatin structure assay (SCSA), Comet assay, Sperm Chromatin Dispersion (HALO) test, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling assay (TUNEL) test, γH2AX evaluation, and in-situ nick translation test (Pizzol et al., 2014).

Comet assay is to assess DNA breakage in sperm which is a single cell gel electrophoresis, performed under neutral or alkaline conditions. Sperm cells are embedded in agarose gel and lysed. It will then subjected to horizontal electrophoresis

and the DNA is visualized using DNA-specific fluorescent dye. Fragmented DNA will migrate away from the central nucleus, creating a tail and resembling a comet‟s image. This shape is due to the migration and accumulation of the short DNA fragments and the intensity of the tail represent the amount of DNA fragmentation (Singh et al., 1988). The DNA damage is quantified by measuring the displacement between the genetic material of the nucleus comet head and the resulting tail using specific image analysis software.

Defective sperm chromatin packaging can be assessed by sperm DNA protamination techniques which could indicate protamine deficiency or aberrant chromatin packing in sperm. The techniques include aniline blue stain, toluidine blue stain and Chromomycin A3 (Dadoune et al., 1988; Manicardi et al., 1995; Erenpreisa et al., 2003).

It is well established that genetic causes account for 10% to 15% of the infertility cases that affects many physiological processes of male reproduction at different levels, such as hormonal homeostasis, spermatogenesis and sperm quality (O'Brien et al., 2010). Hence, before entering an assisted reproductive program, genetic test are routinely included in the diagnostic work up of infertile males. Chromosomal aneuploidies in general are responsible for a significant portion of unsuccessful pregnancy. Fluorescence in-situ hybridization (FISH) is an emerging technology in aneuploidy analysis using chromosome-specific DNA probes to detect numerical chromosomal abnormalities in decondensed sperm (Hassold & Hunt, 2001).

Specific gene mutations could also affect the quality of sperm produced and be responsible for male infertility. No consensus or standardized genes has been properly named to practice as prognostic marker, thus this has generated increasing interest over the years in finding genes that could act as candidate gene for sperm quality assessment (Pizzol et al., 2014). *Protamine 1* (*PRM1*) and *protamine 2* (*PRM2*) genes that are

responsible to ensure compact chromatin packaging has been frequently associated with male infertility. The relative quantities of both genes are normally equally expressed but found to be abnormal in some groups of infertile men (Carrell & Liu, 2001; Carrell et al., 2007). Similarly, hypermethylation of *cAMP response element modulator* (*CREM*) gene has been implicated as a contributing factor in male infertility cases, found to correlate negatively with sperm concentration, motility, and normal morphology (Nanassy & Carrell, 2011). Other studied genes that been associated with sperm quality are *estrogen receptor 1* (*ESR1*), *estrogen receptor 2* (*ESR2*) (Safarinejad et al., 2010; Ge et al., 2014), *follicle-stimulating hormone beta* (*FSHB*) (Tüttelmann et al., 2007), *transition nuclear protein 1* (*TNP1*), *transition nuclear protein 2* (*TNP2*) (Miyagawa et al., 2005) and *androgen receptor* (*AR*) (Yong et al., 2003) among others. One of the molecular techniques to detect the expression level of these genes is quantitative Polymerase Chain Reaction (qPCR) or Real Time PCR. It is used to monitor the amplification of a specific DNA molecule and quantify to determine any irregular expressions. Two common methods of qPCR are non-specific fluorescent dyes like SYBR Green that intercalates with any double stranded DNA and sequence specific DNA probes. Although probe based assay is more expensive than SYBR Green method, it is far more superior in specificity as it will bind only to the sequence of interest. Probe consists of a fluorophore or dye reporter that is attached to the 5‟-end of the oligonucleotide probe and a quencher at the 3‟-end. The quencher acts to quench the fluorescence emitted by the flourophore when excited. The quencher will inhibit any fluorescence signals as the fluorophore and quencher are in close proximity. Probes are designed to anneal to the template that will be amplified by a specific set of primers. In the elongation step of qPCR, taq polymerase will synthesize a new strand extending from the primer and in the process, degrade the probe that has annealed to the template. The degradation of the probe will break the close proximity of the fluorophore and

quencher, which subsequently allows the fluorophore to fluoresce. The fluorescent detected by quantitative thermal cycler is directly proportional to the PCR product produced and the amount of DNA template present and ultimately the mRNA levels (Arya et al., 2005).

The quality of RNA sample used is prerequisite to a reliable and reproducible result. The quality of RNA comprises of both purity and integrity. The purity includes the absence of protein, DNA, carbohydrates, lipids, and other compounds like residual of RNA extraction reagents. The RNA samples must be of the same, good quality when compared to each other to get accurate results. The 260/280 nm ratio is used to assess the purity with the accepted ratio between 1.8 to 2.0 (Fleige & Pfaffl, 2006).

Apart from these, it is critical to check the efficiencies of the primers before performing the experiment for qPCR assay validation. A standard curve provides amplification efficiency value which is important in gene expression analysis using Pfaffl (2001) and Vandesompele (2002) methods. The PCR efficiency can be estimated from the slope of the standard curve, Efficiency = $10^{(-1/slope)}$-1. The amount of amplification is double with 100% efficiency. Typically, desired amplification efficiency range from 90% to 110% (a slope between -3.1 and -3.6). The efficiency value can also be calculated from the efficiency percentage and factored in the data analysis, E = (%Efficiency X 0.01) +1. Apart from that, a standard curve helps in determining the detection limit of the assay and the appropriate amount of DNA to be used in the experiment.

During qPCR experiment, the fluorescence emitted by the reaction is recorded concurrently providing an amplification curve. The amplification curve is a plot of the detected fluorescence VS PCR cycles. The curve is used for setting the quantification point or cycle threshold (CT). The cycle at which the fluorescence signal is sufficiently above background or threshold are met or exceeded are called the CT value. This value

is used for quantification and comparison between samples of real-time PCR (Fraga et al., 2008). In this study, the expression levels were measured using qPCR through relative quantification. This method determines the changes in expression level of the target genes across multiple samples or treatments and expresses it relative to the levels of one or multiple reference genes (Fraga et al., 2008). The relative expression level of the target genes were determined by normalization to reference genes, Beta-actin (*ACTB*) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Optimal reference genes must be equally tested under the same experimental conditions and between all samples.

Normalization is required to reduce experimental variations, such as differences in the amount and quality of starting material. This is to ensure that biological changes in the mRNA levels are revealed. The use of more than one reference gene is thought to even out the variations in the expression of these genes. The stability of reference genes used for data normalization is imperative to ensure accurate result. A stable reference gene indicates results that does not vary with experimental or treatment conditions (Vandesompele et al., 2002).

CHAPTER 3: METHODOLOGY

3.1 Materials

All chemicals and solvent used in this research were of analytical or molecular biology grade. The name of all the chemicals and their sources are listed in Appendix A.

3.1.1 Experimental Animal

The experimental animals were male Sprague Dawley rats (specific pathogen-free) aged 7-9 week and weighed 200 – 250 g. The rats were obtained from the University of Malaya Medical Center (UMMC), University of Malaya.

3.1.2 Ethics, Safety and Health Issues

The experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of University of Malaya. [PASUM/30/12/2015/FDR (R)] (Appendix B). Gloves and facial mask were used at all time during each experiment. Animal cages were cleaned and beddings replaced once a week to maintain hygiene and reduce risk of infections.

3.2 Methods

3.2.1 Rearing and Maintenance of Animal

The rats were reared in the animal house of Centre for Foundation Studies in Science, University of Malaya. Prior to the commencement of treatments, rats were acclimatized

to the experimental conditions for one week. The animals were placed in pairs inside translucent polypropylene cages (40 cm x 25 cm x 16 cm) with steel wire tops. The surrounding environment was maintained at room temperature under standard conditions of 12 hour light and 12 hour dark cycles. At the base of the cages, sawdust was used as bedding to absorb urine and water spillage. The food in the form of dry chow pellet (Specialty Feeds, Australia) and water were available ad libitum throughout the experimental period.

3.3 Preparation of Treatment Solution

3.3.1 Preparation of Normal Saline

Normal saline 0.9% was prepared by dissolving sodium chloride powder (NaCl) in distilled water (Appendix C1). Saline was administered via force feeding according to rats' body weight.

3.3.2 Preparation of Nicotine

Liquid nicotine (L-nicotine, 99+%, Acros Organics, Morris Plains, NJ, USA) was diluted before use. A working solution 10 mg/ml was prepared by diluting the nicotine with saline and stored at 4°C (Appendix C2). The dose of nicotine used in this study was 5 mg/kg, based on preliminary studies and previous researches (Mahaneem et al., 2007; Fairuz et al., 2011) although the LD_{50} of nicotine in rats is 50 mg/kg body weight (Okamoto et al., 1994). Nicotine was administered by subcutaneous (s.c.) injections given under the loose folds of skin of the animal, directly below the dermis and epidermis using a 1 ml syringe and 27G needle to mimic transdermal nicotine patches.

Nicotine administration through inhalation is not feasible as the dose of nicotine between animals varies and is difficult to keep constant (Benowitz et al., 2009).

3.3.3 Preparation of Thymoquinone

Thymoquinone (Sigma-Aldrich, St. Louis, MO, USA) arrived in crystalline powder form and was dissolved in hot saline (90-100°C). The solution was mixed well and the bottle covered with an aluminum foil and stored in a dark, dry and ventilated cabinet at room temperature (Appendix C3). The rats were force fed using a 1 ml syringe attached to a bulb tipped gastric gavage needle (Cadence Science, Staunton, USA). The rats were held at the base of their necks and forearms to ensure that the force feeding process was done without any harm. The dose of TQ used in this study was 5 mg/kg based on previous study (Mabrouk & Ben Cheikh, 2016) as it produced adequate effects without showing any undesirable clinical signs. The LD_{50} of TQ in rats though oral ingestion is 794.3 mg/kg body weight (Al-Ali et al., 2008).

3.4 Preparation of Sperm Medium

3.4.1 Toyoda–Yokoyama– Hosi (TYH) Medium

The TYH was used to serve as the medium for sperm incubation and for dilution. The TYH medium was prepared a day before the rats were sacrificed and stored at 4°C (Appendix D**)**.

3.5 Euthanization

Rats were given treatment from day 1 to day 60 and euthanized on day 61 by an overdosed of ketamine-xylazine provided by the Institutional Animal Case and Use Committee (IACUC), University Malaya through intraperitoneal injection (i.p).

3.6 Sample Preparation

3.6.1 Harvesting of Sperm and Testis

Upon euthanization, cauda epididymis was excised from the rat testis, cut opened and placed into a tube containing pre-warmed 1 ml of TYH medium. The tube was placed in CO_2 incubator with 5% CO_2 at 37°C for 30 minutes. The tissues were then discarded out of the tubes prior to sperm evaluation. Following sacrifice, testes were decapsulated and cut into 50 – 100 mg pieces. It was then placed in 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, MA, USA), immediately snap-frozen in liquid nitrogen and stored at -80°C for gene analysis.

3.7 Sperm Count

Sperm concentration was determined using a Makler Counting Chamber (Sefi-Medical Instruments Ltd., Haifa, Israel). The device is designed with a 1 mm^2 grid on its lower surface that is subdivided into 100 squares of 0.1 x 0.1 mm each. Thus, when the cover glass is placed on the four tips that are 10 microns above the surface level of the disc, the space bounded is uniform and in a row of 10 squares is exactly one millionth of a ml (Makler, 1980). A total of 10 µl of sperm suspension was placed onto the Makler Chamber stage and observed under a light microscope (Olympus, Japan) at 40x

magnification. The number of sperm heads counted in 10 squares indicated their concentration in millions/ml.

3.8 Sperm Motility

Motility evaluation was also performed using the Makler Counting Chamber where 10 µl of sample was dropped onto the chamber and evaluation performed immediately to avoid errors due to tendency of sperm to migrate. The non-motile sperm within the grid were first counted, and then the motile sperm were counted (Makler, 1978). In this study, sperm was considered motile when it displayed any movement. Five replicates were done for each sample and the percentage of motile sperm was determined.

3.9 Sperm Cell Membrane Integrity Assessment by Hypo-Osmolality Test (HOST)

A hypo-osmotic solution was used to evaluate the functional integrity of the sperm membrane based on the presence of coiled or swollen tail (HOST positive), which indicated sperm with intact cell membrane. Ionic strength of 0.15 resulted in an identifiable swollen sperm when an influx of water results in an expansion of cell volume. Sperm with compromised membrane (HOST negative) was not able to hold the fluid and remained in its original shape (Jeyendren et al., 1984).

3.9.1 Preparation of HOST Solution

The HOST solution acts as a medium to induce a hypo-osmolality environment towards the sperm. A hypo-osmotic solution of 150 mOsm/l [7.35 g of sodium citrate

($C_6H_5Na_3O_7.2H_2O$) and 13.51 g fructose in 1000 ml of distilled water] was prepared. The prepared solution was kept at room temperature and warmed at 37°C before use (Appendix E).

3.9.2 Incubation of Sperm and Slide Preparation

Fifty µl of sperm suspension in a tube was incubated with 500 µl of 150 mOsm/l HOST solution at 37°C for 45 minutes. Subsequently, 10 µl of the mixture was smeared onto a slide and air dried at room temperature (Vaez et al., 2014).

3.9.3 Staining

The dried slide was stained using the Diff-Quik stain (Dade Behring, Deerfield, IL, USA) which consisted of three solutions. Firstly, the slide was immersed in Diff-Quik Fix for 5 minutes, then Diff-Quick I for 5 minutes and followed by Diff-Quik II for another 5 minutes. The slides were then rinsed with distilled water until excess stain was cleared.

3.9.4 Analysis

Two hundred sperm per sample were counted under a light microscope (Olympus, Japan) at 40x magnification and the number of HOST positive sperm (sperm with coiled or swollen tail) was recorded and represented by percentage.

3.10 Mitochondrial Function Assessment by MitoTracker Red FM

3.10.1 Preparation of MitoTracker Red FM

MitoTracker Red FM (Molecular Probes, Eugene, Oregon, USA), a far red fluorescent dye was used in this study. The dye is suitable for multicolour labeling experiments because their red fluorescence is well resolved from the green fluorescence of other probes. Stock solution was prepared by dissolving the lyophilized MitoTracker (50 µg/vial) in dimethylsulfoxide (DMSO). Serial dilutions of the dye were tested and the working concentration of MitoTracker used was 500 nM in TYH medium. The mixture was protected from light throughout the process and used immediately after preparation (Appendix F).

3.10.2 Preparation of 4',6-Diamidino-2-Phenylindole (DAPI) Stain

DAPI is a DNA stain that emits blue fluorescence upon binding to the AT region of DNA. It is commonly used as a nuclear counterstain in fluorescence microscopy, flow cytometry, and chromosome staining. The dye's spectral properties make it ideal for use with red fluorophores in multicolor experiments. Stock DAPI (Molecular Probes, Eugene, Oregon, USA) was made by adding deionized water directly to the vial to make 5 mg/ml (14.3 mM) stock solution. The mixture was aliquoted to avoid frozen-thawed damage and protected from light. The stock solution is viable for 6 month of storage when kept at 2 - 6°C and for long term storage at -20°C. Working DAPI solution 200 µg/ml was prepared fresh by diluting the stock in phosphate buffered saline (PBS) (Appendix G).

3.10.3 Sample Staining

Live sperm suspension was diluted in pre-warmed medium (10 million of sperm/ml), centrifuged and the supernatant was removed. Sperm was incubated in 200 µl of 500 nM MitoTracker RedFM and 5 µl of 200 µg/ml of DAPI at 37°C for 30 minutes. Subsequently, the sperm were washed and resuspended in 200 µl fresh pre-warmed medium.

3.10.4 Slide Preparation and Analysis

The sample, 50 µl was then pipetted on a slide and covered with a coverslip. The slides were examined under a fluorescent microscope (Axiovert 200M, Carl Zeiss, Germany). Two hundred sperm per sample was counted and number of MitoTracker labeled sperm (MitoTracker positive) was recorded and represented by percentage (Ramalho-Santos et al., 2007). MitoTracker positive sperm are sperm stained red at the midpiece region which indicates active mitochondria activity and MitoTracker negative sperm demonstrates reduced staining because of lack or absence of functional mitochondria.

3.11 DNA Integrity Assessment by Comet Assay

Integrity of sperm DNA was assessed by performing the comet assay as reported by Jamaludin et al. (2017) with some modifications. The assay involves four main steps: slide preparation, lysis of sperm, neutral electrophoresis, and staining of the slides.

3.11.1 Preparation of Comet Assay Buffers

3.11.1.1 Lysis Buffer

Lysis buffer contains aqueous salt and non-ionic detergent to remove cell membranes, cytoplasm, nucleoplasm and dissolves the nucleosomes. Only the DNA of the cell remains in the form of a nucleiod to fill the cavity in the agarose that the whole cell formerly filled. Two stocks of lysis buffer were prepared. The first buffer was prepared and kept at 4°C. Second lysis buffer was prepared similar to the first buffer with addition of 0.5 mg/ml proteinase K and incubated at 37°C. Both lysis buffers were adjusted to pH 10.3 (Appendix H1).

3.11.1.2 Electrophoresis Buffer

Electrophoresis buffer was prepared one hour before used and kept in the chiller. This buffer contained ion to carry the current and to maintain the pH at a constant value (Appendix H2).

3.11.1.3 Neutralizing Buffer

Neutralizing buffer is important to neutralize the excess alkali from electrophoresis procedure. The buffer was adjusted to pH 7.4 and kept at 4°C (Appendix H3).

3.11.2 Preparation of Agar

Normal Melting Agar (NMA) and Low Melting Agar (LMA) of agarose (Appendix I) were prepared to ensure that the cells were embedded in the agarose gel and

maintained on the slide. Both mixtures were placed on a hot plate (75-85°C) to maintain its liquid form throughout slide preparation.

3.11.3 Preparation of SYBR Green I Dye

For visualization, SYBR Green I (Molecular probes, Invitrogen, USA) was used to stain the DNA. A working SYBR Green I solution was prepared fresh at 1:10000 dilution in pH 7.5 to 8.0 buffer.

3.11.4 Slide Preparation

Frosted slides (StatLab Medical Product Inc., Texas) were covered with 200 µl of 1% (w/v) normal melting agar (NMA) and a coverslip was placed promptly on top of it to allow it to solidify at 4°C for 10 minutes. After the agar solidified, the coverslip was carefully removed. Following this, 100 µl of sperm sample containing 1×10^5 sperm per ml was suspended in 100 µl of 0.5% (w/v) low melting agarose (LMA) and 50 µl of the suspension pipetted onto the pre-coated NMA frosted slides. Fresh coverslips were placed and left at 4°C for 10 min. A second layer 1% LMA was added and allowed to solidify.

3.11.5 Sperm Lysis

The coverslips were then removed and the slides were immersed in the first lysis buffer at 4°C for 1 hour and subsequently transferred to the second lysis buffer at 37°C and left overnight. Following cell lysis, the slides were washed three times with distilled water for 20 minutes to remove residual lysis buffer from the gels.

3.11.6 DNA Fragment Separation by Electrophoresis and Neutralization

Slides were then placed in a horizontal electrophoresis tank (Sub-Cell Model 192 cell Horizontal Electrophoresis System, Bio-Rad, Hercules, CA, USA) filled with fresh electrophoresis buffer. The samples were allowed to equilibrate for 20 minutes before electrophoresed. Electrophoresis was performed at room temperature and conducted at 25V and 300 mA for 20 minutes. The slides were then transferred in three changes of neutralizing buffer for 20 minutes each.

3.11.7 DNA Staining with SYBR Green I

The slides were stained with 20 µl of diluted SYBR green I (1:10000) for 1 hour and cover slip was placed on it. The slides were then observed under a fluorescent microscope.

3.11.8 Comet Image Analysis

The fluorescent-labeled DNA was visualized using an inverted fluorescent microscope (Axiovert 200M, Carl Zeiss, Germany) at 40x magnification and captured using camera AxioCam MRm (Carl Zeiss, Germany). Comet Assay Software Project (CASP) lab Version 1.2.3b1 (Free Software Foundation Inc., Boston, MA, USA) was used to analyse the comet images. One hundred comet images were randomly analysed per slide and scored for tail length (TL), tail moment (TM), olive tail moment (OTM), and % tail DNA. TL is the maximum distance that the damaged DNA migrates from center of cell nucleus. TM is a product of TL and fraction of total DNA in tail. OTM is the product of percentage DNA and median migration distance. Percentage tail DNA is the total of DNA migrates from nucleus into comet tail.

3.12 Molecular Analysis of *Transition Nuclear Protein 2* (*TNP2*) and *Protamine 1* (*PRM1*)

3.12.1 RNA Isolation

The RNA isolation was performed using a conventional method with TRIzol reagent (Thermo Fisher Scientific, MA, USA). Benches and all equipment were cleaned with RNaseZap (Invitrogen; Thermo Fisher Scientific, MA, USA), a RNase inhibitor, to prevent degradation of RNA by RNase contamination.

3.12.1.1 Homogenization

Frozen testis sample (50 – 100 mg) was homogenized in 1 ml of TRIzol reagent using a power homogenizer (IKA T10 basic ULTRA-TURRAX, IKA Works Inc, USA). The homogenized tissue was then incubated at room temperature for 5 minutes.

3.12.1.2 Aqueous Separation

The process was performed according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). An additional isolation step to remove insoluble material from the homogenate was performed by centrifugation at 12000 x g for 10 minutes at 4°C. The supernatant was transferred to a new tube and mixed vigorously for 15 seconds following the addition of 200 µl of chloroform. The sample was incubated for 2 to 3 minutes at room temperature and centrifuged at 12000 x g, 4°C for 15 minutes. Following centrifugation, the mixture separated into the lowest pink organic phase, phenol-chloroform phase (interphase) and a colourless upper aqueous phase.

3.12.1.3 RNA Precipitation

The aqueous phase containing the RNA was carefully removed and placed in a new 1.5 ml microcentrifuge tube. Isopropyl alcohol, 500 µl was added into the aqueous phase, mixed gently before centrifugation at 12000 x g, 4°C for 10 minutes. The RNA pellet appeared barely visible at the base of the tube.

3.12.1.4 RNA Wash

The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol in diethyl carbopyronate (DEPC) treated water. The sample was mixed gently and centrifuged again at 7500 x g for 5 minutes at 4°C. The ethanol was discarded and the pellet was left to air dry for 5 minutes.

3.12.1.5 Elution of RNA

The RNA pellet was then completely dissolved in 100 µl DEPC treated water by incubating at 55 - 60°C for 10 minutes. Isolated RNA was then kept on ice for further analysis.

3.12.2 Quality and Quantity Assessment of RNA

3.12.2.1 Purity and Quantity Measurement

The quantity (ng/µl) and purity of RNA was measured using a NanoPhotometer P300 (Implen, München, Germany) and manually recorded. The elution solution, DEPC treated water was used as the blank measurement.

3.12.2.2 Assessment of RNA Integrity

Integrity of RNA was determined by electrophoresis on 1% (w/v) agarose gel. A typical pattern indicative of undegraded RNA is sharp ribosomal RNA bands with the 28s (~5kbp) band about two-fold more intense than the 18s (~2kbp) band.

A. 1% Agarose gel preparation

Gel was prepared by mixing 0.25 g agarose powder with 1X TBE buffer. The mixture was heated in a microwave for 1 minute until fully dissolved. The bottom of the flask was then cooled under running tap water. One µl of GelStain (Transgen Biotech, Beijing, China) was added into the warm mixture, swirled and poured into the gel casting tray with a pre-set comb. The comb was removed when the gel had hardened. The gel was then transferred into an electrophoresis tank and 1X TBE buffer was poured till it covered the gel.

B. Ladder and sample preparation

Ladder mixture was prepared by mixing equal amount of RNA loading dye (2X RNA Gel Loading Dye, Thermo Scientific, USA) and RNA ladder (RiboRuler High Range RNA Ladder, Thermo Scientific, USA), then incubated at 70°C for 10 minutes before chilled on ice. Four µl of the ladder was pipetted into the well of the gel, started from the first lane. Sample was prepared by mixing 3 µl of each with 3 µl of RNA loading dye. Six µl of mixture was then pipetted into the wells respectively. Electrophoresis (Mupid-One Electrophoresis System, Advance, Tokyo, Japan) was performed at 100V for 30 minutes. After completion of electrophoresis, the gel was visualized using a gel documentation system (MultiImage II AlphaImager HP, Alpha Innotech, San Leandro, CA, USA).

3.12.3 Reverse Transcription PCR

As RNA cannot serves as template in RT-qPCR, RNA must be converted into complementary DNA (cDNA) in order to detect the mRNA transcript. The total RNA was reverse transcribed into cDNA using QuantiNova Reverse Transcription kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol by utilizing 2 µg total RNA in a final volume of 20 µl (Table 3.1). The kit comprised of two main steps: removal of the genomic DNA (gDNA) and reverse transcription (RT).

Table 3.1: gDNA elimination and RT temperature protocol

Step	Temperature	Time	Description
gDNA elimination reaction	45°C	2 minutes	Place sample in thermal cycler
	25°C	Pause cycler	Remove sample, place on ice, add RT components
Reverse transcription reaction			
• Annealing	25°C	3 minutes	Place sample in thermal cycler
• Reverse transcription	45°C	10 minutes	
• Inactivation of reaction	85°C	5 minutes	

3.12.3.1 Removal of Genomic DNA

Prior to RT, the genomic DNA removal reaction was prepared on ice (Table 3.2). Purified RNA sample was incubated with QuantiNova gDNA Removal Mix at 45°C for

2 minutes in order to effectively reduce contaminating gDNA and immediately placed on ice.

Table 3.2: Genomic DNA removal reaction components

Component	Volume / Reaction
gDNA removal mix	2 µl
Template RNA, 2 µg	2 µl
RNase free water	11 µl
Total reaction volume	15 µl

3.12.3.2 Reverse Transcription

Complementary DNA (cDNA) was synthesized by the components supplied by the kit. In addition to reverse transcription enzyme, the kit contained reverse transcription mix which included combination of oligo-DT and random primers, Mg^{+2} and deoxynucleotide triphosphates (dNTPs). Reverse transcription master mix was prepared on ice (Table 3.3). The freshly prepared master mix was then added into each tube containing 15 µl RNA template. The mix was incubated at 25°C for 3 minutes and subsequently at 45°C for 10 minutes. Next, the tube was incubated for 5 minutes at 85°C to inactivate the reverse transcriptase enzyme. The reverse transcription reaction or cDNA was stored at -20°C until further analysis.

Table 3.3: Reverse transcription reaction mastermix

Component	Volume/ Reaction
Reverse transcription enzyme	1 µl
Reverse transcription mix	4 µl
Template RNA	15 µl
Total reaction volume	20 µl

3.12.4 Gene and Primer Sequence Verification Using Conventional PCR and DNA Sequencing

3.12.4.1 Conventional PCR

The genes tested were *TNP2*, *PRM1*, Beta-actin (*ACTB*) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers were designed using Primer3 software (Appendix J) and constructed by Integrated DNA Technologies. Each reaction was set up in a 25 µl reaction volume containing 1.0 µl cDNA (~100 ng), 1X PCR buffer, 0.2 mM dNTPs and 2.5 units/µl Taq DNA Polymerase (TransBionovo Co., Beijing, China) (Table 3.4).

Table 3.4: Mastermix for conventional PCR

Component	Volume 1x
PCR Buffer (10X)	2.5 µl
dNTPs (2.5mM)	2 µl
Taq Polymerase (5U/µl)	0.5 µl
Primer – Forward (10 µM)	0.5 µl
Primer – Reverse (10 µM)	0.5 µl
Template DNA	1 µl
Sterilised distilled water	18 µl
Total volume	25 µl

All the PCR components except cDNA were mixed thoroughly and 24 µl of the mixture was added to labeled PCR tubes. One µl of the template was then pipetted accordingly into the tubes. Sterilized distilled water was added instead of cDNA as a negative control reaction. The amplification process was conducted using a thermal cycler (Aeris Thermal Cycler, Esco Micro Pte. Ltd., Singapore), program as shown in Table 3.5.

Table 3.5: Thermal cycler program

Cycle step	Temperature °C	Time	Cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	35
Annealing	60	30 seconds	
Extension	72	1 minute	
Final extension	72	5 minutes	1
Hold	12	∞	

End point outcome of conventional PCR have to be observed after electrophoresis. Electrophoresis of the amplified DNA was performed on 1% (w/v) agarose gel as was explained earlier (refer to 3.12.2.2). A 100 bp DNA ladder (Promega, Madison, USA) was used to identify the approximate size of the amplified DNA. After the completion of electrophoresis, the gel was visualized using a gel documentation system (MultiImage II AlphaImager HP, Alpha Innotech, San Leandro, CA, USA).

3.12.4.2 Gel Purification and DNA Sequencing

DNA sequencing was performed to verify the amplified product by determining the order of its bases and comparing it with the gene sequence in the National Center for Biotechnology Information (NCBI) database. The bands visualized under UV light were

excised carefully and placed into a 1.5 ml microcentrifuge tube. The piece of gel was then weighed and purified using Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) to isolate the DNA from the gel. Membrane Binding Solution, 10 µl was added per 10 mg per slice and incubated at 55°C until it was completely dissolved. The dissolved gel mixture was then transferred into a mini-column and collection tube assembly. Subsequently, the tube was incubated for 1 minute at room temperature before centrifugation at 16,000 x g for 1 minute. The flow through was discarded and the mini-column reinserted into the collection tube. Seven hundred µl of Membrane Wash Solution was added and the tube centrifuged at 16,000 x g for 1 minute. The flow through was discarded and the mini-column was reinserted before adding 500 µl of Membrane Wash Solution. The tube was then centrifuged at 16,000 x g for 5 minutes. The collection tube was emptied once again and the column assemble was recentrifuged for 1 minute. The tube was then left at room temperature for 30 minutes to allow evaporation of any residual ethanol. Next, the mini-column was transferred to a clean 1.5 ml microcentrifuge tube and 30 µl of nuclease free water was added. The tube was left for 10 minutes at room temperature and then centrifuged 16,000 x g for 1 minute. The mini-column was discarded and the eluted DNA was stored at -20°C until ready to send for sequencing service by 1st Base Sdn. Bhd. (Selangor, Malaysia).

3.12.5 Quantitative PCR (qPCR) or Real-Time PCR

Custom designed primers and probes as listed in Table 3.6 were synthesized by Integrated DNA Technologies (Appendix K). Fluorophore used for the probe in this study was 6-FAM and the quenchers were ZEN and Iowa Black FQ (double-quenched probe). In identifying possible changes of the selected mRNA expression level between control and treatment groups, two target genes, *TNP2* and *PRM1* genes were analysed

together with two reference genes, *ACTB* and *GAPDH*. The gene expression analysis was carried out by QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers were used at 250 nM and probes at 250 nM in a 20 µl reaction of PrimeTime Gene Expression Master Mix (Integrated DNA Technologies, Coralville, IA, USA).

Table 3.6: Sequence information for primers and probes used in this study

Gene and accession number	Oligo	Sequences (5'-3')	Amplicon size (bp)
PRM1 NM_001002850.1	*PRM1* Forward *PRM1* Reverse *PRM1* Probe	TGGCCAGATACCGATGCTG CCTAAAGGTGTATGAGCGGC CGAAGATGTCGCAGACGGAGGA	140
TNP2 NM_017057.2	*TNP2* Forward *TNP2* Reverse *TNP2* Probe	GGAGAAGAGGAGGAGGAGGA ATTGGTGTGACTTTGAGGCC ATGGACACCAAGATGCAGAGCCTT	127
ACTB NM_031144.2	*ACTB* Forward *ACTB* Reverse *ACTB* Probe	TCTTCCAGCCTTCCTTCCTG CACACAGAGTACTTGCGCTC AGGAGATTACTGCCCTGGCTCCTA	238
GAPDH NM_017008.4	*GAPDH* Forward *GAPDH* Reverse *GAPDH* Probe	GAGACAGCCGCATCTTCTTG TGACTGTGCCGTTGAACTTG AGATGGTGAAGGTCGGTGTGAACG	224

3.12.5.1 Preparation of qPCR Reaction Mix

Microcentrifuge tubes were labeled and the number of reactions was calculated accordingly for each of the components. Amplification reactions were prepared in triplicate and no-template control (NTC) was included. The components as listed in Table 3.7 were added in the tube except for cDNA and then capped and mixed.

Table 3.7: Mastermix for qPCR reaction

Component	Volume 1x
Primer Gene Master Mix (2X)	10 µl
Primer – Forward (10µM)	0.5 µl
Primer – Reverse (10µM)	0.5 µl
Probe (10 µM)	0.5 µl
Template DNA	2 µl
Sterilized distilled water	6.5 µl
Total volume	20 µl

3.12.5.2 Preparation of qPCR Reaction Tube

Eighteen µl of the PCR reaction mixture was pipetted into the real-time PCR 0.1 ml tube strips accordingly. Subsequently, 2 µl of cDNA or nuclease–free water (NTC reactions) was added to the tube to make a total volume of 20 µl for each reaction. The tubes were then capped and spin down to remove bubbles.

3.12.5.3 Performing qPCR Reaction

The thermal cycling condition of the instrument was set at initial denaturation of 3 minutes at 95°C followed by 40 cycles of 95°C for 5 seconds and primer annealing/extension temperature at 60°C for 30 seconds (Table 3.8).

Table 3.8: Fast cycling condition of real time qPCR

Cycle step	Temperature °C	Time	Cycles
Initial denaturation	95	3 minutes	1
Denaturation	95	5 seconds	40
Annealing/ Extension	60	30 seconds	
Hold	4	∞	1

3.12.6 Data Analysis of qPCR

3.12.6.1 Quantification

Normalized expression level of the samples was calculated according to Pfaffl (2001) and Vandesompele et al. (2002) methods, using the geometric mean of two reference gene quantities and factoring in of primer efficiency. The data was processed using qBase+ Version 3.0 (Biogazelle, Zwijnaarde, Belgium) for statistical analysis. Utilizing the "Stat Wizard" function, a one-way ANOVA was performed to test for significant differences of expression level between the groups.

3.12.6.2 Normalization

Stability of *ACTB* and *GAPDH* was tested by graph plotting the mean CT values of the genes for all the experimental groups.

3.12.6.3 qPCR Efficiency Determination Using Standard Curve

In performing standard curve of qPCR efficiency, qPCR reactions were set up to amplify different amounts or serial dilutions of the same DNA sample. Ideally, efficient primers will observe a proportional dose-response curve. The experiment was performed as explained earlier (refer 3.12.5) using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The standard curve and efficiency percentage were generated by the instrument. Ten-fold serial dilutions were prepared for *TNP2*, *ACTB* and *GAPDH* genes. While, two-fold serial dilutions prepared for *PRM1* as the gene has low expression, thus a narrower range of curve was required. Each point was prepared in triplicate.

A. Ten-fold (1:10) serial dilution

With the known concentration of cDNA as the first tube, four more tubes were labeled. Sterilized distilled water, 18 µl was added into tube 2 to 5. Subsequently, 2 µl of cDNA from tube 1 was transferred to tube 2. The mixture was mixed and 2 µl of it transferred in tube 3. The process was repeated till tube 5 (Figure 3.1). The tubes were spin down and kept on ice for qPCR analysis.

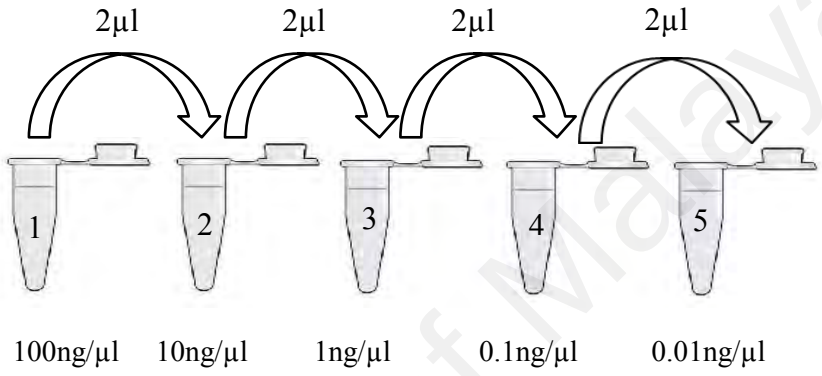


Figure 3.1: Ten-fold serial dilutions of cDNA

B. Two-fold (1:2) serial dilution

Sterilized distilled water, 10 µl was added into tube 2 to 5. Subsequently, 10 µl of cDNA from tube one was transferred to tube two. The mixture, 10 µl was transferred into tube 3. The process was repeated till tube 5 (Figure 3.2). The tubes were spin down and kept on ice for qPCR analysis.

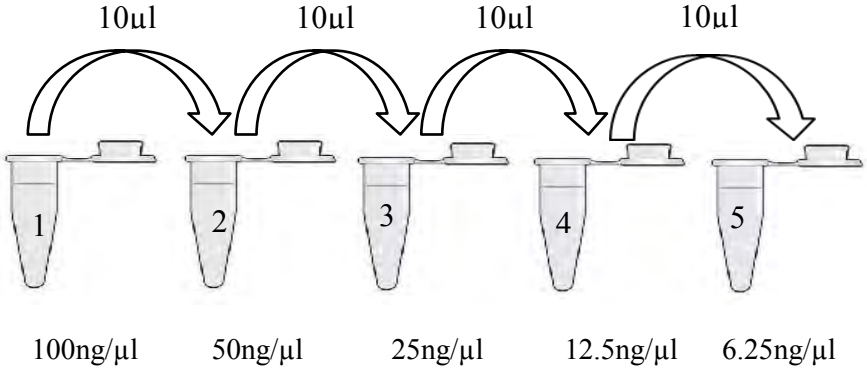


Figure 3.2: Two-fold serial dilutions of cDNA

3.13 Statistical Analysis

The data of control and treatment groups were analysed using the analysis of variance (ANOVA) and Duncan post-hoc test with a statistical significant level of $p<0.05$. Correlation analysis was performed between MitoTracker positive sperm percentage and DNA damage (tail DNA percentage) in the experimental groups using Pearson correlation analysis. Statistical analyses for all parameters were conducted using the Statistical Package for Social Science (SPSS) (Version 23.0) program except for the molecular analysis experiment.

3.14 Experimental Design

The study design was reviewed and approved by the IACUC of University of Malaya. Rats were randomly assigned into 4 groups (8 rats for each group): Control (C), Nicotine (N), Thymoquinone (TQ) and, Nicotine and Thymoquinone (Nicotine-TQ). The body weights were measured before treatment and volume of solution administered was based on the dose and weight of rat (Appendix C). The length of treatment was 60 days (the duration of complete spermatogenic cycle for rats).

3.14.1 Experimental Groups

Rats of control group were force fed with normal saline for 60 days using a bulb tipped gastric gavage needle. Nicotine group rats were injected with 5 mg/kg nicotine subcutaneously for 30 days and force fed with normal saline in the next 30 day. Rats in TQ group were force fed with normal saline for 30 days, followed by 5 mg/kg TQ in the next 30 days. Lastly, the rats of nicotine-TQ group were subcutaneously injected with 5

mg/kg nicotine for 30 days and subsequently force fed with 5 mg/kg TQ in the following 30 day (Table 3.9 and Figure 3.3).

Table 3.9: Dosage and number of rats in each group

Treatment Group	Number of rats (n)	Dosage
Control (C)	8	0.9% (p.o.)
Nicotine (N)	8	5 mg/kg (s.c.)
Thymoquinone (TQ)	8	5 mg/kg (p.o.)
Nicotine-Thymoquinone (Nicotine-TQ)	8	5 mg/kg (s.c.) & 5mg/kg (p.o.)

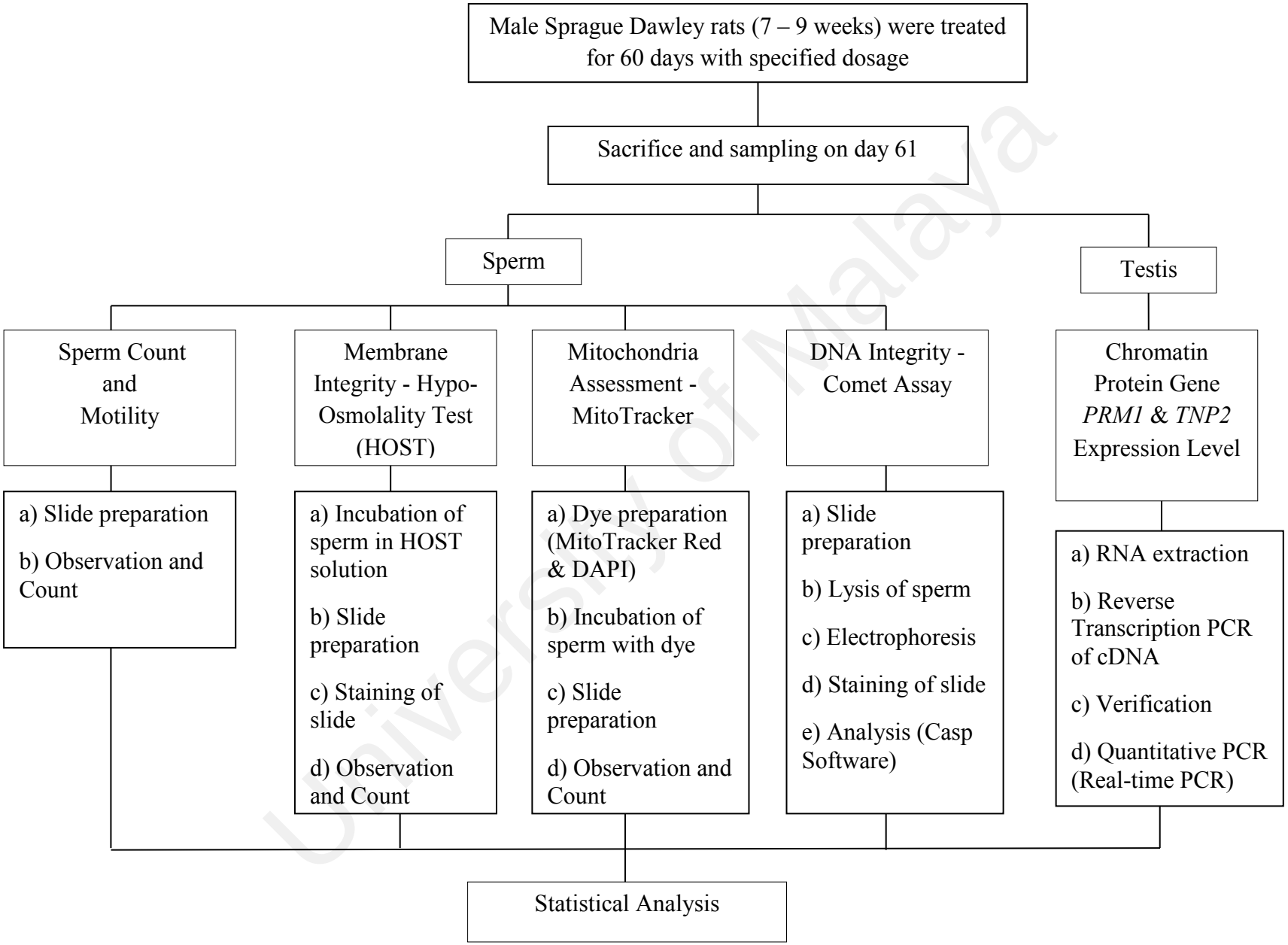


Figure 3.3: Flowchart of research experimental design

CHAPTER 4: RESULTS

4.1 Sperm Count and Motility

The analysis of variance showed that sperm count significantly decreased ($p<0.05$) in nicotine group ($26.72 \pm 1.64\ 10^6$/ml) in comparison to control group ($31.75 \pm 0.80\ 10^6$/ml); whereas the number of sperm were significantly higher ($p<0.05$) in nicotine-TQ group ($30.97 \pm 0.88\ 10^6$/ml) compared to nicotine group (Table 4.1 and Figure 4.1). Motility evaluation demonstrated similar patterns with the number of motile sperm being higher in nicotine-TQ group (85.02 ± 2.24 %) compared to nicotine group (66.24 ± 1.01 %) (Table 4.1 and Figure 4.2).

Table 4.1: Sperm count and percentage of motile sperm of rats treated with nicotine and thymoquinone

Parameter / Group	Sperm Count x 10^6/mL (Mean ± S.E.)	Motile Sperm % (Mean ± S.E.)
Control	31.75 ± 0.80^{b}	83.81 ± 1.93^{bc}
Nicotine	26.72 ± 1.64^{a}	66.24 ± 1.01^{a}
Thymoquinone (TQ)	31.28 ± 1.12^{b}	79.01 ± 1.06^{b}
Nicotine-Thymoquinone (nicotine-TQ)	30.97 ± 0.88^{b}	85.02 ± 2.24^{c}

[abc] Data with different superscripts within the same column show significant difference ($p<0.05$). N = 8 rats per control and treatment groups

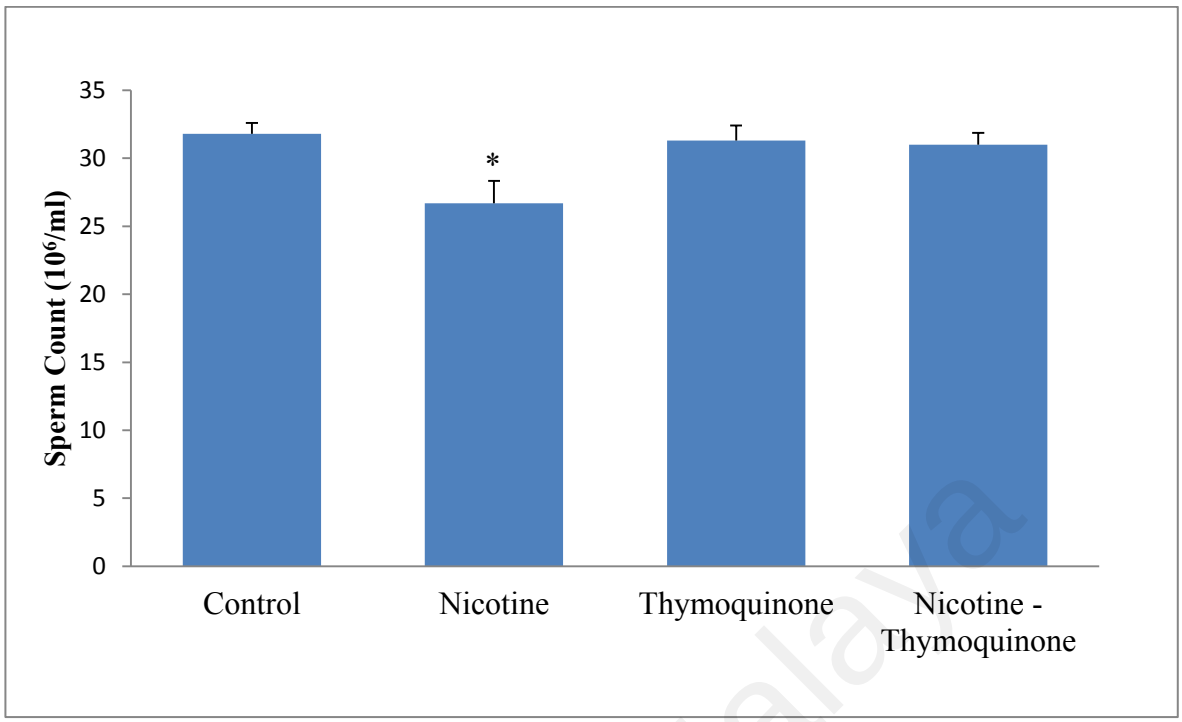


Figure 4.1: Histogram of sperm count in different groups. Data is expressed as mean ± S.E with N=8 in each group.*Significantly different compared to control, thymoquinone and nicotine-thymoquinone groups ($p<0.05$)

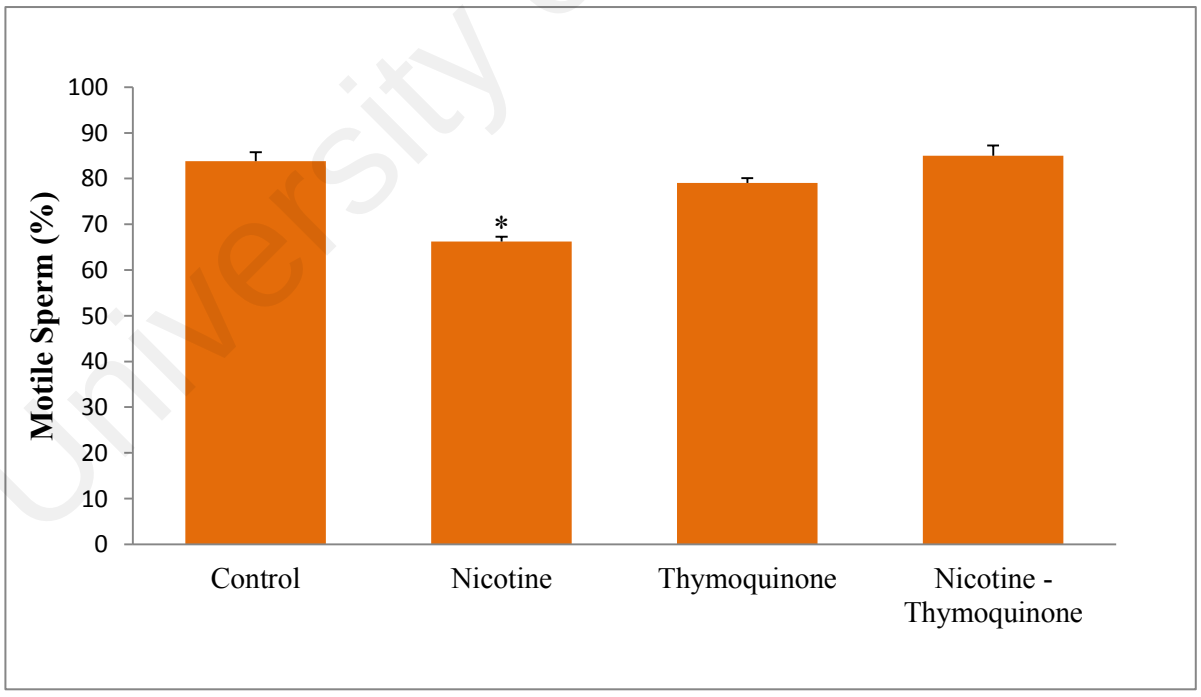


Figure 4.2: Histogram of motile sperm percentage in different groups. Data is expressed as mean ± S.E with N=8 in each group. *Significantly different compared to control, thymoquinone and nicotine-thymoquinone groups ($p<0.05$)

4.2 Membrane Integrity Assessment

The membrane integrity of the sperm is represented by the percentage of HOST positive cells, exhibiting tail coiling (Figure 4.3). As anticipated, there was a significant reduction ($p<0.05$) of intact sperm membrane in nicotine group (47.34 ± 0.69 %) compared to control group (65.98 ± 1.03 %). The percentage of membrane integrity notably increased ($p<0.05$) in nicotine-TQ group (52.58 ± 1.14 %) (Table 4.2 and Figure 4.4).

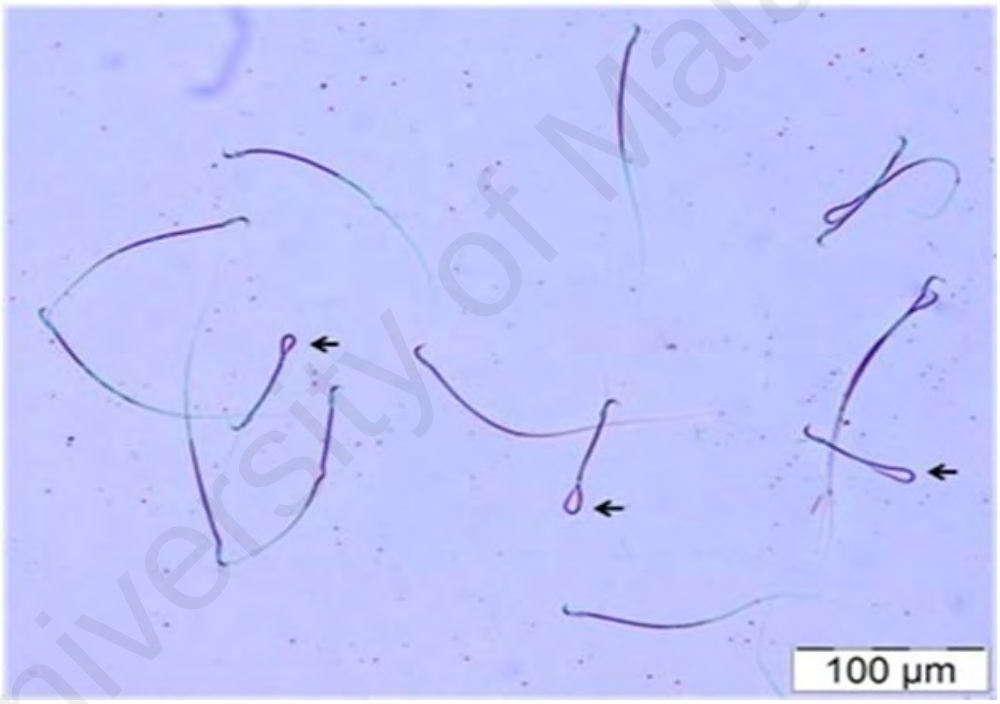


Figure 4.3: A typical photomicrograph of rat sperm after exposure to hypo-osmotic solution in the Hypo-Osmotic Swelling Test (HOST). Sperm with intact membrane or HOST positive exhibited tail coiling (arrowhead). Samples observed under a light microscope (Olympus, Japan) at 40x magnification

Table 4.2: Percentage of HOST positive sperm of rats treated with nicotine and thymoquinone

Parameter / Group	HOST positive % (Mean ± S.E.)
Control	65.98 ± 1.03^{c}
Nicotine	47.34 ± 0.69^{a}
Thymoquinone (TQ)	54.61 ± 1.16^{b}
Nicotine-Thymoquinone (nicotine-TQ)	52.58 ± 1.14^{b}

[abc] Data with different superscripts within the same column show significant difference ($p<0.05$). N = 8 rats per control and treatment groups

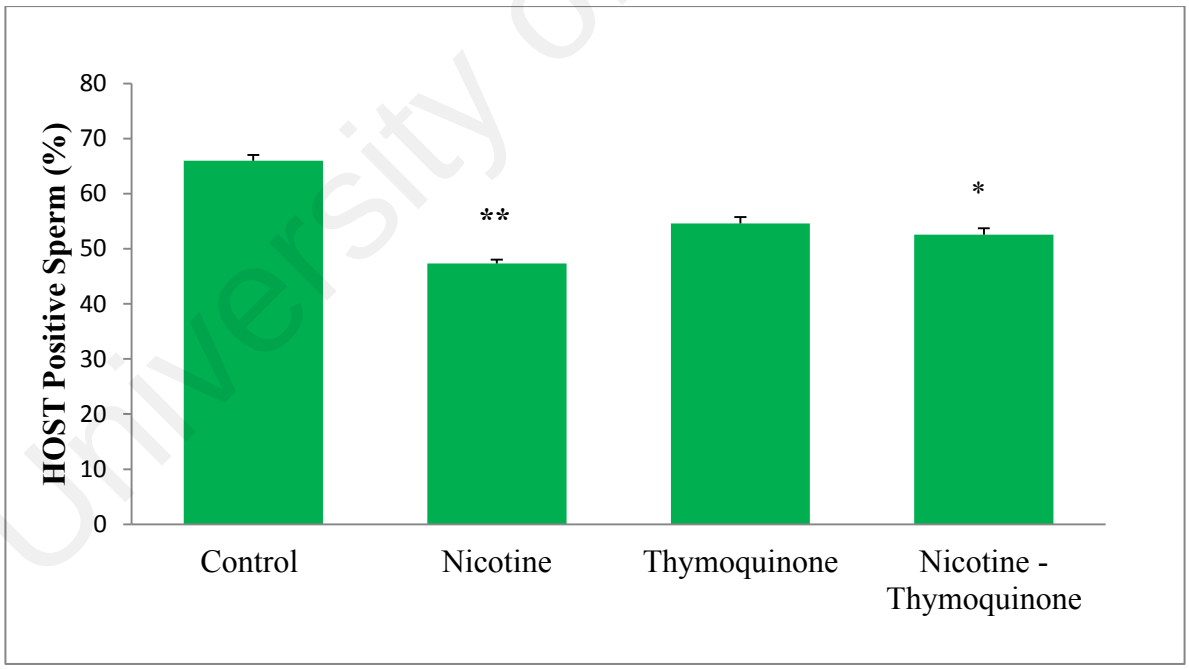


Figure 4.4: Histogram of the percentage of HOST positive sperm in different groups. Data is expressed as mean ± S.E with N=8 in each group. *Significantly different with lower value as compared to control group ($p<0.05$). **Significantly different compared to control, thymoquinone and nicotine-thymoquinone groups ($p<0.05$)

4.3 Mitochondrial Function Assessment

The analysis of variance for the percentage of MitoTracker positive sperm (Figure 4.5) indicated that treatments significantly affect ($p<0.05$) the mitochondrial function. The nicotine group has significantly the lowest mean ($p<0.05$) of MitoTracker positive sperm (75.68 ± 0.90 %) compared to control (87.04 ± 0.88 %), thymoquinone and nicotine-TQ groups (79.08 ± 0.74 %) (Table 4.3 and Figure 4.6).

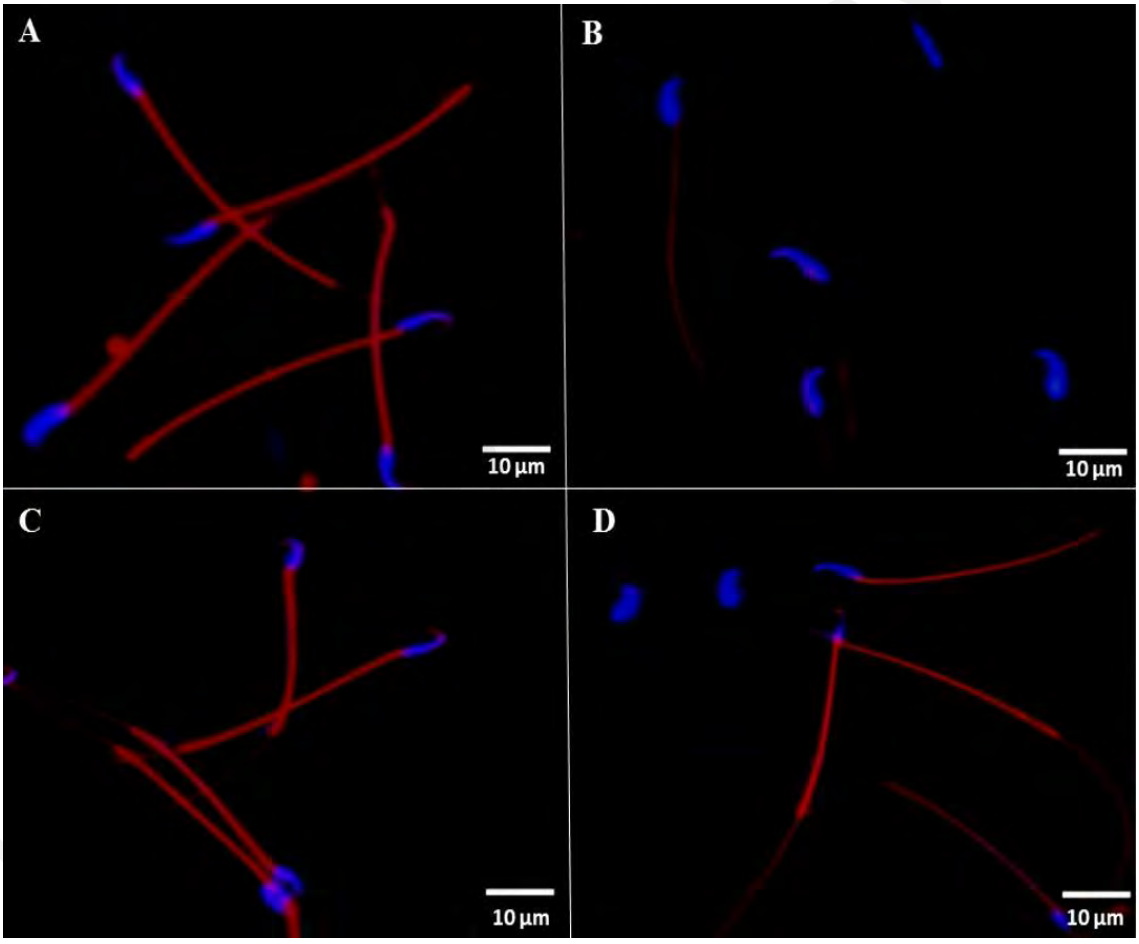


Figure 4.5: A typical photomicrograph of rat sperm stained with MitoTracker Red FM at 40x magnification. For imaging purposes, nuclear DNA was stained with the DNA dye DAPI (blue). Sperm with active mitochondria labeled red at the midpiece region. (A) Control group. (B) Nicotine group. (C) TQ group. (D) Nicotine-TQ group

Table 4.3: Percentage of MitoTracker positive sperm of rats treated with nicotine and thymoquinone

Parameter / Group	MitoTracker +ve % (Mean ± S.E.)
Control	87.04 ± 0.88[c]
Nicotine	75.68 ± 0.90[a]
Thymoquinone (TQ)	88.0 ± 0.84[c]
Nicotine-Thymoquinone (nicotine-TQ)	79.08 ± 0.74[b]

[abc] Data with different superscripts within the same column show significant difference ($p<0.05$). N = 8 rats per control and treatment groups

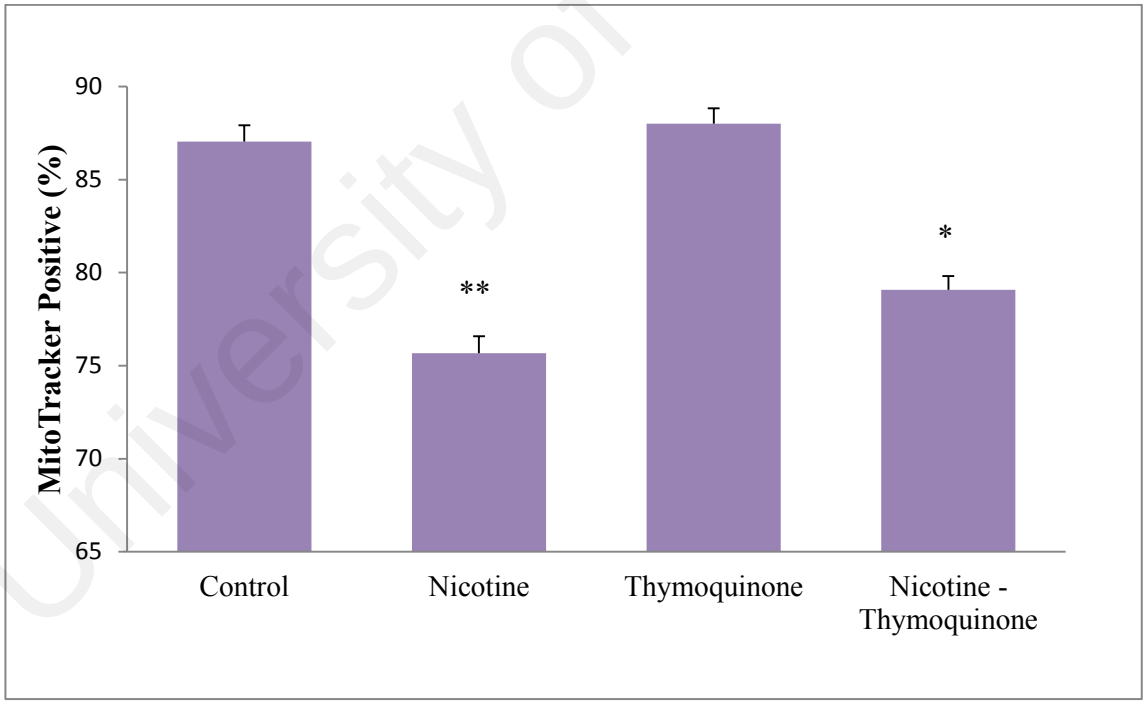


Figure 4.6: Sperm mitochondrial assessment using MitoTracker Red FM. Histogram of the percentage of sperm stained with MitoTracker in different groups. Data is expressed as mean ± S.E with N=8 in each group. *Significantly different with lower value as compared to control and thymoquinone groups ($p<0.05$). **Significantly different compared to control, thymoquinone and nicotine-thymoquinone groups ($p<0.05$)

4.4 DNA Integrity Assessment

The DNA damage caused by nicotine was evidenced by the increase in the comet assay profiles (Figure 4.7) such as TL, TM, OTM and % tail DNA compared to control and TQ groups ($p<0.05$; Table 4.4). The extent of fragmentized DNA migrated away from the head of the comet (nuclear core) is directly proportional to the DNA damage. The DNA damage was smaller in nicotine-TQ group compared to nicotine group in all parameters.

Figure 4.7: Comet images of sperm cells processed using single cell gel electrophoresis (Comet) assay, stained with SYBR Green. (A) Control group: intact cell with undamaged DNA (without comet tail). (B) Nicotine group: sperm with damaged DNA, most of the DNA has migrated to tail. (C) TQ group: sperm with no DNA damage. (D) Nicotine-TQ group: sperm with moderate DNA damage. (E) and (F) Enlargement figure of comet from control group and nicotine group, respectively (enlargement of 200%)

Table 4.4: Sperm DNA damage assessment of rat treated with nicotine and thymoquinone by Comet assay

Parameter / Group	Tail length (arbitrary unit) (Mean ± S.E.)	Tail moment (arbitrary unit) (Mean ± S.E.)	Olive tail moment (arbitrary unit) (Mean ± S.E.)	% tail DNA (arbitrary unit) (Mean ± S.E.)
Control	18.98 ± 0.28[a]	1.45 ± 0.09[a]	1.52 ± 0.09[a]	1.17 ± 0.07[a]
Nicotine	31.24 ± 1.12[c]	4.73 ± 0.20[c]	5.21 ± 0.26[c]	7.06 ± 0.36[c]
Thymoquinone (TQ)	20.46 ± 0.31[a]	1.57 ± 0.10[a]	1.72 ± 0.10[a]	1.23 ± 0.08[a]
Nicotine – Thymoquinone (nicotine-TQ)	22.59 ± 0.40[b]	2.89 ± 0.10[b]	3.04 ± 0.14[b]	2.40 ± 0.16[b]

[abc] Data with different superscripts within the same column show significant difference ($p<0.05$). N = 8 rats per control and treatment groups

4.5 Correlation between Mitochondrial Function and DNA Damage

A significant negative relationship ($p<0.01$) was found between percentage of the MitoTracker positive sperm and DNA damage (Tail DNA percentage) in the experimental groups using Pearson correlation analysis, r = -0.480 (Figure 4.8). This analysis demonstrated that the number of sperm with functional mitochondria was inversely proportional to the DNA damage.

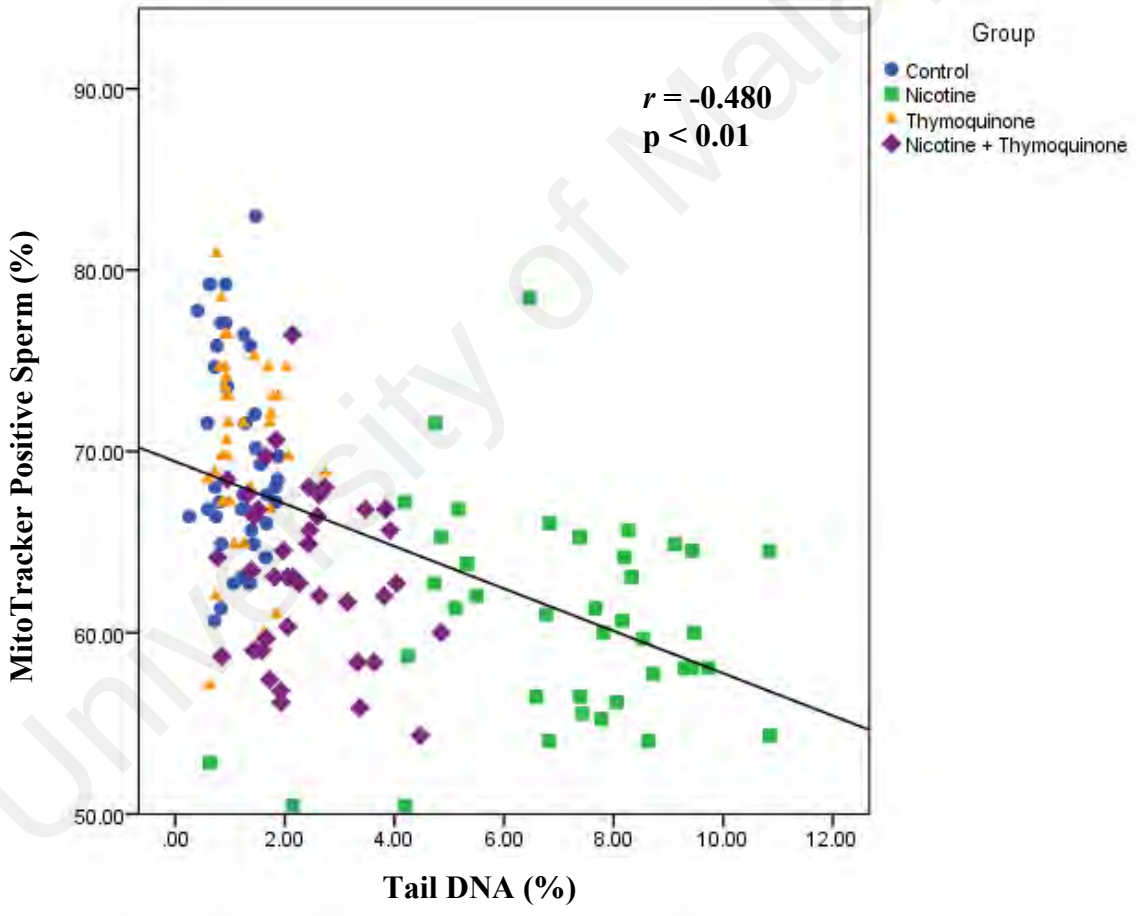


Figure 4.8: Scatter plot shows the correlation of functional mitochondria (percentage of MitoTracker positive) and DNA damage (percentage of tail DNA of nuclei) in sperm between experimental groups

4.6 Molecular Analysis of *Transition Nuclear Protein 2* (*TNP2*) and *Protamine 1* (*PRM1*)

4.6.1 RNA Quality Assessment by Gel Electrophoresis

The quality of RNA samples in each group by means of its integrity were checked by agarose gel electrophoresis. The total RNA quality were assessed on the basis of 18S and 28S rRNA. Results showed two defined ribosomal RNA bands with the 28S band approximately two-fold more intense than the 18S band indicative of intact isolated RNA (Figure 4.9).

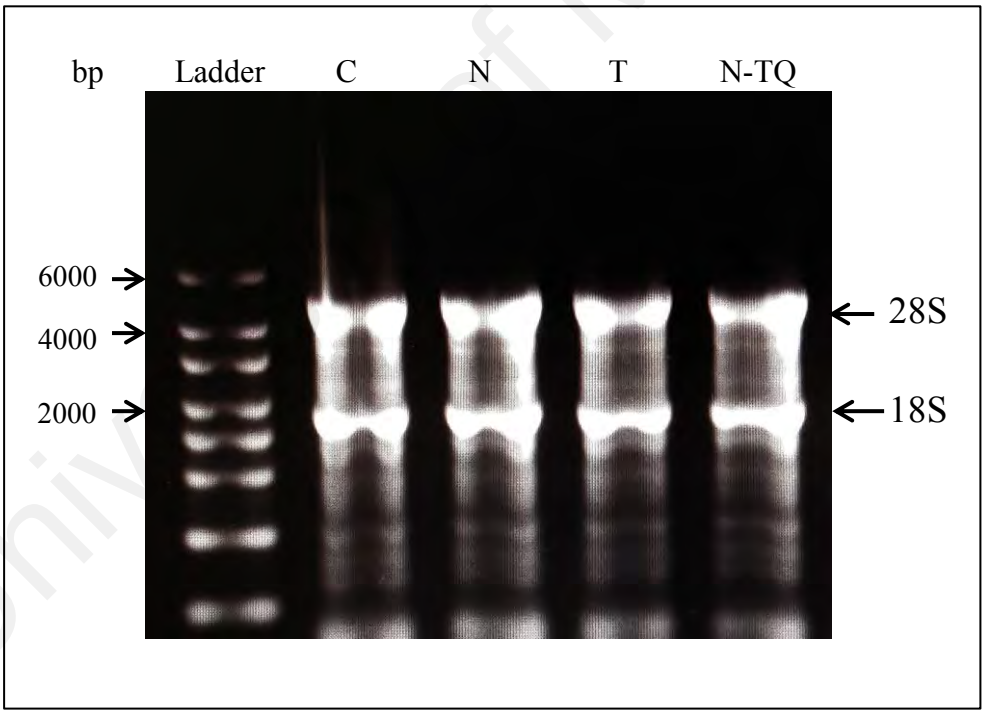


Figure 4.9: Determination of RNA integrity by 1% agarose gel electrophoresis. Total RNA isolated from control group (lane C), nicotine group (lane N), TQ group (lane T) and nicotine-TQ group (lane NTQ). All sample lanes show 18S (~2kbp) and 28S (~5kbp) rRNA bands

4.6.2 Gene and Primer Sequence Verification Using Conventional PCR and DNA Sequencing

4.6.2.1 Conventional PCR

Conventional PCR was performed prior to gene expression level analysis to confirm the presence of particular genes in the tissue sample and to assess the efficacy of primers designed. The genes tested were target genes: *TNP2* and *PRM1* and reference genes: *GAPDH* and *ACTB*. Agarose gel electrophoresis showed the expression of all the genes in the testis through comparison of its known fragment size to the DNA ladder (Figure 4.10).

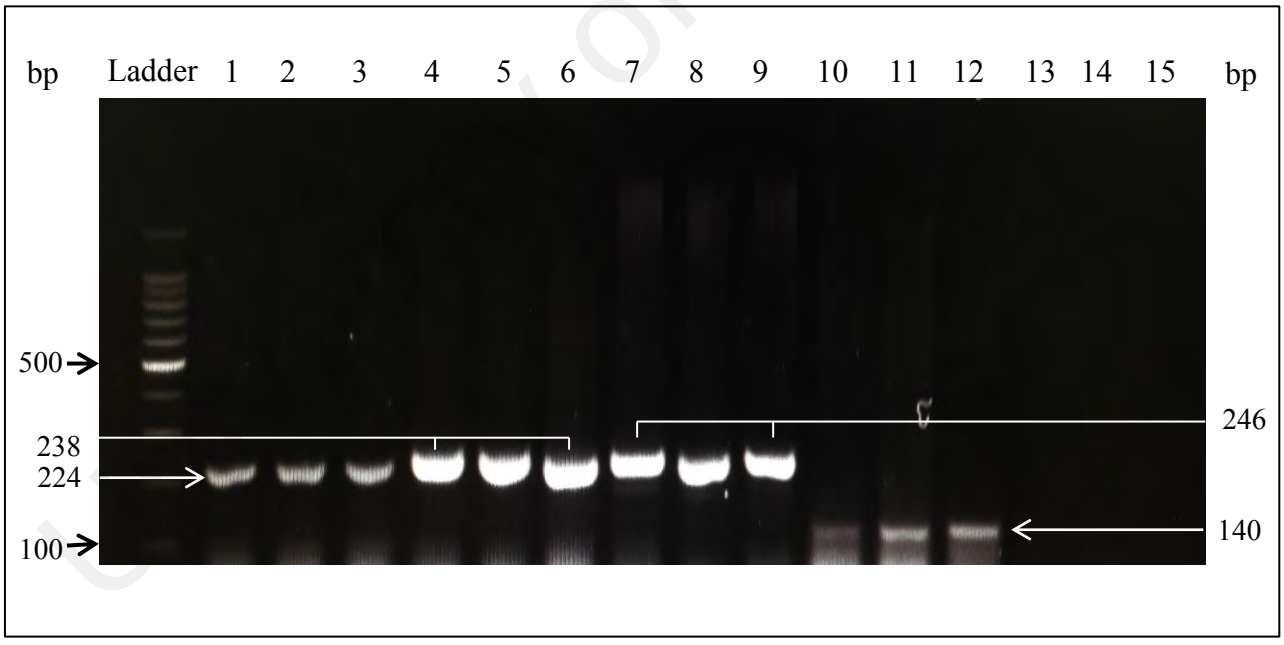
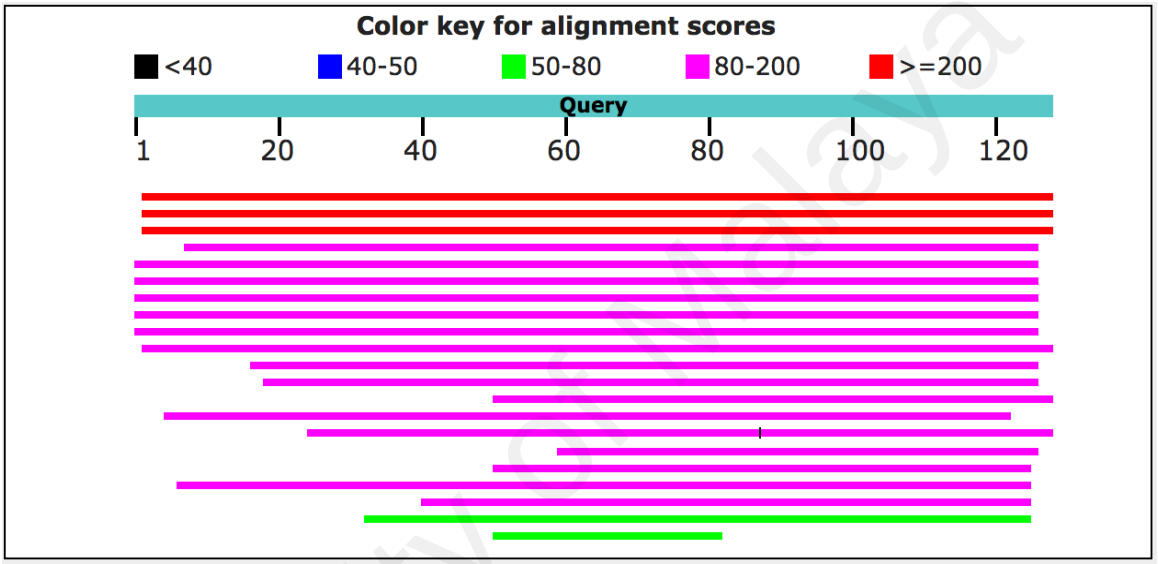


Figure 4.10: Agarose gel electrophoresis of reverse transcription-PCR product. Lane 1 – 3: *GAPDH* (224 bp); Lane 4 – 6: *ACTB* (238 bp); Lane 7 – 9: *TNP2* (246 bp); Lane 10 – 12: *PRM1* (140 bp); Lane 13 – 15: negative control

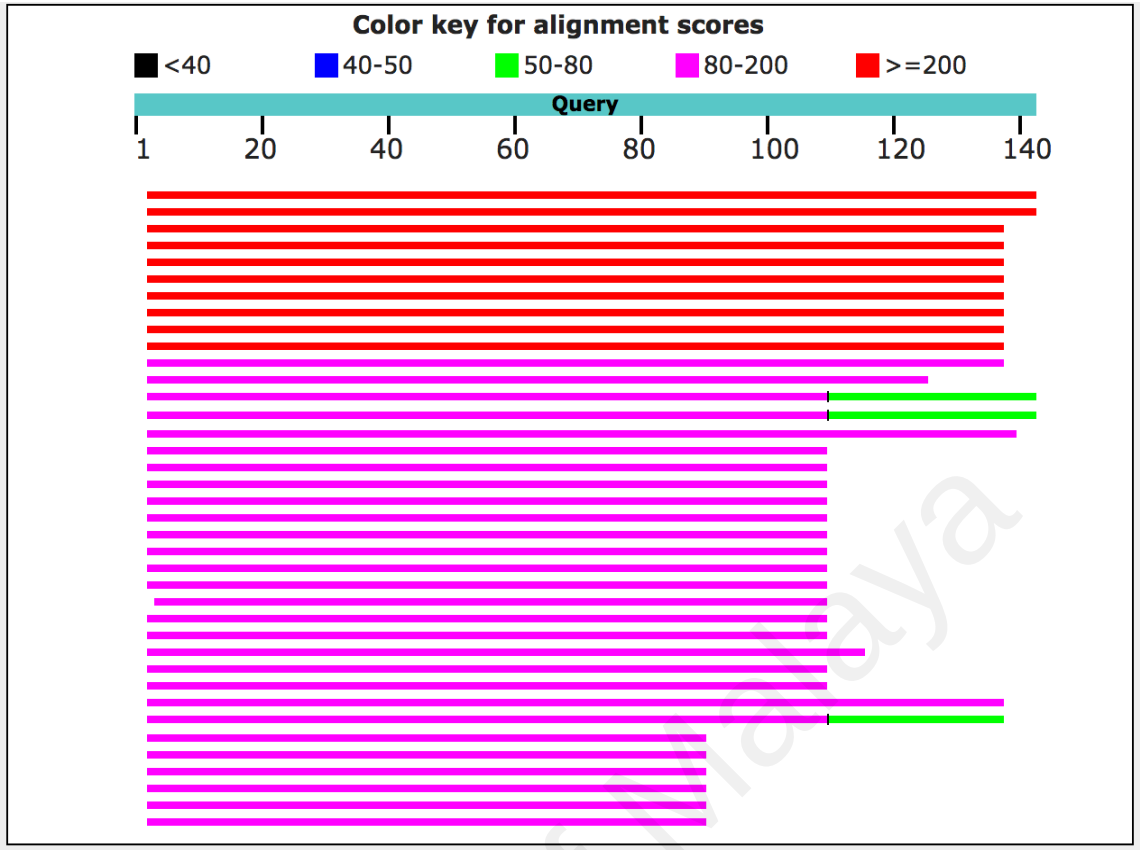
4.6.2.2 DNA Sequencing

The sequence of the amplified product was verified by BLAST from NCBI (Figure 4.11 and 4.12). Refer to Appendix L for sequencing results.



	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	TPA: Rattus norvegicus transition protein 2 (Tnp2) gene, complete cds	235	235	99%	2e-58	100%	BK006521.1
☐	Rattus norvegicus transition protein 2 (Tnp2), mRNA	235	235	99%	2e-58	100%	NM_017057.2
☐	R.norvegicus PRM1, PRM2, PRM3 and TNP2 genes	235	235	99%	2e-58	100%	Z46939.1
☐	PREDICTED: Mus caroli transition protein 2 (Tnp2), mRNA	196	196	92%	1e-46	97%	XM_021185460.1
☐	Mus musculus transition protein 2 (Tnp2), mRNA	191	191	98%	5e-45	94%	NM_013694.4

Figure 4.11: Alignment of *TNP2* combined sequence with gene sequence from NCBI database using Basic Local Alignment Search Tool (BLAST) confirmed *TNP2* from *Rattus sp.*



	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Rattus norvegicus protamine 1, mRNA (cDNA clone MGC:156539 IMAGE:7134211), complete cds	252	252	98%	2e-63	99%	BC126070.1
☐	Rattus norvegicus protamine 1 (Prm1), mRNA	252	252	98%	2e-63	99%	NM_001002850.1
☐	Mus musculus protamine 1 (Prm1), mRNA	209	209	95%	1e-50	95%	NM_013637.5
☐	PREDICTED: Mus caroli protamine 1 (Prm1), mRNA	209	209	95%	1e-50	95%	XM_021184985.1
☐	Mus musculus protamine 1, mRNA (cDNA clone MGC:58255 IMAGE:6773822), complete cds	209	209	95%	1e-50	95%	BC049599.1

Figure 4.12: Alignment of *PRM1* combined sequence with gene sequence from NCBI database using Basic Local Alignment Search Tool (BLAST) confirmed *PRM1* from *Rattus sp.*

4.6.3 Reference Genes Stability

Stable reference genes that does not vary between experimental or treatment groups are required for effective normalization. The reference genes selected for this study showed stable expressions across the groups (Figure 4.13).

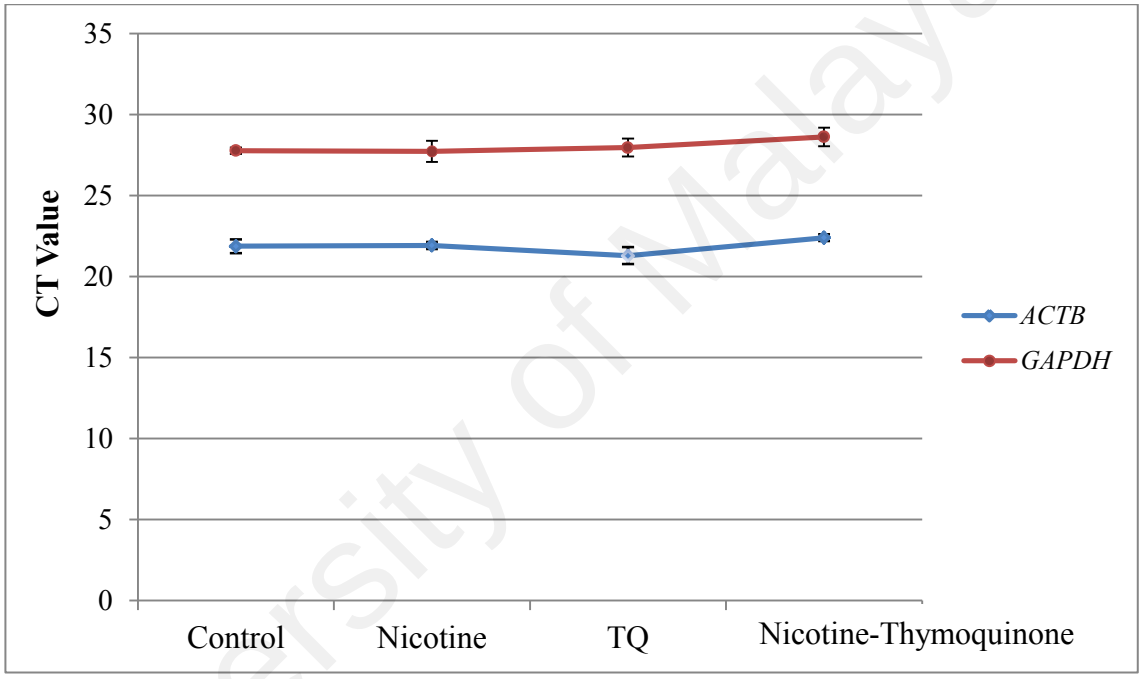


Figure 4.13: The reference genes, *ACTB* and *GAPDH* expression levels in rat testis between experimental groups

4.6.4 Efficiency of qPCR by Standard Curve

The amplification efficiency values were generated from QuantStudio 12K Flex Real-Time PCR Software, determined by analyzing standard curve. The value is important in gene expression level analysis using Pfaffl and Vandesompele methods. The desired amplification efficiency range from 90% to 110% (a slope between -3.1 and -3.6) were achieved for all primers tested (Figure 4.14 - 4.17).

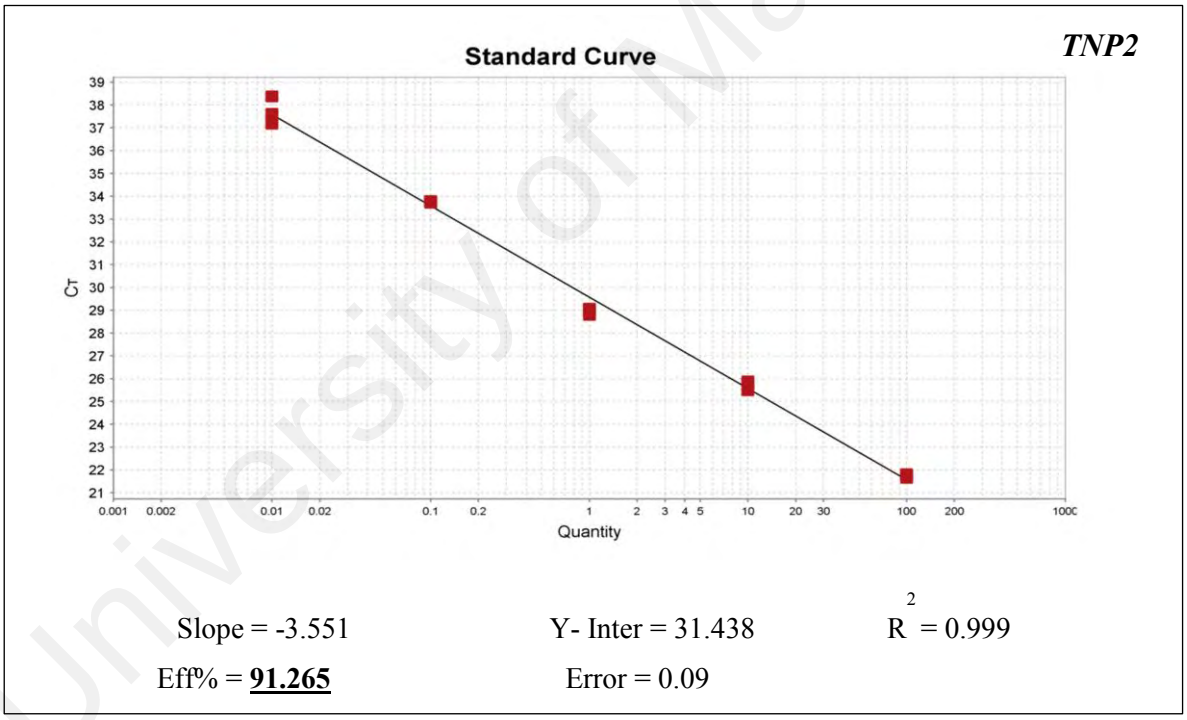
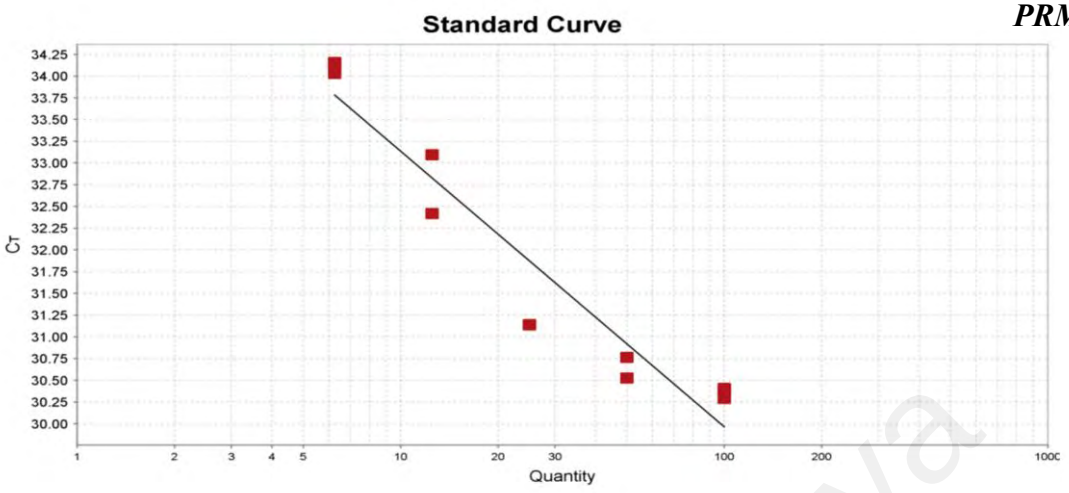


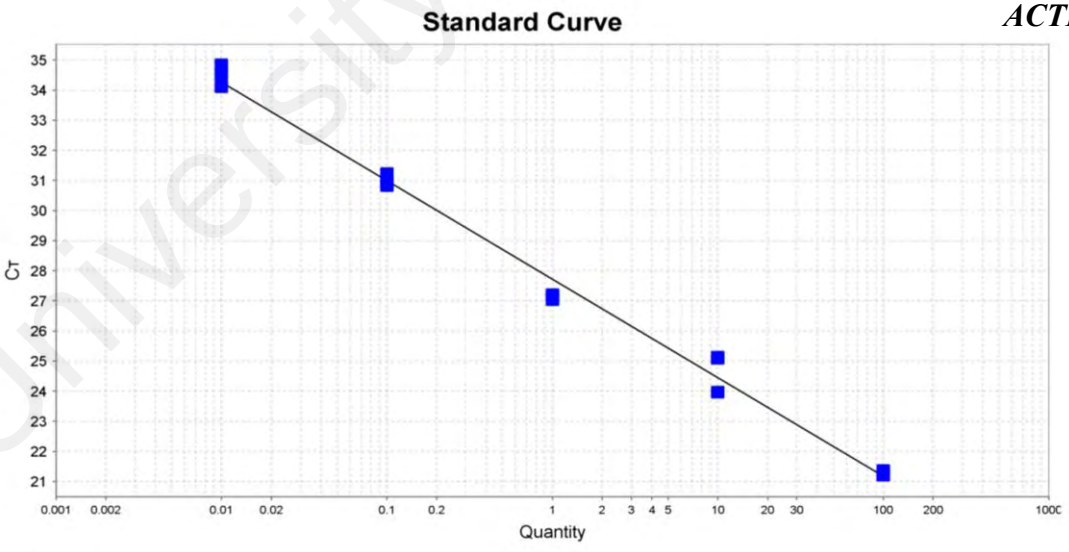
Figure 4.14: A 10 fold-serial dilution of cDNA was used to generate a standard curve for *TNP2* from rat testis by qPCR assay. The efficiency percentage was calculated from the standard curve by QuantStudio 12K Flex Real-Time PCR Software. (Eff% = 91.265)



Slope = -3.17 Y- Inter = 36.304 R^2 = 0.97

Eff% = **106.779** Error = 0.302

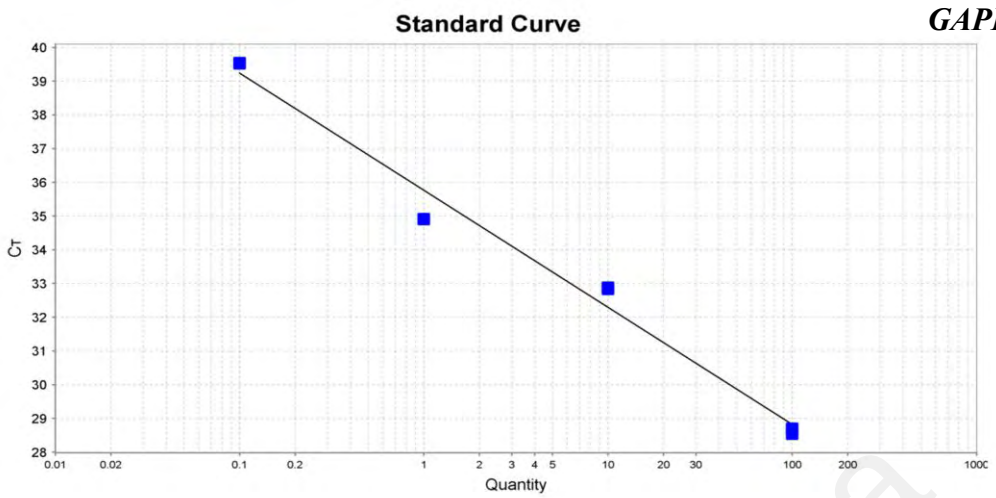
Figure 4.15: A 2 fold-serial dilution of cDNA was used to generate a standard curve for *PRM1* from rat testis by qPCR assay. The efficiency percentage was calculated from the standard curve by QuantStudio 12K Flex Real-Time PCR Software. (Eff% = 106.779)



Slope = -3.272 Y- Inter = 27.722 R^2 = 0.992

Eff% = **102.127** Error = 0.083

Figure 4.16: A 10 fold-serial dilution of cDNA was used to generate a standard curve for *ACTB* from rat testis by qPCR assay. The efficiency percentage was calculated from the standard curve by QuantStudio 12K Flex Real-Time PCR Software. (Eff% = 102.127)



Slope = -3.473 Y- Inter = 35.768 R^2 = 0.982

Eff% = **94.075** Error = 0.168

Figure 4.17: A 10 fold-serial dilution of cDNA was used to generate a standard curve for *GAPDH* from rat testis by qPCR assay. The efficiency percentage was calculated from the standard curve by QuantStudio 12K Flex Real-Time PCR Software. (Eff% = 94.075)

4.6.5 Expression Analysis of *TNP2* and *PRM1* Genes

Analysis of *TNP2* and *PRM1* genes were performed using qPCR (Appendix M). The *TNP2* gene showed no expression level changes in thymoquinone group but was significantly down regulated in nicotine group (0.047 ± 0.009) and nicotine-TQ group ($p<0.05$). Whereas, the expression level of *TNP2* in nicotine-TQ group demonstrated significant higher expression (0.111; $p<0.05$) compared to nicotine group (Figure 4.18). The expression level of *PRM1* was unaffected by nicotine and thymoquinone as there were no significant fold changes between treatment and the control groups (Figure 4.19).

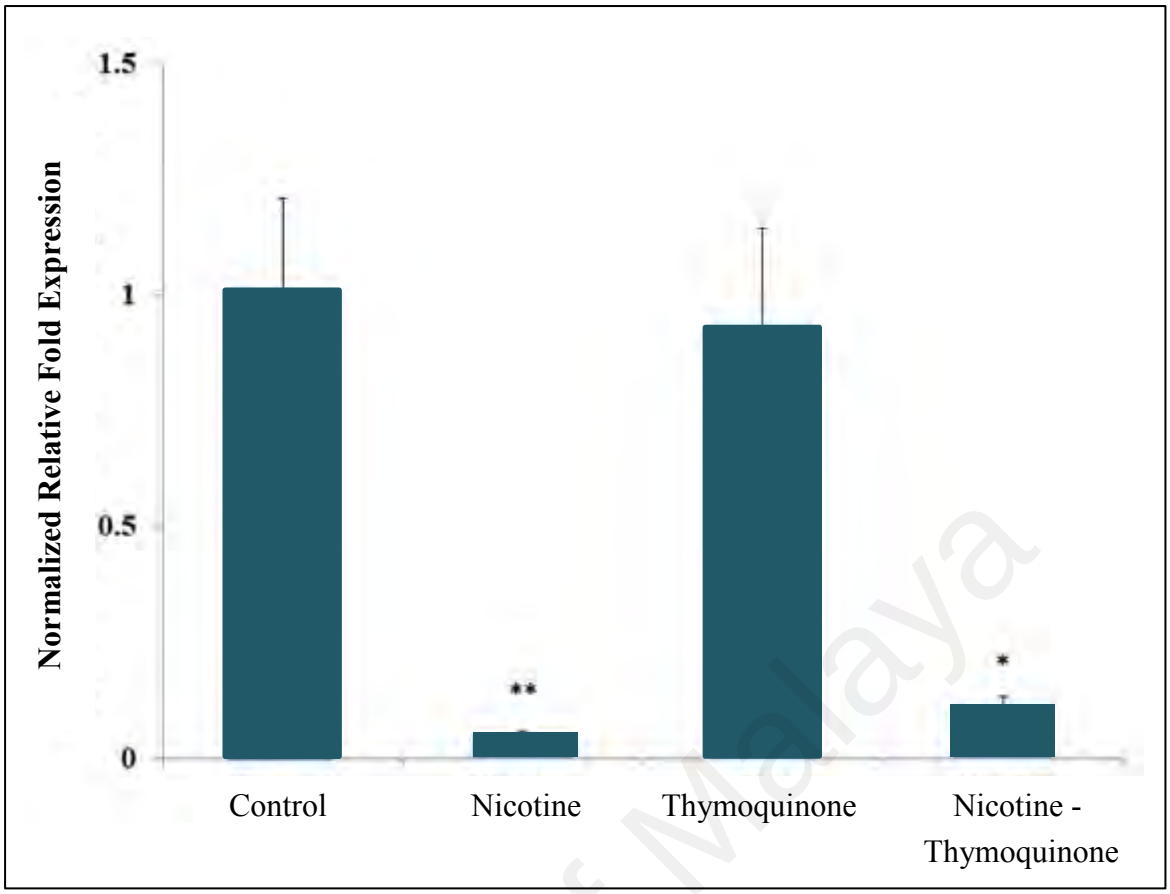


Figure 4.18: Effects of nicotine and thymoquinone on the expression of *TNP2*, one of the genes responsible for sperm chromatin condensation in rat testis. Expression level were normalized against expression of *ACTB* and *GAPDH*. Results are expressed as normalized relative fold expression ± S.E. *Significantly different compared to the control group ($p<0.05$).**Significantly different compared to the control and nicotine-thymoquinone groups ($p<0.05$)

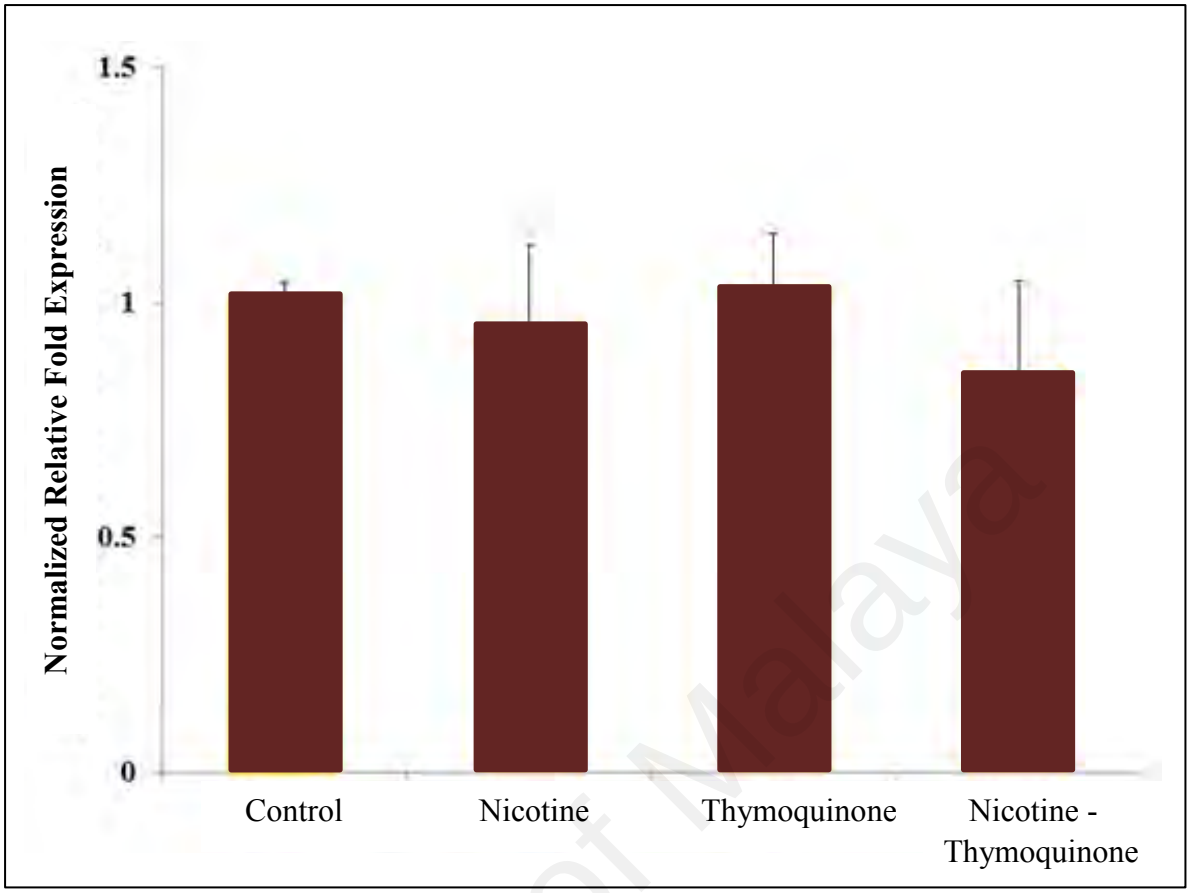


Figure 4.19: Effects of nicotine and thymoquinone on the expression of *PRM1*, one of the genes responsible for sperm chromatin condensation in rat testis. Expression level were normalized against expression of *ACTB* and *GAPDH*. Results are expressed as normalized relative fold expression ± S.E.

CHAPTER 5: DISCUSSION

5.1 Rat as a Research Animal Model

Rats have been used as animal models dated back to before 1850s (Lindsey & Baker, 2006). Now, it is one of the most widely used models in biomedical research to study human diseases. According to Jacob (2009), the research community began to move away from dogs towards rats in 1960s. Consequently, many of the rat strains have been extensively studied which has led to well characterized physiology of rats across all major organ systems and the animal being ideal for work related to biology systems. Amann (1982) regarded the rodents as the most ideal species in reproductive studies followed by rabbits. The higher sperm production efficiency in its males compared to human males may be advantageous as they allow a great range of responses. Rats are presumably preferable to mice and hamster because of their well characterized reproductive processes, convenient size and its general use in toxicological studies. Moreover, the reproductive organs of rats are large enough to observe quantification changes, even after 50% to 75% reduction in weight. Sprague Dawley rats are widely used in reproductive studies because of its high fertility and consistent breeding characteristics with low incidence of spontaneous tumor. Furthermore, Wilkinson et al. (2000) showed that Sprague Dawley has the heaviest body weight, largest seminal vesicle and highest sperm count as compared to the Wistar or Dark Agouti rats.

In the present study, Sprague Dawley rats were used and 5 mg/kg of nicotine given as the dose showed adequate disruption of spermatogenesis and induced injurious effects on the reproductive functions (Mahaneem et al., 2007; Fairuz et al., 2011) which is necessary to observe ameliorative potential of thymoquinone (TQ). For TQ, the

dosage of 5 mg/kg was used as it demonstrated therapeutic effects without showing any adverse clinical signs and the dose is far below its LD_{50} (Mabrouk & Ben Cheikh, 2016).

There are a vast number of techniques available in evaluating semen and sperm quality which includes sperm count, motility, morphology and DNA integrity. In this study we focused on sperm count, sperm motility, sperm membrane integrity, sperm mitochondrial function and DNA damage assessments were examined.

5.2 Sperm Count Evaluation

Sperm count and sperm motility are parts of semen analysis testing in estimating fertility. These assessments are widely used in semen quality measurements since low number of sperm and abnormal sperm motility would lower the chances of successful fertilization. In humans, the tests assist a physician in determining if a man is infertile and ascertaining whether sperm's health is the reason behind the infertility (Khatun et al., 2018).

The present study showed that there was a significant decrease in the sperm count of nicotine group compared to control in rats. The findings corroborated the negative impact of nicotine towards sperm count by numerous studies (Trummer et al., 2002; Mostafa, 2010; Egesie et al., 2013). In a study by Egesie et al. (2013), male rats were administered with 2 mg/kg body weight (b.w.), 4 mg/kg b.w. and 6 mg/kg b.w. of nicotine for 30 days. The result showed that nicotine caused significant reduction of sperm count compared to control group. When ICR male mice were intraperitoneally

injected with b.w. of nicotine for 35 days, sperm count of nicotine treated group decreased in dose dependent manner (Kim et al., 2008).

The decrease in the sperm count could be associated with nicotine's detrimental effects on testosterone production (Segarra & Strand, 1989). Testosterone is essential for spermatogenesis in the testis and sperm maturation in the epididymis (Sharpe et al., 1992; Takamiya et al., 1999). This is supported by Oyeyipo et al. (2010) where nicotine was given orally to male rats for 30 days at 0.5 and 1.0 mg/kg b.w. The serum levels of testosterone for both groups were significantly lower in a dose dependent manner. Nicotine effects on testosterone was first demonstrated by Kasson and Hsueh (1985) who revealed the inhibition of androgen biosynthesis by nicotinic cholinergic agonists on the primary culture of testicular cells derived from hypophysectomized rats. Nicotinic cholinergic agonists are drugs such as nicotine that imitate the action of acetylcholine on the nicotinic receptors located in the nervous system, muscles and many other tissues (Kasson & Hsueh, 1985). The findings of that study showed that the inhibition occurred selectively on the 17α-hydroxylase activity, an enzyme involved in androgen biosynthesis. Research by Yeh et al. (1989) showed that nicotine and cotinine inhibited 17α-hydroxylase, 17,20-lyase and 17-ketosteroid reductase which are important enzymes in multiple stages of testosterone synthesis from the steroid precursor, cholesterol.

Previous studies also suggested that nicotine might alter the testosterone level through hypothalamus-pituitary-adrenal (HPA) axis. Smoking and nicotine activates the release of Corticosteroid Releasing Hormone (CRH) that leads to the release of Adrenocorticotropic hormone (ACTH) from the anterior pituitary (Matta, 1987). The ACTH will stimulate the secretion of glucocorticoids such as cortisol from the cortex of adrenal gland which in turn inhibits gonadotropins [follicular stimulating hormone (FSH) and luteinizing hormone (LH)] secretion from the pituitary gland (Weisberg,

1985; Vine, 1996). This inhibition could possibly occur as the rise in plasma levels of glucocorticoids suppresses the sensitivity of the gonadotroph cells in the pituitary towards gonadotropin-releasing hormone and subsequently, may prevent gonadotropin secretion. Lack of gonadotropin will decrease testosterone production and eventually causes an arrest of spermatogenesis as well as affecting the maintenance of structural morphology and normal physiology of testis.

In fact, Jana et al. (2010) showed that chronic exposure of nicotine significantly reduced the plasma concentrations of LH and FSH in rats, demonstrating the inhibitory action of nicotine on gonadotropin release from the pituitary gland.

In addition, nicotine could affect the sperm count by directly promoting testicular peroxidation through the generation of free radicals. Mosbah et al. (2015) demonstrated that administration of 1 mg/kg b.w. of nicotine for 2 months in male rats induced degeneration and atrophy of testicular tissue. The researchers ascribed the results to nicotine-induced oxidative damage indicated by the elevation of lipid peroxidation by-product, Thiobarbituric Acid Reactive Substances (TBARS) and significant reduction in the activities of the antioxidant enzymes. Nicotine interacts with oxygen molecules and increases the generation of the reaction by-products called free radicals which are unstable atoms or molecules with extra or unpaired electrons. Its unstable state would make the free radicals constantly seek to relieve itself of the unpaired electron resulting in oxidation of other molecules and negatively affecting the molecule (Tremellen, 2008). High levels of unsaturated fatty acids in the membranes make the testis tissue susceptible to peroxidative decomposition by free radicals (Crowley-weber et al., 2003). This may contribute to testicular injury and would interfere with sperm production and hormonal secretions of androgens since the major roles of testis are spermatogenesis and steroidogenesis.

As expected, sperm count was significantly higher in nicotine-TQ group compared to nicotine group in this study (Table 4.1). This result showed the ability of TQ to improve sperm count against the harmful effects of nicotine and was in agreement with several reports which showed that TQ improved low epididymal sperm count after various treatments or conditions (Al-Zahrani et al., 2012; Ng et al., 2014; Mabrouk & Ben Cheikh, 2016; Salahshoor et al., 2018). Mabrouk and Ben Cheikh (2016) showed that supplementation of 5 mg/kg b.w. TQ for 5 weeks to male rats exposed to 2000 parts per million (ppm) of Pb (lead) acetate in drinking water, significantly improved the decreased epididymal sperm count caused by lead. This was in agreement with a report by Salahshoor et al. (2018) in which male mice were intraperitoneally injected with 20 mg/kg b.w. of morphine and orally administered with TQ in various doses. Male reproductive parameters which included sperm count showed significant improvement in the TQ group and co-administration of TQ and morphine treated groups compared to morphine group.

The increment of the sperm count could be attributed to the effects of TQ on the reproductive hormones secretion. Improvement of the sperm count by TQ and *Nigella sativa* were often reported along with increased plasma testosterone level and Leydig cells number which would likely facilitate spermatogenesis process and increased the sperm count. Mosbah et al. (2016) showed that *Nigella sativa* oil, conceivably through its active component TQ was reported to be able to increase testosterone, LH and FSH biosynthesis in rats when administered alone and improved the hormone level imbalances induced by Chylorpyfiros, an insecticide. Similarly, Aithal et al. (2016) showed that diabetic rats treated with *Nigella sativa* seed powder (300 mg/kg b.w) and TQ (4 mg/kg b.w) have higher testosterone level compared to diabetic-induced group. These effects may result from the activation of hypothalamic–pituitary–testicular axis which then increased the steroidogenesis and spermatogenesis processes (El-Khasmi et

al., 2011). It was also reported that *Nigella sativa* oil would stimulate the activity of ß-hydroxysteroid dehydrogenase, an enzyme involved in testosterone synthesis (Tawfeek, 2006).

Apart from that, Sankaranarayanan and Pari (2011) reported that administration of TQ exerted marked antioxidant properties in a chemically-induced oxidative stress model. Excess free radicals in the oxidative stress state may cause testicular injury through peroxidation which will affect sperm production as testis is the site of spermatogenesis. Sayed et al. (2014) showed that TQ strengthened antioxidant systems, reduced lipid peroxidation and improved histological damage of rats' reproductive organs caused by cadmium chloride. TQ could improve the antioxidant defense against free radicals generated by nicotine and ameliorate the testicular impairment which consequently would recover the sperm count.

5.3 Sperm Motility Evaluation

The number of motile sperm was significantly decreased in nicotine group compared to control. While the number of motile sperm was higher in nicotine-TQ group in comparison to nicotine group (Table 4.1). These results reflect the unfavorable effect of nicotine and the ability of TQ in improving motile function of sperm.

Various studies revealed that sperm motility of rats decline with nicotine treatment. When male S.D. rats were intramuscularly injected with nicotine at 5 mg/kg b.w. for 100 days and sperm were collected, sperm motility significantly increased as compared with control (Ng et al., 2014). Oral administration of aqueous extract of

Nicotiana tabacum at doses 10 -30 mg/kg b.w. for 21 days adversely affected sperm motility in a dose dependent manner in male rats (Gambo et al., 2013).

Nicotine could impact the motility of sperm by disrupting their membrane integrity through free radicals and reducing its supply of energy, adenosine triphosphate (ATP). The sperm membrane contains abundant amounts of unsaturated fatty acids which gives structural fluidity that is necessary for membrane fusion during fertilization (Hwang & Lamb, 2012). This structure, however, is also more susceptible to free radical attacks and the initiation of lipid peroxidation (Agarwal et al., 2007). Jones et al. (1979) were the first to report on free radical's ability to hamper sperm motility. Experiments were conducted on washed human sperm and treated with as little as 30 nmoles of lipid peroxide/ml containing ascorbate and ferrous ions. The report showed that the sperm became immotile within minutes caused by the rapid peroxidative breakdown of sperm phospholipids and fatty acids. Free radicals or reactive oxygen species (ROS) cause peroxidation of the sperm membrane which will reduce sperm flexibility, tail motion and consequently diminish the sperm movement. Apart from that, direct ROS damage towards the mitochondria causes impediment of energy production and thus decreases the sperm motility (De Lamirande & Gagnon 1992a; De Lamirande et al., 1997). This was supported by De Lamirande and Gagnon (1992b) who discovered significant loss of sperm's ATP levels in the first hour of ROS treatment followed by reduction of intact sperm motility. These authors proposed that rapid immobilization was due to insufficient phosphorylation, a process important for ATP generation as well as protein regulation. This caused damage towards axonemal protein of sperm flagella that is required for movement. These findings were corroborated by Duru et al. (2000) who showed that *in vitro* incubation of sperm with hydrogen peroxide, the major ROS produced in the sperm, affects sperm motion parameters in a dose dependent manner through the depletion of intracellular ATP.

The ameliorative action of TQ towards sperm motility in this study seems mainly due to its potent antioxidant capacity. Thymoquinone's oxidant scavenger system may assisted the neutralization of free radicals and protected the membrane structure from lipid peroxidation thus preserving sperm fluidity and allowed the observed improvement of sperm mobility. Mohammad et al. (2009) showed that oral administration of 300 mg/kg b.w. *Nigella* seed solution to male rats for 60 days significantly increased sperm motility as compared to control rats. Miah et al. (2018) has also demonstrated the ability of *Nigella sativa* oil and TQ as supplements in cryoprotectant to improve sperm cryo-survivability in ovine sperm. Moreover, Nagi et al. (2010) reported that supplementation of TQ completely reversed the decreased of ATP induced by acetaminophen by the improvement of mitochondrial functions which consequently enhanced sperm motility.

5.4 Membrane Integrity Assessment

Membrane integrity is important for sperm metabolism and its properties are associated with sperm capacitation, acrosome reaction and fusion of sperm to the egg surface (Jeyendran et al., 1984). Therefore, the assessment of sperm membrane integrity is a useful indicator of sperm fertilizing capacity. The membrane integrity of sperm in the current study was assessed using hypo-osmolality test (HOST). This test works on the basis of semi-permeability of the intact cell membrane, which causes sperm to swell under hypo-osmotic conditions.

The present study demonstrated a significant higher number of membrane damaged sperm in nicotine group compared to other groups (Table 4.2). The sperm membrane is particularly vulnerable to oxidative damage as a result of large amounts

of polyunsaturated fatty acids (PUFA) in the structure that are substrates for ROS (Agarwal & Prabakaran, 2005). The presence of double bonds in the PUFA may trigger a lipid peroxidation (LPO) cascade during the free radical attack. As a result of the lipid peroxidation, lipid hydroperoxides, alkoxyl and peroxyl radicals are produced which will further cause a chain reaction of lipid peroxidation (Baumber et al., 2000).

According to Bansal and Bilaspuri (2011), the autocatalytic and self-propagating reaction of polyunsaturated fatty acids peroxidation in the sperm cell membrane can lead to cell dysfunction related to the loss of membrane functions and integrity. Furthermore, the loss of membrane integrity will increase its permeability and impair the capacity to regulate intracellular ions involved in the control of sperm mobilization. This present result is in accordance with other studies which proved nicotine's harmful effects on the plasma membrane (Arabi, 2004).

The greater number of sperm with intact membrane in nicotine-TQ group compared to nicotine group, indicated TQ's potent antioxidant activity (Table 4.2). Thymoquinone was reported to be a powerful free radical and superoxide radical scavenger (Mansour et al., 2002). Badary et al. (2003), showed the strong antioxidant potentials of TQ through scavenging ability of different free radicals which is mainly a potent superoxide anion scavenger *in vitro*. The results of Salahshoor et al. (2018) is in agreement with results of the present study on the therapeutic effect of TQ towards sperm membrane. Their experiment was conducted on male mice that were given morphine, 20 mg/kg body weight by intraperitoneal injections to induce damage and TQ through oral administration at various doses (2 mg/kg b.w., 10 mg/kg b.w. and 20 mg/kg b.w.) for 30 days. Based on the study, the reduction of the sperm count in morphine-receiving groups could be the direct result of increased lipid peroxidation due to oxidative stress, thus leading to the loss of sperm transfer to the epididymis.

Thymoquinone acted as an antioxidant to decrease this tendency and neutralized the destructive effects of morphine.

Nigella sativa seed aqueous extract showed protective role towards sperm membrane integrity in a cryopreservation study (Awan et al., 2018). Higher plasma membrane integrity percentage was observed in tris-citric egg yolk extender supplemented with *Nigella sativa* extract for the chilled and thawed spermatozoa compared to control at a concentration dependent manner in buffalo.

5.5 Mitochondrial Function Assessment

Sperm are rich in mitochondria which is a vital organelle for normal sperm functions. Besides ATP production, mitochondria are involved in calcium homeostasis, generation of physiological ROS, the intrinsic apoptotic pathway and steroid hormone biosynthesis (Amaral et al., 2013). Alteration in mitochondrial function is also associated with reduced sperm motility. Mitochondrial function is often assessed by evaluation of inner mitochondrial membrane potential (MMP) which is the electrochemical gradient established during the process of oxidative phosphorylation when the protons are pumped from the inside of the mitochondria to the outside (Agarwal & Prabakaran, 2005).

In this study, the percentage of MitoTracker labeled sperm (MitoTracker positive) was significantly lower in nicotine group compared to other groups indicating reduced number of functional sperm mitochondria (Table 4.3). This result is in agreement with those of Jana et al. (2010) who injected rats with 0.6 mg/kg b.w. of nicotine daily for 12 weeks. The MMP of testicular tissue was determined using the JC-

1 mitochondria fluorescent dye and it was found that rats treated with nicotine have lower MMP than rats in control group. Calogaro et al. (2009) also reported parallel results when sperm obtained from healthy, non-smoking, normozoospermic men were incubated in (cigarette smoking extract) CSE. The findings showed CSE increased the percentage of sperm with low MMP in a dose and time dependent manner.

In the present study, the decreased number of sperm with functional mitochondrion could be caused by nicotine's oxidant potential where it augments ROS level. This is consistent with data by Wang et al. (2003) who collected semen samples from infertile patients and assessed MMP and ROS production in sperm. That study found a significant inverse correlation between MMP and ROS levels in the sperm. Semen samples from patients with abnormal semen parameters had higher levels of ROS and significantly lower MMP compared to samples from healthy donors. According to Mishra and Shaha (2005), high levels of ROS disrupt the inner and outer mitochondrial membranes, and cause oxidation of the mitochondrial pores which thereby causes disturbances in MMP. Direct ROS impairment towards mitochondria could deplete the energy supply, thus affecting sperm motility. As stated previously, study by De Lamirande and Gagnon (1992) showed that incubation of sperm with ROS caused a fall in sperm ATP levels followed by a decrease in sperm motility. The decline in sperm motility in nicotine group in the present study is consistent with this idea.

It was also revealed that impaired mitochondria itself could increase ROS production, which subsequently affects mitochondrial functions in sperm (Evenson et al., 1982). These two mutually interconnected relationships happen when ROS causes damage to the mitochondrial membrane and thereafter the damaged mitochondrial membrane produces more ROS. The elevation of ROS by the impaired mitochondrial membrane will further aggravate the already laden physiological antioxidant defenses.

Similar to the motility results, there was an increased percentage of sperm with functional mitochondria in nicotine-TQ group. This once more can be attributed to the antioxidant properties of TQ which include free radicals scavenging system, lipid peroxidation suppression and sustaining of the antioxidant defense mechanisms during free radical reactions (Sankaranarayanan & Pari, 2011; Basarslan et al., 2012). The antioxidative effects of TQ may be due to the redox properties of the quinone structure of the TQ molecule (Figure 5.1). Quinone has great antioxidant potential as it is able to accommodate electrons in novel ways. The quinone acts as antioxidants by terminating the chain reaction and removing free radical intermediates, thus inhibit other oxidation reactions by being oxidized themselves. The adjacent double bond arrangement of quinone also provides chemical stability as it allows rapid electron movement from one site to another and this makes it efficiently engage in repeated reduction-oxidation reactions or as antioxidants (Rucker, 2011). Thymoquinone which sometimes is referred to as edible quinone is metabolically converted into thymohydroquinone through a two-step one electron reduction or one-step two electron reduction enzymatic reactions. Thymoquinone can also be converted to glutathionylated-dihydrothymoquinone through a non-enzymatic reduction. These reduced end products of TQ have great antioxidant properties as well (Armutcu et al., 2018). Furthermore, TQ has been reported to be able to significantly cross the blood brain barrier (Elmaci & Altinoz, 2016). This is due to its very simple molecular structure and in turn it has an easy access to subcellular compartments (Badary et al., 2003), all of which facilitates its radical scavenging properties.

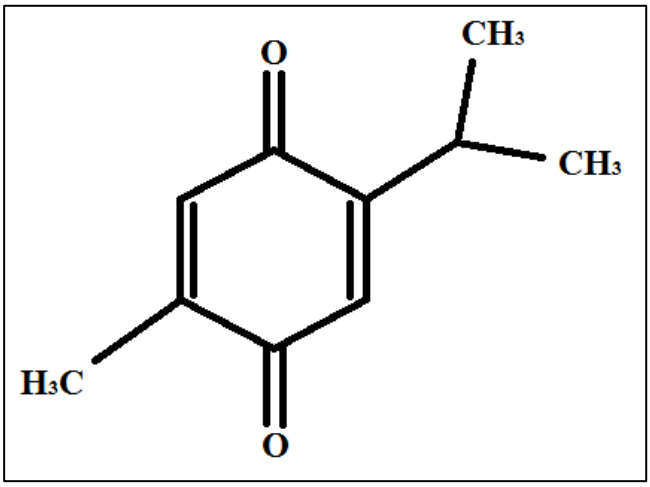


Figure 5.1: Chemical structure of thymoquinone (TQ)

Thymoquinone supplementation has also demonstrated to increase ATP in an experiment conducted by Nagi et al. (2010). In that study, mice were treated with TQ orally for 5 days and intraperitoneally injected with 500 mg/kg b.w. acetaminophen to induce hepatotoxicity. The ATP measured from liver homogenate showed that supplementation of TQ (2 mg/kg b.w.) for 5 days before the acetaminophen administration resulted in higher amount of ATP compared to acetaminophen group. This result reflected those of Sayed-Ahmed and Nagi (2007) where rats were injected with gentamicin (80 mg/kg b.w.) to induce renal failure and then received 50 mg/l of TQ in drinking water for 8 consecutive days. The findings showed that ATP (mmol/g) in kidney tissues of rats supplemented with TQ alone and TQ supplemented to gentamicin-treated rats were significantly greater than in gentamicin group. This may suggest the ability of TQ to enhance the utilization of substrates and oxidative phosphorylation resulting in increased energy production and mitochondrial function.

5.6 DNA Integrity Assessment

Integrity of DNA refers to the construction durability of the DNA to serve its purpose in storing genetic information. A sperm carries the paternal genome and the secure transfer of it to oocyte is imperative. Integrity of the DNA content in sperm is prerequisite for embryo development and offspring production (Gonçalves et al., 2010; Aitken et al., 2010). High sperm DNA fragmentation have been associated with infertility with many studies showing that the chances of successful pregnancy are significantly decreased with damaged sperm DNA (Speyer et al., 2010). When semen samples from patients with a history of unexplained recurrent pregnancy loss by their female partners were collected and analysed for DNA strand breaks, the percentage of sperm DNA fragmentation was significantly higher in patients with recurrent pregnancy loss compared to fertile controls (Brahem et al., 2011). Sperm with a high degree of DNA fragmentation was reported to have a higher miscarriage rate because embryos derived from sperm with damaged DNA could impair the blastocyst development (Simon et al., 2014). Evenson and Wixon (2008) also showed that the rates of conception were statistically lower among couples with elevated percentage of sperm with damaged DNA. Therefore, sperm DNA integrity is vital in determining the functional competence of sperm.

In present study, nicotine group showed the highest in DNA damage profile [tail length (TL), tail moment (TM), olive tail moment (OTM), and % tail DNA] compared to other groups (Table 4.4). The etiology of sperm DNA damage is multifactorial. However, sperm DNA fragmentation may result from three main interrelated mechanisms categorized as abnormal chromatin packaging, apoptosis and oxidative stress (Hekmatdoost, 2009). The sperm chromatin is extremely compact and organized to guarantee a secure transfer of paternal DNA. Abnormally packaged DNA becomes

susceptible to harm against various stressors. Compromised chromatin packing will consequently expose the sperm DNA to fragmentation and lower the chances of fertility.

Oxidative stress is the most common cause of sperm DNA damage (Kodama et al., 1997; Barroso et al., 2000). It is the state of imbalance between the formation of ROS and the ability of antioxidant scavengers to neutralize it. ROS can produce an oxidative environment and have been implicated in DNA fragmentation. It might harm DNA by directly attacking the purine and pyrimidine bases and the deoxyribose backbone specifically through modification of bases, defective frame shifts, DNA cross links, single and double strand DNA breaks and gene mutations (Duru et al., 2000; Aitken & Krausz, 2001). Normally, the chromatin of mature sperm is tightly packaged and stable which protects against DNA damage (Sakkas et al., 1999). Nonetheless, the sperm DNA remains susceptible to ROS and radicals attack, compromising the integrity of the genetic material and its fertilizing potential (Zepeda et al., 2014). Kodama et al. (1997) showed that levels of 8-hydroxy-2′-deoxyguanosine in the sperm DNA, a form of oxidative damage, were significantly higher in male infertile patients than in control patients. Wang et al. (2003) reported that infertile patients had significantly higher levels of ROS and apoptosis markers in the semen compared to normal donors which could lead to sperm DNA damage. Interestingly, Saleh et al., (2002) showed that semen semen samples from infertile men who smoked cigarettes had higher level of seminal oxidative stress indices than infertile non-smokers and fertile non-smoking control. The results also showed increase of sperm DNA damage in infertile smokers compared to other groups indicating possible relationship between oxidative stress and sperm DNA breakages. Aitken et al. (1998) also demonstrated that incubation of sperm in the presence of oxidant agent, hydrogen peroxide caused DNA damage at a concentration

dependent manner. At the highest levels of oxidative stress, extremely high rates of DNA fragmentation were observed.

As stated previously, it is well established that nicotine is able to induce oxidative stress. Seema et al. (2007) reported that rats treated with 0.6 mg/kg b.w. of nicotine for 60 days had increased production of ROS which has been associated with testicular oxidative stress. The increased DNA fragmentations in nicotine group in the current study reinforce the association of nicotine with oxidative stress (Table 4.4). Increased levels of ROS by nicotine could adversely impact DNA and RNA structures. Apart from that, nicotine and its metabolite cotinine that easily reached testis can have a direct cytotoxic effect on sperm and cause DNA fragmentation (Arabi, 2004; Sepaniak et al., 2006).

Furthermore, the effect could exacerbate as nicotine was revealed to be associated with reduced endogenous antioxidants level. The biological system is integrated with antioxidant defense system as means to remove ROS. Diminution of endogenous antioxidants will result in an accumulation of undetoxified ROS and consequently cause more cellular damage. The enzymatic scavengers of ROS include c-glutamyl transpeptidase (c-GT), glutathione peroxidase (GPx), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G-6-PDH). The non-enzymatic antioxidant such as gluthathione (GSH), quenches free radicals and gets oxidized in order to inactivate the free radicals (Murugesan et al., 2007). Jana et al. (2010) reported that rats treated with nicotine for 12 weeks have decreased GPx, GR, GST, c-GT, G-6-PDH activities and GSH level in the testicular cells.

Aside from that, nicotine could enhance sperm DNA damage through initiation of apoptosis (Agarwal & Said, 2003). Apoptosis is a mode of cell death characterized by cellular morphological and biochemical alterations that is tightly regulated as

physiological phenomenon. Apoptosis can be triggered by a normal developmentally regulated process or induced in response to injury or stress. Apoptosis plays two primary roles in spermatogenesis which are to limit the population of germ cells to a number that can be supported by the Sertoli cells and selective depletion of abnormal germ cells. A number of studies have proposed that sperm with damaged DNA is indicative of sperm that escaped from programmed cell death which is referred as abortive apoptosis. Abortive apoptosis occurs when there is incomplete clearance of abnormal sperm through apoptosis and it manages to reach the epididymis (Agarwal & Said, 2003).

The apoptotic pathway that could be of interest in this study is possibly via the caspase cascade activation. Free radicals which may be produced by nicotine can disrupt the inner and outer mitochondrial membranes which results in the release of cytochrome C protein that resides in the inner membrane space of mitochondria into the cytosol. This activates caspase-9 of the caspase cascade which can then cleaves and activates other downstream caspases such as caspase-3 and caspase-7 that constitute a major caspase activity and triggers apoptosis marked by DNA degradation (Moustafa et al., 2004; Villegas et al., 2005). This is consistent with a study by Wang et al. (2003) who showed that semen samples from infertile patients with high levels of ROS had significantly higher levels of cytochrome C, caspase-9 and caspase-3 expressions compared to normal donors. Mosadegh et al. (2017) showed similar results when male rats were given 0.2 mg/kg b.w. and 0.4 mg/kg b.w. nicotine by intraperitoneal injections. The study revealed significant upregulated caspase-3 expression in nicotine-receiving groups, dose dependently, compared to control which in turn would enhance DNA material fragmentation.

Pearson correlation analysis was performed to find the relationship between mitochondrial function and DNA damage occurring among the groups in the present

study (Figure. 4.8). A significant negative relationship was found between the percentage of MitoTracker positive sperm (mitochondrial function) and the percentage of Tail DNA (DNA damage). The two variables were inversely proportional to each other, indicating an increased in DNA damage with impaired mitochondria. These results corroborate the role of mitochondria in the key events of apoptosis and DNA fragmentation.

Addressing the aim of this study, it was found that the deleterious effect of nicotine towards DNA was evidently decreased with TQ treatment. This was demonstrated by significantly lesser DNA fragmentation in nicotine-TQ group in all the parameters compared to nicotine group ($p<0.05$) (Table 4.4). Therefore, a possible curative effect of TQ may be presumed. The present result is in agreement with the study by Kamarzaman et al. (2013) which demonstrated that injection of 10 mg/kg TQ provided protection against cyclophosphamide-induced DNA damage. This was indicated by a significant decrease of DNA fragmentation in cyclophosphamide and TQ co-administration group in comparison to cyclophosphamide group.

Based on the mechanisms of nicotine-induced DNA damage discussed earlier, the favorable effect of TQ could be implicated by its high antioxidant activity. Several studies have described TQ acted as an efficient scavenger of superoxide, hydroxyl radical and single molecular oxygen (Kruk et al., 2000; Nagi & Mansour, 2000; Badary et al., 2003). Administration of TQ has also reported to counteract the increasing level of LPO, indicated by the decreased of malondialdehyde (MDA) level, a by-product of LPO (Alenzi et al., 2010). The potent antioxidant potentials of TQ could reduce direct attacks of free radicals on the DNA molecule and mitochondria which is responsible in triggering the caspase cascade of apoptosis, thus reducing DNA fragmentation. It was also revealed that TQ had significant anti-apoptotic effects by suppression of caspase-3

activity, an executioner of cell apoptosis (Helal, 2010; Ullah et al., 2012; Fouad et al., 2014).

Additionally, TQ is reported to be able to induce endogenous antioxidant enzymes, further assisting the combat against ROS. Antioxidant enzymes are important for neutralizing free radicals or superoxide radicals in order to overcome the oxidative stress. According to various reports, these enzymes play an important role in the actions of TQ. In an experiment conducted by Ismail et al. (2010), male rats were fed with high-cholesterol diet to induce hypercholesterolemia and were given TQ-rich fraction extracted from *Nigella sativa* orally for 8 weeks. It was found that supplementation of TQ caused an upregulation of antioxidant genes, superoxide dismustase, catalase (CAT) and GPx compared to the untreated rats. As a result, the TQ treated rats showed increased liver antioxidant enzyme levels when compared to control rats. Sankaranarayanan and Pari (2011) also reported that administration of 80 mg/kg b.w TQ for 45 days improved the activities of antioxidant enzymes CAT, GPx, glutathione-S-transferase and the levels of low molecular weight antioxidants Vitamin C, Vitamin E in kidney and liver tissues of streptozotocin-induced diabetic rats. Similarly, Sayed-Ahmed and Nagi (2007) noted that reduced antioxidant enzymes level, GSH, GPx and CAT caused by gentamicin-induced nephrotoxicity were completely reversed through concurrent administration of TQ (50 mg/l in drinking water) for 8 consecutive days.

From the results of the present study, it can therefore be suggested that TQ may be of use in ameliorating the DNA damage caused by nicotine possibly through its antioxidant, anti-apoptotic activities and the induction of antioxidant enzymes.

5.7 Expression of *Transition Nuclear Protein 2* (*TNP2*) and *Protamine 1* (*PRM1*) Genes

In order to protect the sperm DNA, sperm chromatin is built extremely condensed and stable (Evenson et al., 2002; Carrell et al., 2008). Changes occur in sperm chromatin compaction during the development of spermatids into mature sperm, histones are replaced by transition nuclear proteins. Subsequently, these proteins are then replaced by a more basic protein called protamines that are responsible for the final condensation of the chromatin (Shirley et al., 2004; D'Occhio et al., 2007). Thus, these proteins are crucial for proper chromatin condensation. Nevertheless, damage to the sperm DNA is still able to occur under certain conditions (Singh et al., 2003). The disruption in expressions and binding of sperm nucleoproteins, the transition nuclear proteins and protamines increases the susceptibility of sperm to DNA injury (Simon et al., 2011; Venkatesh et al., 2011).

Interestingly, in the present study, *TNP2* was significantly affected by nicotine and TQ (Figure 4.18), while nicotine and TQ did not have any influence on the level of *PRM1* expression (Figure 4.19).

TNP2 was significantly downregulated in nicotine group and showed improvements in nicotine-TQ group ($p<0.05$). To the best of our knowledge, there are no related studies on the effects of nicotine on *TNP2* expression level, much less by TQ. Transition nuclear proteins (TNP) are essential nucleoproteins as they participate in the initial condensation of the chromatin, where it transiently replaces the testis-specific histone variants before being replaced by protamines (Balhorn et al., 1984; Carrell et al., 2007).

Various functions of the transition proteins have been suggested by previous literatures which include nuclear shaping, histone removal, transcriptional repression, chromatin condensation and repair of the DNA strand breaks that normally briefly occur during the removal of nucleosomes (Caron et al., 2001). Zhao et al. (2004) reported that a decrease in the total transition protein content has a direct effect on fertility even though histone displacement and protamine deposition proceeded relatively normal. The study suggested that transition proteins are not essential for the exchange process but the absence of both transition protein leads to irregular chromatin condensation and persistence endogenous DNA strand breaks. The *TNP2* showed to be more involved in chromatin condensation better than *TNP1* and it controlled DNA integrity through DNA nick repairing activity (Kierszenbaum, 2001) during spermiogenesis and chromatin condensation in the epidydimis (Meistrich et al., 2003). Temporary nicks occur from topoisomerases activity to relieve torsional stress in assisting histone-protamine exchange (Smith & Haaf, 1998; Gill-Sharma et al., 2011). These nicks need to be fixed otherwise they will persist and evolve into DNA fragmentation in mature sperm (Kierszenbaum, 2001).

TNP2 knockout mice showed relatively normal testis histology, normal number of sperm and still tertile (Zhao et al., 2001). The negative consequences of *TNP2* deficiency were assumed to be minimized by compensation initially by *TNP1* and proceeded by the protamines. Nevertheless, it was reported that even though the effect of null *TNP2* in mice did not have a clear effect on chromatin condensation, it was observed that there were incomplete condensation and increased denaturability of the DNA believed to be resulting from DNA strand breakage (Zhao et al., 2001). Therefore, these results suggest that disruption in *TNP2* expression in the current study which was induced by nicotine may contribute to DNA disintegration.

The molecular mechanisms behind nicotine-induced *TNP2* downregulation is presently unclear. From the present study, it is possible to deduce that the alteration of *TNP2* expression level in nicotine group might be caused by oxidative stress through attack of ROS towards the mRNAs. As an antioxidant agent, TQ showed the ability to slightly improve its expression. The toxic effects of nicotine might also cause mutations or polymorphisms on the *TNP2* gene that would induce conformational changes in the expression, which could alter their incorporation into sperm chromatin.

Hormone inhibition by nicotine might also provide a possible explanation for *TNP2* dysregulation. As mentioned previously, nicotine suppressed the release of gonadotropins and testosterone. The inhibition of these hormones could affect *TNP2* regulation as deprivation of FSH signaling was found to greatly decrease TNP and protamine levels (Xing et al., 2003). Cacciola et al. (2013) also found that downregulation of GnRH occurs with downregulation of *TNP2* mRNA, *follicle-stimulating hormone beta subunit* (*FSHB*) and *follicle-stimulating hormone receptor* (*FSHR*). Thus, these show that androgen deficit could alter the levels of basic nuclear proteins including *TNP2* even though there are no conclusive evidences of *TNP2* being in direct pathway of hypothalamic-pituitary-testicular axis. Hence, the improvement of *TNP2* expression in nicotine-TQ group compared to nicotine group in this current study could also be attributed to the ability of TQ to improve the level of reproductive hormones and consequently increase *TNP2* expression level.

As for protamines, reduced viability of either the *PRM1* or *PRM2* gene in male mice resulted in abnormal chromatin compaction, sperm DNA damage, and ultimately infertility (Cho et al., 2001; Cho et al., 2003). Venkatesh et al. (2009) reported that infertile men with protamine deficiency have poor sperm DNA packaging that makes the sperm genome vulnerable to toxic environmental stimulus and more predisposed to free radical injury which leads to fragmentation. Iguchi et al. (2006) also demonstrated

that individual who carried a mutation in the *PRM1* gene had low sperm count (oligozoospermic) and exhibited a high level of DNA fragmentation in the sperm measured using the TUNEL assay. A study of *PRM1* in sperm DNA of male rats treated with local gin, an alcoholic drink, similarly showed the importance of *PRM1* on sperm DNA integrity (Minari & Salau, 2016).

There were no changes in the expression level of *PRM1* in all the experimental groups compared to control, indicating that nicotine and TQ did not have any influence on the particular gene. However, the result is in contrast to earlier findings where smoking was found to interfere with protamine expression at mRNA level (Yu et al., 2014; Osman et al., 2018). Yu et al. (2014), demonstrated that smokers with normal sperm production (normozoospermic) have increased histone-to-protamine ratios, indicating abnormal histone to protamine transition compared to non-smoking men. The result was found to be closely correlated with sperm motility, viability, counts, and cotinine level. Defects in the chromatin remodeling process are often characterized by an abnormal *PRM1* to *PRM2* ratio which are normally expressed approximately at a 1:1 ratio in healthy men. This ratio has been used in assessing sperm quality and alteration of this ratio is associated with male infertility (Nanassy et al., 2011). Yu et al. (2014) further showed that smoking altered the expression of *PRM1* to *PRM2* ratio in men and verified the results in an *in vitro* study in TM3 cells treated with cigarette smoking condensate. They suggested the involvement of smoking-related reactive oxygen species in the changes of protamine expression.

Aitken and De Iuliis (2010) hypothesized close relationship between abnormal sperm chromatin protamination and oxidative DNA damage. Defective chromatin remodeling process during spermiogenesis results in the production of sperm with reduced protamination efficiency. Hence, this will create a state of vulnerability,

whereby the sperm become susceptible to oxidative damage. The chromatin would then be easily attacked by ROS and subjected to damage.

The results of the current study are further contradicted by Hammadeh et al. (2010) who revealed that oxidative stress induced by cigarette smoking causes poor protamine replacement process. However, the researchers also revealed that smoking affected *PRM1*/*PRM2* expression ratio by under-expression of *PRM2*, suggesting that smoking affects *PRM2* greater than *PRM1* (Hammadeh et al., 2010). The genetic mechanism underlying smoking-induced protamine abnormality is not yet clear. Nevertheless, this might provide an explanation of why *PRM1* in the present study was not affected by the nicotine treatment. Only *PRM1* was examined in the analysis of protamine expression in this current study since rats, the experimental animal of the present study, do not express *PRM2* (Balhorn, 1982; Bunick et al., 1990).

It is uncertain whether the unaltered expression levels of *PRM1* in the current study is due to insubstantial nicotine inducement or nicotine having no effect on *PRM1*. Nonetheless, it can be safely assumed that DNA damage displayed in this study was not a result of irregular *PRM1* expression.

Even so, the findings of the present study raise intriguing questions on why merely *TNP2* was affected by nicotine and not *PRM1*. These results also suggest that *TNP2* and *PRM1* transcription are independent from each other which agree with previous study by Zhao et al. (2001). Since *PRM1* mRNA expression was not affected by nicotine and TQ, normal DNA protamination between the groups were assumed, which further indicated that the gene was not involved in DNA injury. The interesting outcome of *TNP2* expression level could place the gene to be relatively responsible for DNA damage but a review paper by Leduc et al. (2008) reported that DNA fragmentation in double TNP knockout models does not persist and the DNA

condensing impairment may be compensated by protamines. Nevertheless, the extent of compensation is unknown and the real impact of *TNP2* deficiency in this study towards chromatin packaging is uncertain for it to be ruled out. Further studies on protein expression analysis of *PRM1* and *TNP2* on the sperm are required to provide more conclusive results. Figure 5.2 summarizes the interactions of nicotine and TQ on sperm characteristics in this study.

5.8 Limitations of the Present Study

Present study demonstrated the detrimental effects of nicotine on sperm and the ability of TQ to improve it. Nevertheless, there are a few aspects that can be improved upon which include the length of treatment. Previous studies like the present study have shown that the length of time is sufficient for nicotine to induce sperm damage. However with a longer time period, a more extensive impact could possibly be observed. Therefore, better observation on the magnitude of TQ strength against injury could be assessed. Apart from that, protein analysis of the nucleoproteins was unable to be tested in the gene expression portion of the current study. Due to time and financial constraint, decisions were made to narrow down the study whilst aims and relevance of the study was kept preserved. In addition, Bunick et al. (1990) reported that rats do not have the variant *protamine 2* (*PRM2*). Thus, in the present study the ratio of *PRM/PRM2* that is associated with male infertility could not be tested.

5.9 Recommendations for Further Research

This research is an initial study on the beneficial effects of TQ against nicotine on the male reproductive system, specifically the sperm. In the route of entering TQ in clinical trials, the study has much further scope to explore for a better understanding of the mechanisms, finding the right concentrations and to observe the extent of TQ's healing effect on the reproductive functions. The recommendations are as stated below:

1. As most of the possible mechanism in the study involved oxidation, it is recommended to perform oxidative stress test such as TBARS test to have a better understanding of the pathways.

2. It is recommended to test on the level of reproductive hormones such as testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to investigate nicotine and TQ effects on the hypothalamus-pituitary-testicular axis.

3. Microscopic observation of the male reproductive organs specifically testis by histopathological evaluation to widen the scope of the research.

4. Sperm protamination test and protein analysis of nucleoproteins involved in chromatin condensation.

5. Investigation that revolves around *transition nuclear protein* gene could be performed as it has shown an interesting outcome in this study. Further analysis on its gene expression might help to determine the possibility of the mRNA to be utilized as a biomarker.

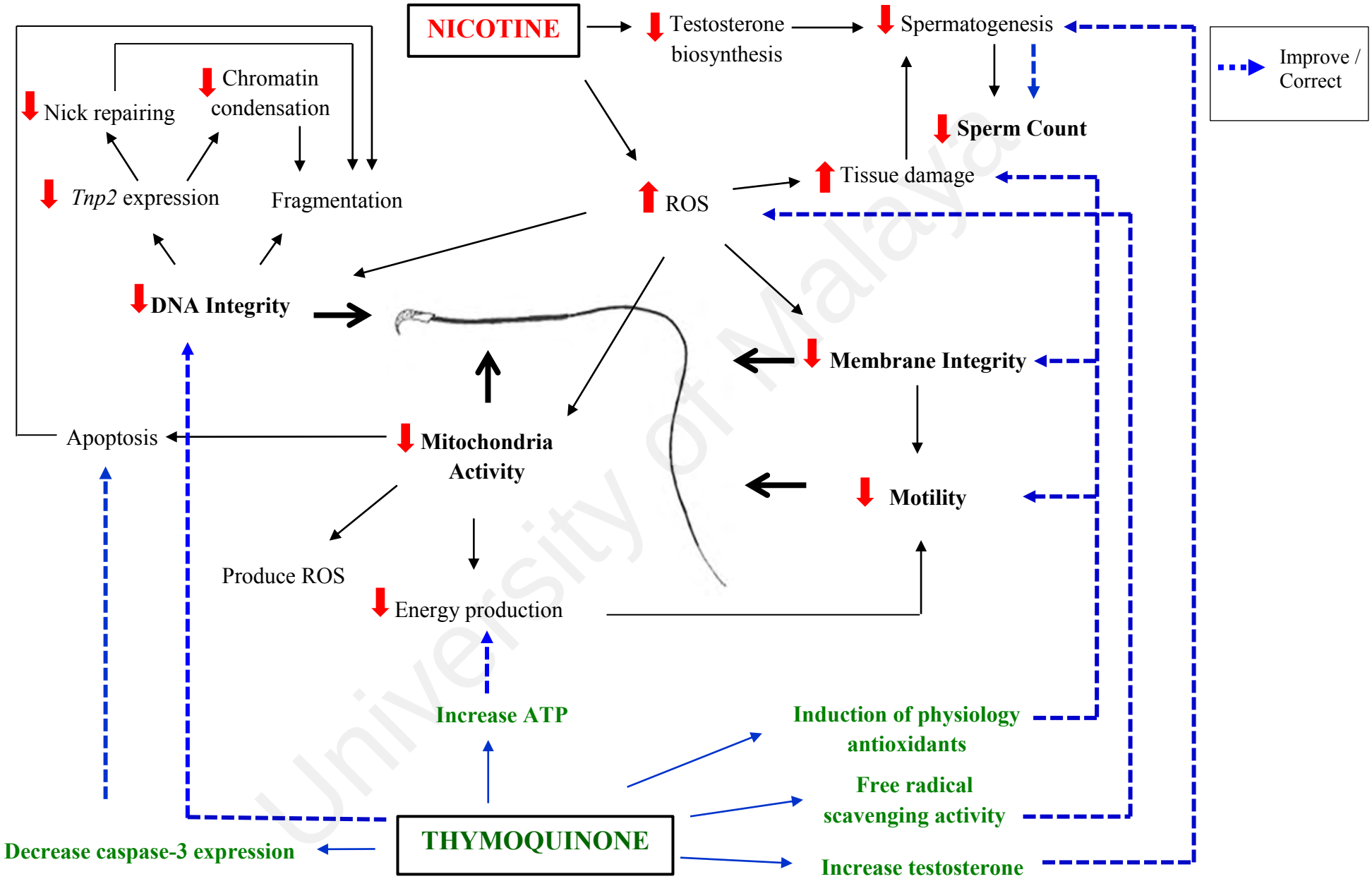


Figure 5.2: Schematic diagram showing the interaction between harmful effects of nicotine and the healing potential of thymoquinone on sperm's structure and function

CHAPTER 6: CONCLUSION

This study set out to demonstrate the potential ameliorative role of thymoquinone (TQ) on nicotine-induced sperm damage in rats. Nicotine usage has increased throughout the years through medium such as cigarette smoking and has been acknowledged for its association with infertility. Sperm quality is imperative in ensuring a successful fertilization process as it is the main cause of male infertility. Through this research, we have hoped to find an ideal natural curative substance through TQ to counter the detrimental effects of nicotine.

This current research showed that TQ was able to increase sperm count and motility despite nicotine treatment. Furthermore, poor sperm membrane integrity and low number of sperm with functional mitochondria incurred by nicotine was improved with TQ. The DNA breakages in sperm caused by nicotine were also reduced by the TQ treatment. It was also found that sperm mitochondrial function and DNA fragmentation in this study was inversely proportional. Apart from that, in the analysis of genes responsible for sperm chromatin condensation, nicotine and TQ treatment demonstrated no effect on the *PRM1* gene. However interestingly, *TNP2* was significantly downregulated in the nicotine group and showed improvements in the nicotine-TQ group. As the study showed nicotine and TQ has no effect on *PRM1* gene expression levels, it is safe to assume that the gene did not influence the DNA damage in this study. Nevertheless, the dysregulation of *TNP2* could have fairly affected condensation and caused fragmentation of the DNA. Further research could be useful in investigating the effects of nicotine and TQ on the gene expression of *PRM1*, *TNP2* and other genes that are involved in spermiogenesis at protein level.

Therefore, the findings of this study demonstrated that nicotine has deleterious effects on the viability of sperm characteristics and TQ showed restorative potential on the injurious state of sperm caused by nicotine. The present study not only adds to the growing body of knowledge on the detrimental effects of nicotine towards sperm but also as far as we are aware, this is the first study to investigate the curative effect of TQ on damaged sperm caused by nicotine. Hence, the findings from this study make several contributions to the current literature on pharmacological benefits of TQ in reproductive functions and lay the groundwork for future research in combating infertility particularly among male smokers.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

No.	Type of Publication	Title	Details
1	Conference Abstract	The effect of Thymoquinone on sperm count and sperm membrane of nicotine treated rats	21st Biological Science Graduate Congress 2016 (15th-16th December 2016)
2	Conference Abstract	Thymoquinone ameliorates nicotine-induced sperm damage in rats	International Conference on Drug Discovery and Translational Medicine 2018 (3rd-5th December 2018) doi: 10.3389/conf.fphar.2019.63.00001
3	Journal Article	Thymoquinone enhances sperm DNA integrity in nicotine-induced infertile male rats	Tropical Journal of Pharmaceutical Research (TJPR)
4	Journal Article	Ameliorative effects of Thymoquinone on sperm parameters and testosterone level of nicotine-treated Sprague Dawley rats	Brazilian Archives of Biology and Technology (BABT)