A STUDY OF CHRONIC TOBACCO *SHISHA* SMOKE EXPOSURE ON SOME REPRODUCTIVE PARAMETERS AND SPERM DNA INTEGRITY IN ADULT MALE WISTAR RATS

BY

Ibrahim SULEIMAN B.Sc.(ABU, 2010); M.Sc.(ABU, 2014)

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DECLARATION

I declare that the work in this thesis, entitled "Effects of Chronic Tobacco *Shisha* Smoke Exposure on Some Reproductive Parameters and Sperm DNA Integrity in Adult Male Wistar Rats' has been carried out by me in the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Health Sciences, Ahmadu Bello University, Zaria under the supervision of Prof. A. Mohammed, Dr M.U. Kawu and Dr Y. Tanko.The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Ibrahim SULEIMAN**Name of Student** **Signature** **Date**

CERTIFICATION

This thesis, entitled "EFFECTS OF CHRONIC TOBACCO SHISHA SMOKE EXPOSURE ON SOME REPRODUCTIVE PARAMETERS AND SPERM DNA INTEGRITY IN ADULT MALE WISTAR RATS" by Ibrahim SULEIMAN meets the regulations governing the award of the degree of Doctor of Philosophyin Human Physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof. A. Mohammed, MBBS., M.Sc., Ph.D.Date
Chairman, Supervisory Committee

Prof. M.U. Kawu, D.V.M., M.Sc., Ph.D. Date
Member, Supervisory Committee

Dr. Y. Tanko, B.Sc., M.Sc., Ph.D. Date
Member, Supervisory Committee

Dr. M.B Akor-Dewu, B.Sc., M.Sc., Ph.D. Date
The Head, Department of Human Physiology,
Faculty of Basic Medical Sciences,
Ahmadu Bello University, Zaria

Prof. S.Z. Abubakar,B.Eng., MSc., Ph.D. Date
The Dean, School of Postgraduate Studies,
Ahmadu Bello University, Zaria

DEDICATION

This thesis is dedicated to Almighty Allah (S.W.T) for His immeasurable guidance and protection throughoutthe course of this project work, without Him I am nobody; and also to my parents,Mal. Salami Ibrahim and Mallama Ibrahim Hajara; and my lovely wife MallamaJamila Suleiman,for their invaluable support throughout thisstudy and to the entire Physiologists in the world.

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ABSTRACT

Currently there are no putative empirical data on the effect of *Shisha*smoking on sperm DNA integrity and some of the available data on the adverse effects of *Shisha*smoking on conventional semen characteristics: sperm count, sperm motility, sperm viability and sperm morphology are contradictory. Despite the well-known deleterious reproductive effects of cigarette smoking, it is relatively unclear whether or not*Shisha*smoking has the same effect on male reproductive parameters. The present study was aimed at determining the effect of chronic *Shisha*smoke exposure on testicular and prostate gland parameters, and sperm DNA integrity in adult male Wistar rats. Twenty-one adult male Wistar rats between the ages of 8-12 weeks, weighing between 160 -180 g were divided by simple random into three groups containing 7 rats per group. Group I rats were kept for 30 minutes daily in the nose-only exposure chamber for a period 13 weeks without exposure to *Shisha* smoke; group II (with water in the *Shisha*jar) was exposed to bonged shisha smoke (BSS) and group III (without water in the shisha jar) was exposed to unbonged *Shisha*smoke (UBSS), respectively for 7 seconds first and fresh air later for 53 seconds, alternatively for 30 minutes daily for a period of 13 weeks. The *Shisha*smoke was drawn from the *Shisha*apparatus outlet by a vacuum compressor at a pressure of 300 kPa into the nose-only exposure chamber where the rats were kept. At the end of the exposure, five animals from each group were randomly selected and anaesthetised with 0.4 mL/100g of combined ketamine and diazepam and blood samples were obtained through cardiac puncture. The result obtained showed that chronic exposure to *Shisha*smoke revealed a significant increase in testicular malondialdehyde(MDA) level, high sperm DNA fragmentation, marked reduction in serum testosterone concentration, sperm count and progressive sperm motility,pronounced necrosis of tissues in the seminiferous tubules in the testicular histology, intense necrosis and

decreased epithelia heights in the prostate gland histological, abnormal sperm cell morphology, and increased serum prostate specific antigen (PSA) concentration were also recorded. The testicular MDA level in the BSS (1.61 ± 0.08 µmol/mg) groupwas significantly higher than the UBSS (1.26 ± 0.04 µmol/mg)and the control (0.44 ± 0.08 µmol/mg) groups. SerumPSA concentration in the BSS exposed group (3.04 ± 0.03 ng/ml) was significantly higher than the UBSS (1.94 ± 0.06 ng/ml) and the control (1.30 ± 0.05 ng/ml) groups. Serum testosterone concentration was considerably lower in the BSS group with value of 25.4 ± 1.16 nmol/L compared to UBSS (31.8 ± 0.58 nmol/L) and control(41.6 ± 0.50 nmol/L) groups. The DNA fragmentation index(DFI) resultsobtained were significantly higher in the BSS (29.0 ± 1.90%) and UBSS(20.0 ± 0.94%) exposed groupsthan the control group (9.6 ± 0.67%). Epididymal sperm counts were significantly lower in the BSS (3.3 ± 0.13 x107cell/ml) and UBSS (4.08 ± 0.08 $x10^7$ cell/ml) groups than the controlgroup(5.62 ± 0.15 $x10^7$ cell/ml). Progressive spermmotility percentage value in the BSS (32.2 ± 1.01%) exposed group was significantly lower than the UBSS (40.0 ± 1.70%) and the control (48.0 ± 2.50%) groups. Thenon-progressive sperm motility percentage values were higher in the BSS (47.0 ± 2.30%) and UBSS (43.6 ± 0.90%) exposed groups than the control group (37.0 ± 3.0%), non-motile sperm cell percentage values were higher in the exposed groups with values of21.4 ± 2.50% and 18.04 ± 1.40% for BSS and UBSS groups, respectively than the control (15.0 ± 2.23%) group.A significant ($r = -0.904$; $P < 0.01$) negative correlation was observed between malondialdehyde and testosterone concentration, and sperm count.While,a significant ($r = 0.881$; $P < 0.01$) positive correlation was observed between malondialdehyde and sperm DNA fragmentation index; serum testosterone concentration and sperm count ($r = 0.939$; $P < 0.01$); and significant negative correlation ($r = -0.922$; $P < 0.01$)between sperm DNA fragmentation index and sperm count.. The present study

observed significant harmful effects of *Shisha* smoking in the testis and prostate gland of the experimental animals as evidenced by the histological damages in these organs. The study also highlighted the possible role of oxidative stress on the adverse effects of *Shisha* smoking on sperm count, motility, morphology and enhanced sperm DNA fragmentation in the *Shisha* exposed groups, which were higher in the bonged group. *Shisha* tobacco smoking as an alternate to the traditional cigarette smoking should be discouraged as the acclaimed less harmful method of smoking does not significantly remove the toxins in the smoke, but rather compounds the harmful effect of tobacco.

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LIST OF ABBREVIATIONS/SYMBOLS

ACSM - Aerodyne aerosol chemical speciation monitor

ACT- α-1-antichymotrypsin

ANOVA - Analysis of variance

APGAR–Appearance, pulse, grimace, activity and respiration

AR - Acrosomal reaction

ASRM - American Society for Reproductive Medicine

ATP - Adenosine triphosphate

BaP - Benzo(a)pyrene

BSS - Bonged *Shisha*smoke

cAMP - Cyclic adenosine monophosphate

CD45 - Cluster of differentiation

CEA - Carcinoembryonic antigen

CO - Carbon monooxide

DFI - DNA fragmentation index

DHA - Docosahexaenoic acid

DHT - Dihydrotestosterone

DNA – Deoxyribonucleic acid

EDTA - Ethylene diamine tetraacetic acid

ELISA - Enzyme-linked immune-sorbent assay

ERC - Excess residual cytoplasm

H & E – Haematoxylin and eosin

H_2O_2 - Hydrogen peroxide

HDL – High-density lipoprotein

ICSI - Intracytoplasmic sperm injection

IVF - *in vitro* fertilisation

KPa - Kilopascal

LDL – Low density lipoprotein

LPO - Lipid peroxidation

MDA - Malondialdehyde

MEK – Mitogen-activated protein kinase

NADH - Reduced nicotinamide adenine dinucleotide

NADPH - Reduced nicotinamide adenine dinucleotide phosphate

NBT - Nitroblue tetrazolium

OH^- - Hydroxyl ion or radical

OR – Odd ratio

OS - Oxidative stress

PAH - Polycyclic aromatic hydrocarbons

PKA - Activation of protein kinase A

PLA2 - Phospholipase A2

PS - Phosphatidylserine

PSA - Prostate specific antigen

PSS - Physiological saline solution

PUFA - Polyunsaturated fatty acid

ROS - Reactive oxygen species

SDFI – sperm DNA fragmentation index

SOD - Superoxide dismutase

TAC - Total antioxidant capacity

TB - Tuberculosis

TBARS - Thiobarbituric acid reactive substances

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling

UBSS -Unbonged shisha smoke

WHO- World Health Organisation

WPS - Water-pipe smoking

ZP - Zona pellucida

CHAPTER ONE

1.0 INTRODUCTION

Sperm function is assessed in terms of the ability of a given population of spermatozoa to fertilize an egg. However, an equally important aspect of sperm function is the ability of the male gamete to support the development of a normal healthy embryo that will develop into a normal blastocyst, implant, establish a placenta, and differentiate into a normal healthy baby (Aitken and De luliis, 2007).*Shisha* also known as Hookah smoking or water-pipe tobacco smoking, is generally viewed as a social activity and a relatively inexpensive way to get together and have fun as shown in Plates III and III below(American Lung Association, 2007). Often, the group shares one pipe and tries different flavours such as apple, bubble gum, chocolate, mint, orange soda, root beer and grape(Primack *et al.*, 2009). It is widely perceived to be less harmful than other forms of tobacco use. The reproductive adverse effects of cigarette smoking have been studied with conflicting and unsatisfactory results (Mostafa, 2010), but there is dearth of data on effects of *Shisha* smoking on the reproductive system



Plate I: Indian women smoking *Shisha*(Kahill, 2014)



Plate II: African women smoking *Shisha*(Kahill, 2014)

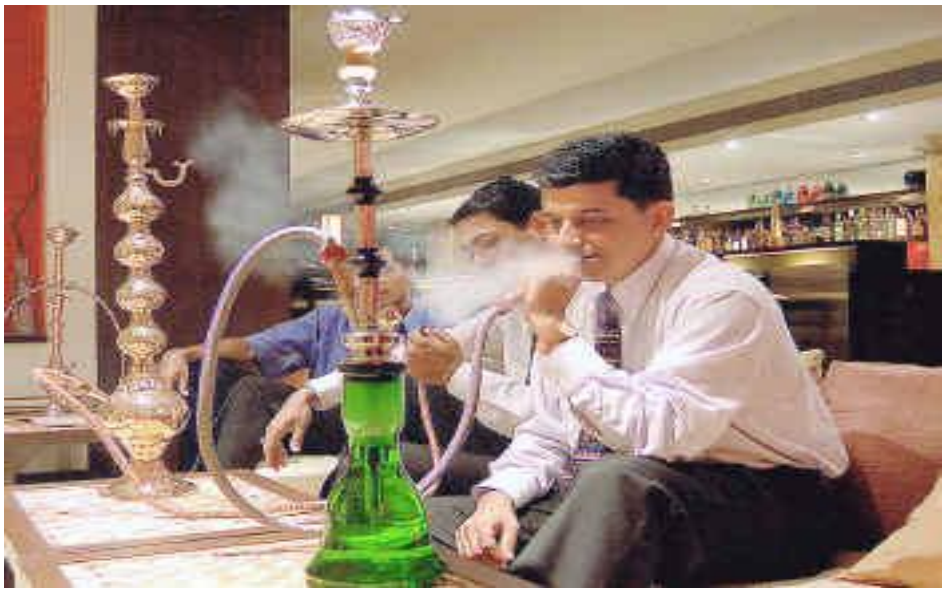


Plate III: Americans smoking hookah (Novac, 2011)

Water-pipe smoking (WPS) is popular in the Middle East and is starting to gain popularity in several Western countries. This old practice has recently sprung up rapidly around bars in college campuses in the United States, Europe and Africa(Caroline*et al*., 2011;Martinasek *et al*., 2013), and has become a practice of concern among students of University campuses in Nigeria.

Hookahs are considered more pleasant and less irritating to the throat by many smokers. This is due to theappealing nature of Hookah smoking and why some people, particularly young people who otherwise would not use tobacco, begin to use hookah (American Lung Association, 2007). Originally created as a theoretically less harmful method of tobacco use, it was suggested that smoke should first be passed through a small receptacle of water so that it would be rendered harmless. Thus, according to World Health Organisation (WHO), the widespread, unsubstantiated belief that the practice is safe is as old as the hookah itself (WHO, 2005).Hookah smoking carries the same health risks as cigarette smoking, which includes lung cancer and heart disease. The smoke contains significant amounts of nicotine, tar and heavy metals such as those found in batteries and rat poison (Asotra, 2005). The WHO estimates that hookah users may inhale as much smoke during one hookah session, as a cigarette smoker would consume 100 or more cigarettes (WHO, 2005).

The heat sources that are applied to burn the tobacco such as wood cinders or charcoal also increase health risks. When the heat sources are burned, they emit their own potentially dangerous chemicals, including carbon monoxide and heavy metals (American Lung Association, 2007). The social aspects of hookah smoking pose even more health hazards, putting users at risk of infectious diseases such as tuberculosis, hepatitis and herpes. Shared mouthpieces and the heated moist smoke can enhance the opportunity for such diseases to spread (American Lung Association, 2007). The truth is, tobacco kills and does not discriminate whether in hookah or cigarette as legibly written on the *Shisha*packet shown on plate IV. Using a hookah to smoke tobacco is not a safe alternative to cigarette smoking (WHO, 2005).



Plate IV: Grape with mint flavour *Shisha*packet (Ibrahim, 2015)

Over the last decade, many studies have confirmed that sperm DNA damage has strong association with every early embryonic developmental problems. These include impaired fertilization, slow early embryo development, reduced implantation, high rate of miscarriage and birth defects in the offspring. Childhood cancers have also been associated with oxidative damage to sperm DNA as a consequence of paternal smoking (Sheena *et al*., 2013). This damage is poorly characterized, but is known to involve hypo-methylation of key genes, oxidative base damage, endonuclease-mediated cleavage and the formation of adducts with xenobiotics and the products of lipid peroxidation. There are many possible causes of such DNA damage, including abortive apoptosis, oxidative stress associated with male genital tract infection, exposure to redox cycling chemicals, and defects of spermiogenesis associated with the retention of excess residual cytoplasm. Physical factors such as exposure to radiofrequency electromagnetic radiation or mild scrotal heating can also induce DNA damage in mammalian spermatozoa, although the underlying mechanisms are unclear. Ultimately, resolving the precise nature of the

DNA lesions present in the spermatozoa of infertile men will be an important step towards uncovering the aetiology of this damage and developing strategies for its clinical management (Aitken and De luliis, 2007).

The Sperm Chromatin dispersiontest measures the presence of DNA damage. The test determines the percentage of sperm with fragmented DNA and the degree of DNA damage and provides a DNA Fragmentation Index (DFI) score to indicate the likelihood of sperm contributing to infertility problems.

Prostate cancer is the leading cancer diagnosis and the second most common cause of cancer death among adult males. It is the fourth most common cancer in men worldwide (Parkin *et al*., 1999). It is the most common cancer in Nigerian males; having overtaken liver cancer. Nigerian males have been shown to have a high prevalence rate of prostate cancer of at least 1046 per 100,000 men of age 40 and above(Stephen *et al*. 2013).Prostate specific antigen (PSA) is a single chain glycoprotein produced by epithelial cells of the prostate gland. Three major forms of PSA exist in the serum: free PSA, bound PSA, and complex PSA. The Prostate Specific Antigen immunoassays are widely used to detect early-stage prostate cancer, to evaluate disease progression, and to assess therapeutic response. Serum total PSA measurement is the most useful and widely used test for the early diagnosis of prostate cancer. The majority of immunoreactive PSA in serum is complexed to α-1-antichymotrypsin (ACT); Armbruster, 1993. In addition to the total serum PSA level, the ratio of free to total PSA has become an important variable for distinguishing between males with benign and malignant prostate. The percentage of free serum PSA is lower in males with prostate carcinoma than in those with benign prostate hyperplasia or with no apparent prostate disease (Stenman *et al*., 1991).

Recent studies have reported that serum percentage-free PSA is lower in men with cancer than in the men with benign prostates and have proposed that its measurement can be used to save 20% of biopsies in men with total serum PSA levels of 4.0–10.0 ng/ml, while missing <5% of the cancers (Catalona *et al*., 1998) . Although the biological reason for the differences in percent-free PSA levels among men with and without prostate cancer is not known, it has been suggested that malignant epithelial cells produce more ACT than benign epithelial cells, leading to an increased proportion of cellular PSA that is bound to ACT in cells (Wu *et al*., 1998).

1.1 STATEMENT OF RESEARCH PROBLEM

According to data from the WHO (2009), there are about 2.4 billion people worldwide that consume tobacco in the forms of smoking, chewing, snuffing or dipping. In Nigeria, the prevalence of tobacco use among adults (12.3% males <1% in females) is generally lower than in more developed countries.However, prevalence among youth tends to be higher than among adults.A mean lifetime smoking prevalence of 26.4% was reported among secondary school students, with values ranging from 7.2% to 42.9% (Odukoya *et al*., 2013).The WHOalso estimates that tobacco-related deaths was 6.4 million in 2015, will amount to 8.3 million in 2030 and one billion deaths by the end of 21st century (Mathers and Loncar, 2006; WHO, 2008; WHO, 2009).

Male infertility is the commonest cause of infertility (Sheena, 2013). It contributes nearly 50% of infertility in couples of reproductive age. This occurs as a result of population ageing and adverse changes in lifestyles, including smoking (De Mouzon *et al*., 2006).

It has been reported that more than 100 million people worldwide indulge in water pipe smoking (WPS) daily (Fahed*et al*., 2011). Initially, WPS was common mainly in the Middle East, Turkey,

India, and Pakistan. With globalisation and immigration from these countries, WPS spread to Western countries, notably among the youth. It has been estimated that 20% to 40% of college students in the United States have experienced WPS. A recent study found that WPS use was not restricted to any single racial, ethnic, or cultural group (Dugas *et al*., 2010). Among the reasons for the growing popularity of WPS are low cost, easy access, the accompanying social interaction and sweetened flavour that can mask the taste of tobacco (Chaouachi, 2009).

A high level of DNA fragmentation may compromise the possibility of a successful pregnancy no matter what fertility treatment is used. The DNA fragmentation occurs when there is an alteration to the bases or a physical break in one or both of the DNA strands which may result from oxidative stress induced by smoke. Sperm DNA fragmentation is the physical breaking of one or both DNA strands in sperm chromosomes.If this occurs within a gene in a sperm that fertilized the egg, the consequence may be the death of the resulting embryo or miscarriage(Sheena *et al*., 2013).

A 2008 report by the American Society for Reproductive Medicine (ASRM) states that, while routine DNA integrity testing in the evaluation of infertility is not done, sperm DNA damage is more common in infertile men and may affect reproductive outcomes in selected couples, including those with recurrent spontaneous miscarriage or idiopathic infertility(Zhang *et al*., 2014).

1.2 JUSTIFICATION

Smoking with a water pipe (WP), also known as a hookah, *shisha, goza, narghile, and hubble-bubble*, has been practised extensively for about 400 years. *Shisha*smoking, commonly known as water-pipe smoking (WPS), is considered by the public to be less harmful than cigarette smoking, leading to tolerance of this practice(Chaouachi, 2009). The misperception of its impact

on health, including the idea that WPS is less addictive than cigarette smoking; and the lack of public health warnings on the use of WPS is a subject of concern.

Although research on the effects of cigarette smoking on male fertility has yielded some contradictory results, most of the reported studies seem to suggest that it can adversely influence the reproductive function in both laboratory animals (Oyeyipo *et al*., 2013), and humans (Abbott andWinzer-Serhan,2012). There is paucity of reports in the literature regarding the possible adverse effects of *Shisha*on male reproductive system in humans or laboratory animals. Information to be obtainedfrom this studymay reveal the potential reproductive hazardsinducible by the exposure of the youth to hookah smoking in the name of social interaction and having fun.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

The aim of this study was to determine the effect of chronic*Shisha*smoke exposure on testicular and prostate gland parameters, and sperm DNA integrity in adult male Wistar rats.

1.3.2Specific objectives: To determine in adult male Wistar ratsthe effects of chronic *Shisha*smoke exposure on the following, viz:

(i) Testicular MDA level.

(ii) Serum testosterone levels.

(iii)Serum prostate specific antigen (PSA) level.

(iv)Sperm DNA fragmentation.

(v) Semen characteristics including sperm count, sperm motility and sperm morphology.

(vi) Histology of testesand prostate glands.

1.4 NULL HYPOTHESIS

Chronic *Shisha*smoke exposure does not significantly affect testicular and prostate gland parameters and sperm DNA integrity in adult male Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Smoking and Infertility

Epidemiological data show that human reproductive disorders are a common problem worldwide, affecting one-sixth of all couples. Infertility is thus considered a social disease by the World Health Organization (WHO). Recently, a number of reports have been published concerning the considerable decrease observed in male fertility (Cissen *et al*., 2016).The low sperm count of contemporary males, apart from the deterioration of the quality of the semen, reduces the chance of reproduction, constitutes a major problem for public health, and may be among the causes of the negative birth rates in many countries (Jørgensen*et al*., 2012; Esteves and Chan, 2015).

A significant positive association has been found between active smoking and sperm DNA fragmentation as well as axonemal damage. Smoking is also associated with a decreased sperm count. Fraga *et al*. (1996) found the level of 8-hydroxy-2'-deoxyguanosine(2-OH-8-dG) – a marker of DNA fragmentation – to be 50% higher in smokers compared with non-smokers. It is believed that smoking affects spermatogenesis by increasing the production of norepinephrine, which increases the conversion of testosterone to estrogen causing decreased testosterone levels (Argawal, 2005).

2.2 Composition of Cigarette Smoke

Cigarette smokecontains about 4000 active compounds belonging to a variety of chemical classes known to be toxic, including polycyclic aromatic hydrocarbons (PAH) [e.g.

benzo(a)pyrene (BaP), acenapthelene, phenanthrene, pyrene and chrysene], nitrosamines, heavy metals [e.g. cadmium (Cd), lead and cobalt], alkaloids (nicotine), aromatic amines and so forth.Large volumes of oxidants in cigarette smoke increase the level of oxidative stress in smokers(Ding *et al*., 2005).

2.3 Composition of *Shisha* Smoke

Aerosol chemical composition measurements of *Shisha*smoke using the recently developed Aerodyne Aerosol Chemical Speciation Monitor (ACSM), revealed the presence of total particle mass, individualcompounds or classes of compounds (such as nicotine,polycyclic aromatic hydrocarbons, or aldehydes), or tar(nicotine-free dry particulate matter), metals, such as arsenic, lead, nickel, cadmium and chromium (Ng, *et al*., 2011; El Sayed*et al*., 2015).

2.4 *Shisha*Smoking Epidemiology around the Globe

The prevalence of Shisha smoking is peaking in United States and is reported to reach up to 40% from 2005 – 2008 (Smith *et al*., 2011). A Study conducted in two universities of United States revealed the prevalence of life time hookah users as 27.8% (Brockman *et al*., 2012). Another survey conducted at the University of San Diego, USA unveiled that the prevalence of *Shisha*smoking to be 24.5% (Smith *et al*., 2011). Another study also reported that out of the whole sample, there were more than 1/3rd (40.3%) of people who had smoked tobacco from a *Shisha*. Maziak (2011), reported that *Shisha*smoking is continuing to spread among populations worldwide and perhaps represents the second global epidemic since that of cigarette; the prevalence being 6-39% (Maziak, 2011; Sutfin *et al*., 2011). In another analysis conducted at Wayne State University, USA, prevalence of *Shisha*smoking was 10% in students, while another study evaluated it to be 26% (Jamil *et al*., 2011). Jordan and Delnevo (2010) reported prevalence

of 9.7% among the 3,010 New Jersey high school students, while Grekin and Ayna (2008) reported that 15% of students had used *Shisha* once in their life time (Grekin and Ayna, 2008; Jordan and Delnevo, 2010).

Prevalence of *Shisha* smoking is on the rise Worldwide. In Lebanon, the global youth tobacco survey conducted in 2005 included 13–15 years old.This study concluded that 59.8% of them had smoked shisha at least once in the past month as opposed to only 10% of them being cigarette smokers (Nakkash *et al*., 2011). Studies spanning three different colleges of Saudi Arabia indicate the prevalence of 12.6% *Shisha*users (Taha *et al*., 2010). Study conducted in two rural schools of Qualobia and Management and Science University in Malaysia unveiled the prevalence of *Shisha* smokers being 19% and 20% (Al-Naggar and Bobryshev, 2012). A study conducted in Beirut, in adolescents of 13–20 years of age, reported that 60% of respondents had smoked *Shisha*at least once in their life time, while a study in Syria including University students, revealed that 62.6% males and 29.8% females were regular smokers of *Shisha* (Afifi *et al*., 2010).

2.5 Sexand Age Distribution

A Study held in two large universities of the United States showed that *Shisha*smokers are mostly males of ages between 15–25 years (Brockman *et al*., 2012). In Saudi Arabia the situation is similar, as 63.8% of students started smoking *Shisha*at ages of 16–18 years; the male gender being dominant (Taha *et al*., 2010). In Syria, mostly the age of commencement of this habit is found to be 19.2 years for males and 21.7 years for females, on average. Jawaid*et al*. (2012) reported males as dominant users of *Shisha* (53.6%). Similarly, Jaffary *et al*. (2012) reported male predominance in *Shisha*smoking with a mean age of 21 years (Jaffri *et al*., 2012). Surveys conducted in all Middle Eastern countries also revealed that males are the predominant*Shisha*-

users. Similar findings were also reported in Egypt and Qualabia (Amin *et al*., 2010). However in Jordan, the trend was found to be totally opposite, as more females were reported to be *Shisha* smokers compared to males (Dar-Odeh *et al*., 2010).

2.6 Reason for *Shisha* Smoking

Chaouachi, in the year 2009, compiled all assorted reasons why people smoke *Shisha*. He listed about 15 reasons for*Shisha*smoking (Chaouachi, 2009) viz: global tourism and migration flows, a new hassle-free lighting system (new easy to light charcoal), relative acceptance by non-smokers, unexepected backlash effect of anti-tobacco campaigns (viewed as safer than cigarette smoking), filtration of some noxious substances (some carcinogens, among others, may be filtered out), a light dependence (seen as easy to quit), the influence of television (Egyptian movies have featured hookah smokers for decades), the rise of individualism in modern societies (socialising needs and the search for new forms of sociability), conviviality (social smoking, sharing the hose (ludens), talking, long time passing), a powerful symbolism (dream, art, mysticism, peace pipe), a transverse social, sexual, religious and inter-generational practice (social and cultural melting pot), flavours (tobamel [muassel], a flavoured tobacco [or no-tobacco]-honey/molasses based mixture), the cultural status of honey, a highly sensory experience and rebellion values.

2.7 Attitudes and Beliefs Regarding *Shisha* Use

Attitudes and belief about tobacco use are the singular main factors affecting people's behavior towards *Shisha*use. Till now, very few studies have investigated attitudes and beliefs towards *Shisha*use. Markets even in the developed countries promote the fallacy that *Shisha*is less hazardous than smoking and it is the main driving factor behind its current popularity. Out of the

few relevant studies conducted, one states that 30% of university students believe in the fallacy about *Shisha*being less deleterious than cigarette. A study in Egypt reported that 21% out of 206 male *Shisha*smokers reported that they preferred *Shisha*for the its perceived less toxic effects. A study in Pakistan revealed that 60% of the population considered cigarettes to be more deleterious. The same belief was also reported in a study in Egypt, Malaysia and Jordan (Sameerur *et al*., 2012).

Research however has proven otherwise, suggesting three additional risks to health of water pipe smoking over cigarette smoking. The first being that, as *Shisha*is smoked over, coal this adds to the already many harmful toxins of the smoke. Secondly, a *Shisha*smoker inhales up to 200 times more smoke in a single session as compared to cigarette smokers. Thirdly it is linked to high rates of second hand smoking due to its high social acceptance (Aljarrah *et al*., 2009).

The famous misperception that the *Shisha*is filtered due to the presence of water in the smoking apparatus seems to be one main belief justifying it being less injurious.However,it is well-known that making air bubbles pass through water does not change their content, and since the volatile carcinogen of tobacco smoke and other particles will stay within the air bubbles during their passage through water, it does not render *Shisha*any less harmful than cigarette (Aljarrah *et al*., 2009).

2.8 Comparison of *Shisha* Smoke with Cigarette Smoke

Based on smoking regimen consisting of 171 puffs each of 0.53 l volume and 2.6 s duration with a 17 s inter-puff interval, the following results were obtained for a single smoking session of 10 g of mo'assel tobacco paste with 1.5 quick-lighting charcoal disks applied to the narghile head *Shisha*. The smoke was observed to contain 2.94 mg nicotine, 802 mg tar, 145 mg carbon monoxide (CO) and relative to a smoke of single cigarette, greater quantities of chrysene,

phenanthrene and flouranthrene (Shihadeh and Saleh, 2005). It is also a fact that the number of puffs and their volume from using shisha are about 10 times higher than cigarette, and higher concentration of metals, while burning temperature for *shisha* is about 900°C as compared to 450°C for cigarette (Aljarrah *et al*., 2009).

Peak concentration of nicotine in cigarette and *Shisha*are same, but the relatively long duration of the *Shisha*use results in considerably greater effective nicotine exposure. The use of a single-compartment pharmacokinetics model with linear clearance kinetics, and a nicotine clearance constant of 0.0333 min^{-1} obtained by fitting an exponential decay curve to the average nicotine concentrations at 5, 15, 30, and 45 minutes post–cigarette initiation ($R2 = 0.98$); the nicotine AUC observed in a study conducted by Eissenberg and Shihadeh (2009), was 243 ng/ml-min for the cigarette and 418 ng/ml-min for *Shisha*. Relative to a cigarette, *Shisha*smokers were exposed to 1.7 times the nicotine dose (Eissenberg and Shihadeh, 2009).

2.9 Cardiovascular effects of shisha smoking

The injurious effects of *Shisha* smoking also affectthe cardiovascular system. Studies have shown that after 45 minutes of *Shisha*use, heart rates are found to be significantly increased (Maziak, 2011). Similarly,other studies have reported a mean increase in systolic and diastolic blood pressure and heart rate in*Shisha*smokers after a*Shisha*smoking session. Unlike cigarette smoking, little is known about health effects of *Shisha*use. One acute effect is dysfunction in autonomic regulation of the cardiac cycles, such as reduction in heart rate variability. Reduced heart rate variability is associated with inhalation exposure induced oxidative stress and increase in heart rate and blood pressure. Similarly, it has been reported that serum concentration of HDL, Apo A in *Shisha*smokers was significantly lower than in non-smokers. However LDL-

cholesterol, Apo B and triglycerides were significantly higher in smokers (Al-Numair *et al*., 2007; Eissenberg and Shihadeh, 2009).

2.10 Effect of *Shisha* Smoke on Pregnancy Outcome

Female smoking prevalence differs greatly from country to country. It depends on educational and cultural atmosphere. It has been evaluated that smoking one or more *Shisha*per day during pregnancy is associated with at least 100 g reduction in body weight. Furthermore, the risk of delivering low birth weight babies almost triples among those who take up the practice of *Shisha*smoking during the 1st trimester.Mothers who smoke shisha had low birth weight infants compared with non-smoking mothers (OR = 2.4, 95%CI, 1.2-5). In addition, other problemsassociated with *Shisha*smoking include lower APGAR score and increased pulmonary problems at birth (Tamim *et al*., 2007).

2.11 Effect of *Shisha* Smoking on Plasma Nicotine Concentration and Respiratory Rate

Eissenberg and Shihadeh (2009), reported that for a *Shisha*smoker, mean pre-smoking plasma nicotine concentration was 2.0 ± 0.2 ng/ml, which increased to 6.1 ± 1.1 ng/ml at 5 minutes, 6.4 ± 0.8 ng/ml at 15 minutes,7.9 ± 1.0 ng/ml at 30 minutes, and 8.5 ± 1.0 ng/ml at 45 minutes, respectively, after smoking. The higher nicotine associated with water-pipe tobacco smoking relative to cigarette smoking was significant at 45 minutes (Eissenberg and Shihadeh, 2009). Mean carboxy-haemoglobin (CO Hb) is increased by 4 times for starting 5 min of shisha smoking than by entire cigarette (Sutfin *et al*., 2011). Respiratory rate also increases upto 2.0 ±2.0 breathes/min. (Shaikh *et al*., 2008). A single session of *Shisha*smoking produces four to five times higher carbon II oxide (CO) than that produced from smoking a cigarette. When breath of heavy tobacco smoker was measured, CO level of 30-40 ppm were found. One session of hookah

smoking caused elevation of mean CO value by almost eight folds higher than that of cigarette smoking (Khan *et al*., 2011).

2.12 Oral Effects of *Shisha* Smoking on the Oral Cavity and Respiratory Tract

Shisha smoking has many negative effects on the mouth, including staining of teeth, dental restorations and reduced ability of smell and taste. *Shisha* is one of the notable risk factors for periodontal bone loss, dry sockets and oral squamous cell carcinoma (Dangi *et al*., 2012).

A case control study carried out in Beirut reported that the incidence of benign lesion of the vocal cords and vocal folds in a*Shisha*smoking group is 21.5%, with edema being the most common at 16% followed by cyst presenting at 4.8% (Hamdan *et al*., 2010).

2.13*Shisha*Smoking and Metabolic syndrome

Shisha smokers are found to be significantly more likely to have hypertryglycaemia, hyperglycemia, hypertension and abdominal obesity, probably due to its disruptions of metabolic hormones activities (Hafique *et al*., 2012).

2.14Carcinogenic Effect of *Shisha* Smoking

A *Shisha* smoker is exposed to hundreds of potentially dangerous materials present in *Shisha*smoke at one time. The major *Shisha* smoke constituents are carbon monoxide and nicotine (Maziak *et al*., 2011). Studies have shown that the concentration of carcinoembryonic antigen (CEA), known as marker of malignant transformation and chronic inflammation, is increased in tobacco smoke causing a variety of cancers (Sajid *et al*., 2008).

Health effects of *Shisha*have been a matter of debate among researchers as different investigator have varied points of view. Composition of tobacco smoke in *Shisha*is variable and not well

standardised. It is evident that the smoke emerging from the water pipe contains numerous toxicants known to cause cancer. Carcinogenic substances present in tobacco smoke include: naphthalene, acenaphthylene, acenaphthene, flourne, phenanthrene, anthracene, flouranthene, pyrene, benzo[a]anthracene, chyrsene, benzo[b + k]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene, dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene (Sepetdjian *et al*., 2010). The levels of carbon monoxide and carboxyhaemoglobin were higher among *Shisha*smokers than cigarette smokers or non-smokers. Analysis of mainstream aerosol found that *Shisha*smoke contains significant amounts of nicotine, tar and heavy metals (Hafiz *et al*., 2014). In a standard heavy protocol of 100 puffs of 3 sec, 2.25 mg of nicotine and 242 mg of nicotine free dry particulate were obtained. Along these, high levels of arsenic, chromium and lead were found in *Shisha*smoke. Increased puff frequency causes increase in nicotine free dry particulate matter, whereas the remaining water from the bowl increases the amount of nicotine (Shihadeh, 2003).

Several data (outside Nigeria) suggest that *Shisha*smoking is as toxic as cigarette smoking. A *Shisha* smoker may absorb large amounts of its harmful substances because of the mode of smoking, depth of inhalation, length of smoking session and frequency of puffing. Hazards of tar and its carcinogenicity were directly related to working temperature and not only combustion and pyrolysis. Hookah smoking is associated with increased risk of lung cancer in ethnic Kashmiri population with the risk being 6 times more as compared to non-smokers (Koul *et al*., 2011).

A study comparing 35 healthy *Shisha*users with 35 healthy, non-exposed controls, showed that *Shisha*use was associated with a significant increase in frequency of chromosomal aberrations

and sister chromatid exchanges, while the frequency of satellite associations and the mitotic index was significantly higher in water pipe users, relative to controls (Yadav and Thakur, 2000). However, in a different study conducted with cigarette and hookah, hookah does not generate side stream smoke from the charcoal topping of the bowl for charring the *Shisha* particles,the only smoke suggested be taken is the one which filtered into the water inside the bowl; inhaled bysmokers into the lung through the hose-pipe, however, the filtered smokes were reported less toxic than cigarette smoke (Sajid *et al*., 2008). Another study also showed that *Shisha*smoke contains particles that are three times less concentrated than cigarette smoke; 74.4×10^9 for a 1000 ml and 9.24×10^9 for a 45 ml of particles for *Shisha* smoke puff and cigarette smoke puff, respectively (Zaga and Gatta-vecchia, 2002). However,*Shisha*smokers inhaled much more smoke than cigarette smoker in a session of smoking, lasting up to 6 hours. Sajid *et al*. (2008), reported that carcinogenic embryonic antigen (CEA) levels in heavy smokers (spending up to 6 hours per day in 3 to 8 smoking sessions of a tobacco weight equivalent to about 60 cigarettes) are very much at risk than medium (up to 2 hrs per day in 1 to 3 smoking sessions) and light smokers. Traditional hookah smoking has produced fewer carcinogenic effect than cigarette smoking, but it still produces worrisome smoke in long term exposure (Sajid *et al*., 2008).

2.14.1 Shisha smoking and oesophageal carcinoma

Although cigarette smoking is an established risk factor for esophageal carcinoma, there is little information regarding its association with *Shisha*. Dar*et al*. (2012) reporteda significant association between esophageal carcinoma and *Shisha* Smoking.A similar association has also been reported in studies conducted in China, India and Iran (Khan *et al*., 2011; Mao *et al*., 2011).

2.14.2 Shisha smoking and Pancreatic cancer

Cancer of the pancreas is a fatal disease and is significantly associated with tobacco smoking. The disease is caused by damage to the DNA, with smoking being a significant risk factor. Cigars and *Shisha*are known to increase the risk of developing pancreatic cancer (Jarosz *et al*., 2012).

2.14.3 Effect of *shisha* smoking on Bladder carcinoma

Shisha smoking normally involves the use of burning charcoal. The smoke inhaled by the user contains constituents originating from the charcoal, in addition to those from the tobacco which is also one of the main initiating factors for carcinoma. In a study of 100 cases of bladder cancer, 5% of the patients with bladder carcinoma were *Shisha*users (Nusrat *et al*., 2001).

2.14.4 Keratoacanthoma and squamous cell carcinoma of lip effects of shisha smoking

EI-Hakim (1999), reported a significant association between *Shisha*smoking and squamous cell carcinoma and keratoacanthoma of the lip (El-Hakim and Uthman, 1999).

2.15Risk of Infection due to Sharing of *Shisha* Smoking Pipe

It was evaluated that the practice of sharing a water-pipe mouth piece poses a serious risk of transmission of communicable diseases including tuberculosis (TB) and hepatitis. The water pipe and the water inside the *Shisha* apparatus can become an abode for bacteria such as those causing TB, resulting in the spread and transmission of the disease. Pipe sharing with someone with pulmonary TB, can lead to a greater risk of TB transmission (Chandir *et al*., 2010).

2.16Consequences of Excessive Generation of Reactive Oxygen Species in Sperm Cell

Oxidative stress (OS) is considered a major contributory factor to infertility. Oxidative stress is the result of imbalance between the reactive oxygen species (ROS) and antioxidants in the body

which can lead to sperm damage, deformity, and eventually male infertility. There is evidence to demonstrate that low and controlled concentrations of these ROS play an important role in sperm physiological processes such as capacitation, acrosome reaction, and signaling processes that ensure fertilization (Amrit, and Bilaspuri, 2011).

Spermatozoa are vulnerable to ROS because their plasma membrane and cytoplasm contain large amounts of polyunsaturated fatty acids. Excessive generation of ROS in semen by leukocytes as well as by abnormal spermatozoa could be a cause of infertility. Hydrogen peroxide is the major ROS producer in human spermatozoa. Moderately-elevated concentrations of hydrogen peroxide do not affect sperm viability but cause sperm immobilization, mostly via depletion of intracellular adenosine-triphosphate (ATP) and the subsequent decrease in the phosphorylation of axonemal proteins. High concentrations of hydrogen peroxide induce lipid peroxidation and result in cell death (Misro *et al*., 2004).

Gomez *et al*. (1998) demonstrated that levels of ROS produced by spermatozoa were negatively correlated with the quality of sperm in the original semen.They reported that the levels of antioxidants in seminal plasma from infertile men were significantly lower than in fertile controls.They suggested that the pathological levels of ROS detected in the semen of infertile men are more likely to be a result of increased ROS production rather than reduced antioxidant capacity of the seminal plasma.Poor sperm quality is linked to increased ROS generation as a consequence of the presence of excess residual cytoplasm. Spermatozoa undergo a remarkable transformation during the final stage of sperm differentiation, and shed their cytoplasmic processes to become mature spermatids (Aziz *et al*., 2004). Following spermiation, any residual cytoplasm associated with spermatozoa is retained in the mid-piece region as an irregular cytoplasmic mass (Figure 2.1)

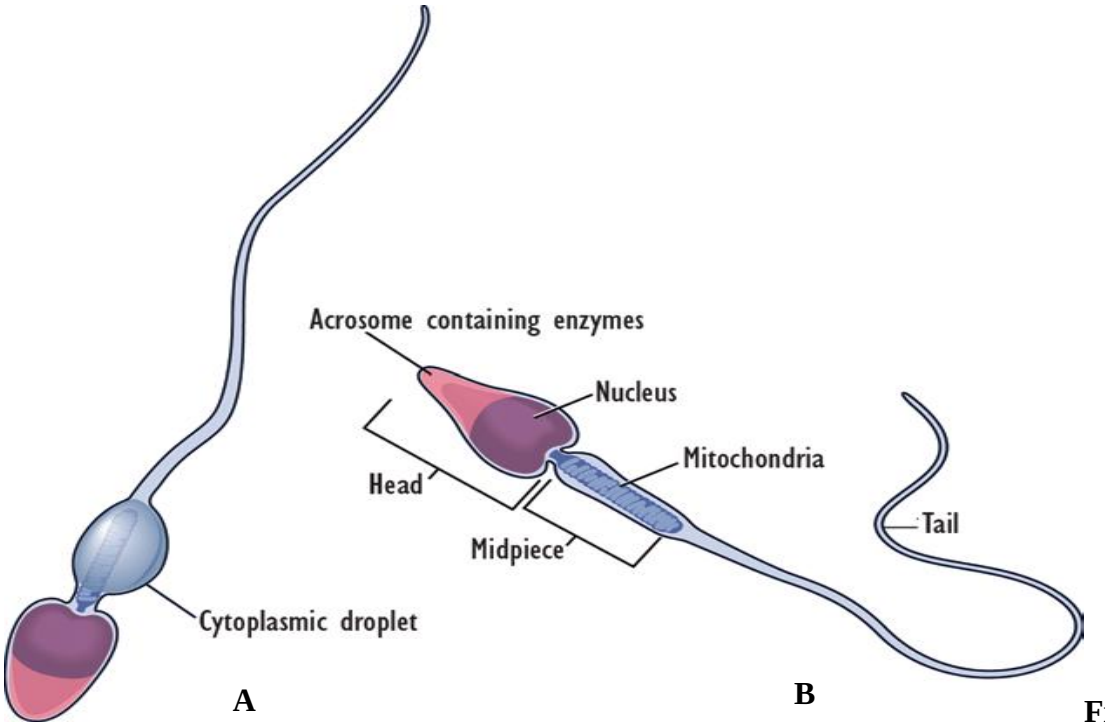


Figure 2.1: A: Spermatozoon showing cytoplasmic droplet in the mid-piece of the tail segment. B: Schematic representation of a mature spermatozoon (Aziz *et al*., 2004)

2.16.1Origin of reactive oxygen species in male reproductive system

In males, two ROS generating systems are possibly involved, a hypothetical NADH oxidase (figure 2.2) at the level of sperm membrane, and low sperm diphorase (mitochondrial NADH-dependent oxidoreductase). In bovine semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Sariözkan *et al*., 2009).

Leucocytes and immature spermatozoa are the two main sources of ROS. Leucocytes particularly neutrophils and macrophages have been associated with excessive ROS production and they ultimately cause sperm dysfunction (Aitken and Baker, 1995; Garrido *et al*., 2004).

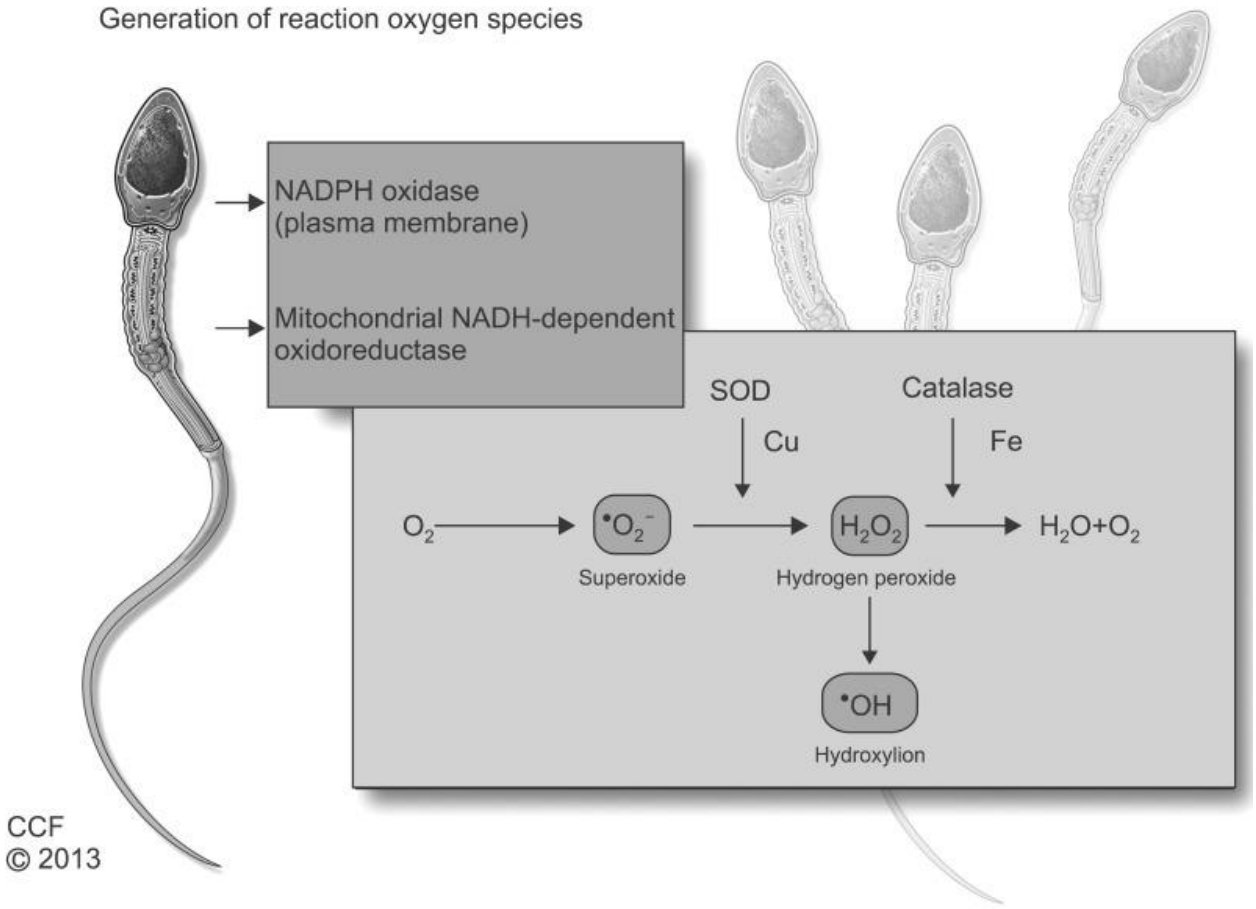


Figure 2.2: Generation of reactive oxygen species in sperm cells (Chen*et al*., 2013)

2.16.1.1Endogenous sources of reactive oxygen species

2.16.1.1.1Reactive oxygen species generation from Leucocytes

Peroxidase-positive leucocytes include polymorphonuclear leucocytes (50%~60%) and macrophages (20%~30%). A large proportion of these peroxidase-positive leukocytes originate from the prostate and seminal vesicles. When these major sources of ROS are activated by various intracellular or extracellular stimuli, such as infection or inflammation, they can discharge up to 100 times more ROS than normal, and increase the reduced nicotinamide adenine dinucleotide phosphate (NADPH) production via the hexose monophosphate shunt (Lavranos *et al*., 2012). An increase in proinflammatory cytokines, such as interleukin (IL)-8,

and a decrease in the antioxidant superoxide dismutase (SOD) can result in a respiratory burst, production of high levels of ROS, and ultimately, oxidative stress (OS). OS will cause sperm damage if seminal leukocyte concentrations are abnormally high as is the case in leukocytospermia (Lu *et al*., 2010), which the WHO defines as the presence of more than one million peroxidase-positive cells per milliliter of semen (WHO, 2010).

Over the years, extensive research has been carried out to establish a link between the presence of leukocytes in the ejaculate and a male factor as the cause of infertility. Various studies point to a correlation between decreased sperm function and seminal plasma with abnormally elevated levels of ROS, interlukin (IL)-6, IL-8, and tumor necrosis factor, all of which result in increased sperm cell membrane lipid peroxidation (Nandipati *et al*., 2005; Lavranos *et al*., 2012).

2.16.1.1.2 Reactive oxygen species generation from immature spermatozoa

During spermatogenesis, developing spermatozoa extrude their cytoplasm in order to prepare for fertilization. However, damaged spermatozoa retain excess cytoplasm around the midpiece due to an arrest in spermiogenesis; this condition is known as excess residual cytoplasm (ERC). The ERC activates the NADPH system by means of the hexose-monophosphate shunt, which spermatozoa use as a source of electrons for ROS generation and potentially, OS. Hence, ERC ultimately affects sperm motility, morphology, and fertilization potential, which may lead to male infertility (Rengan *et al*., 2012).

2.16.1.1.3 Reactive oxygen species generation from varicocele

Varicocele is defined as an abnormal dilation of veins in the pampiniform plexus around the spermatic cord. Since varicocele is detected in about 40% of male partners of all infertile couples, it is considered the leading cause of male factor infertility (Will *et al*., 2011). It has been

shown that the level of seminal ROS is associated with the grade of varicocele; that is, the higher the grade of varicocele, the greater is the level of ROS detected (Shiraishi *et al*., 2012).

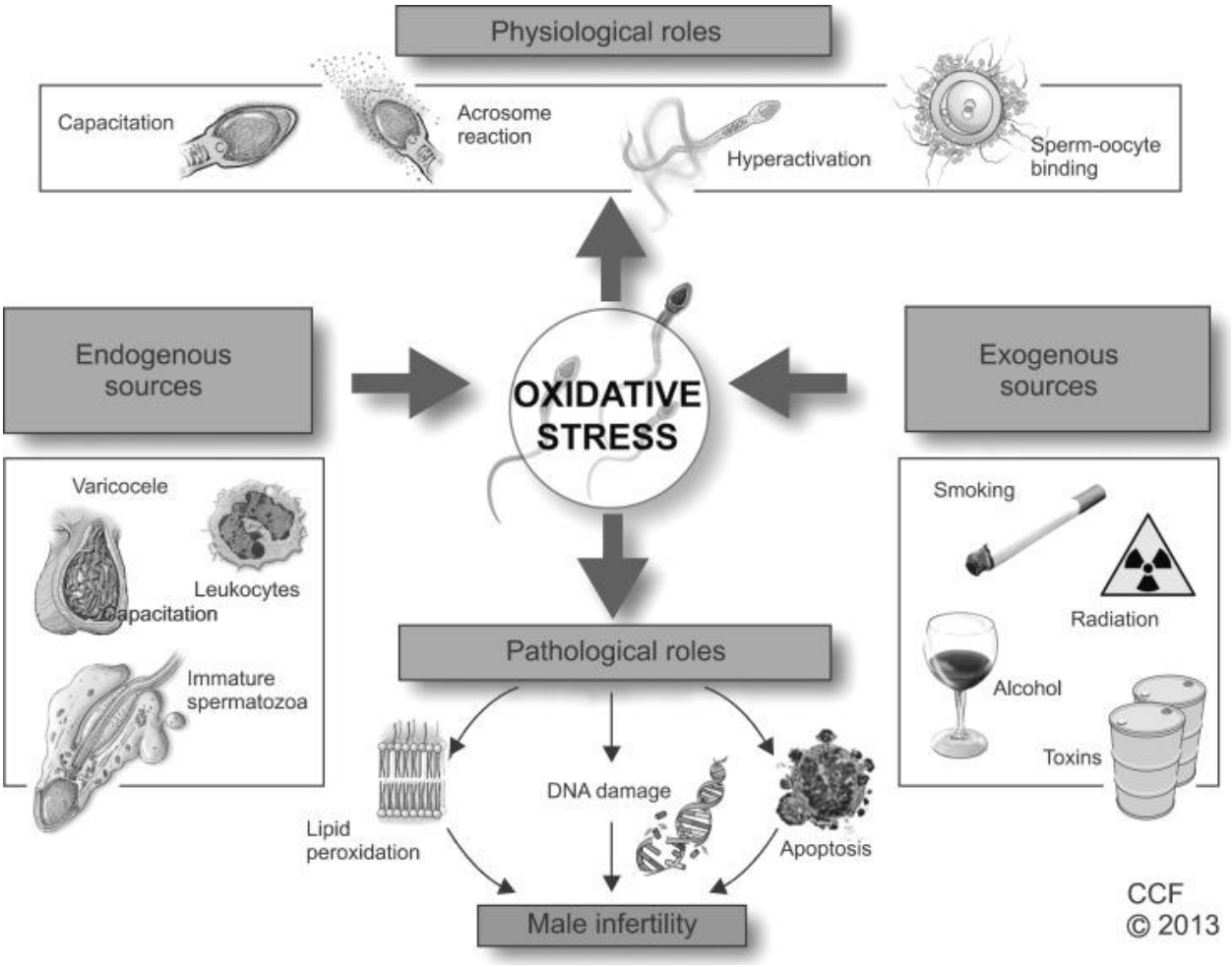


Figure 2.3: Various sources of reactive oxygen species and their effects on sperm cells (Tremellen, 2008)

2.16.2 Exogenous sources of reactive oxygen species

2.16.2.1 Effect of reactive oxygen species from radiation on sperm cells

Radiation, a natural source of energy, has significant clinical effects on humans. With respect to male reproductive health, several studies have implicated radiation emitted from mobile phones for the increase in production of ROS and impaired semen quality in humans (Agarwal *et al*.,

2008). *In vitro* studies have demonstrated that electromagnetic radiation induces ROS production and DNA damage in human spermatozoa, which further decreases the motility and vitality of sperm cells as well as their concentration depending on the duration of exposure to radiation (De Iuliis *et al*., 2013). These radiofrequency electromagnetic waves can negatively affect the electron flow along the internal membranes of the cell as a result of the numerous charged molecules within the cytosol, thus disrupting normal cellular and organelle function (Lavranos *et al*., 2012).

2.16.2.2Effect of reactive oxygen species fromsmoking on sperm cells

Tobacco use is known to be one of the major preventable causes of death worldwide. Cigarettes contain more than 4,000 chemical compounds including alkaloids, nitrosamines, and inorganic molecules. Some of the chemicals were shown to cause an imbalance between ROS and antioxidants in the semen of smokers (Lavranos *et al*., 2012). This ROS and antioxidant disproportion affects the overall semen quality. Smoking has been shown to result in a 48% increase in seminal leucocyte concentrations and a 107% increase in seminal ROS levels. Moreover, smokers have decreased levels of seminal plasma antioxidants such as vitamin E and vitamin C, placing their sperm at the additional risk of oxidative damage. This has been confirmed by a significant increase in the levels of 8-OHdG, another biomarker of oxidative damage, in the seminal plasma of smokers (Kiziler *et al*., 2007). A study on the semen profiles of smokers versus non-smokers showed that spermatozoa from smokers were significantly more sensitive to acid-induced DNA denaturation than those of non-smokers resulting in higher levels of DNA strand breaks. Another study performed on smokers revealed that the increased cadmium and lead concentrations in their blood and semen led to increased ROS production with an accompanying decrease in sperm motility. Furthermore, prolonged exposure to tobacco

smoke has been linked to an increase in sperm DNA damage and apoptosis, leading to increased male infertility (Kiziler *et al*., 2007).

2.17Physiological Roles of Reactive Oxygen Species in Seminal Plasma

Reactive oxygen species can have beneficial or detrimental effects on sperm function depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS.The ROS cause sperm pathologies (ATP depletion) in the form of inadequate axonemal phosphorylation or LPO, resulting in a loss of sperm motility and viability. During epididymal transit, sperm acquire the ability to move progressively.However, they acquire the ability to fertilize in the female tract through a series of physiological changes called capacitation. Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosomal reactionand sperm-oocyte fusion in order to ensure appropriate fertilization (Saleh and Agarwal, 2002). Superoxide anion appears to play a role in this process. Studies have indicated that male germ cells at various stages of differentiation have the potential to generate ROS (Agarwal *et al*., 2005).

2.18Beneficial Role of Reactive Oxygen Species in Sperm Cell Capacitation

Capacitation is the penultimate process in the maturation of spermatozoa and is required to render them competent to successfully fertilize the ovum (Choudhary *et al*., 2010). Controlled ROS production occurs in spermatozoa during the capacitation process, initiating various molecular modifications. The first step involves an increase in cyclic adenosine 3',5'-monophosphate (cAMP). The cAMP pathway is necessary for many living organisms and life processes as it can activate enzymes and regulate gene expression (Tsai *et al*., 2013). This pathway involves the activation of protein kinase A (PKA) and the phosphorylation of PKA substrates (arginine, serine, and threonine). This subsequently leads to the phosphorylation of

MEK (extracellular signal regulated kinase)-like proteins and threonine-glutamate-tyrosine, and finally tyrosine phosphorylation of fibrous sheath proteins. This increase in cAMP causes hyperactivation of the spermatozoa. Only hyperactivated spermatozoa have increased motility to undergo acrosome reaction and acquire the characteristics required for successful fertilization (Kothari *et al*., 2010; Chen *et al*., 2013).

2.18.1 Role of reactive oxygen species in hyperactivation of sperm cell

Hyperactivation is a specific state of sperm motility when spermatozoa become highly motile. The process of hyperactivation is essential for successful fertilization and is considered a subcategory of capacitation. Hyperactive spermatozoa exhibit features of high amplitude, asymmetric flagellar movement, increased side-to-side head displacement, and non-linear motility (Suarez, 2008). The role of ROS in the initiation of hyperactivation has been well documented *in vitro* as shown when spermatozoa were incubated with low concentrations of OHradical (Agarwal *et al*., 2014).

2.18.2 Reactive oxygen species aid sperm acrosome reaction

When a hyperactivated spermatozoon passes the cumulus oophorus, it binds to the zona pellucida (ZP) of the oocyte and initiates an exocytotic release of proteolytic enzymes, creating a pore in ZP's extracellular matrix. The spermatozoa then penetrate this physical zona barrier and fuse with the oocyte (De Lamirande and O'Flaherty, 2008). The molecular events of the acrosome reaction overlap substantially with those of capacitation, including phosphorylation of similar tyrosine proteins, influx of Ca^{2+}, and increased cAMP and PKA levels. The role of ROS in the in vivo acrosome reaction involves the spermatozoa's actions on the ZP via phosphorylation of three plasma membrane proteins. *In vitro* activation of the acrosomal reaction (AR) was also

observed when physiological concentrations of O_2-, H_2O_2, and NO were added to the seminal plasma (Bansal and Bilaspuri, 2010).

2.18.3 Reactive oxygen species facilitate sperm-oocyte fusion

For successful fertilization, the spermatozoa must penetrate the ZP and fuse with the oocyte. High amounts of poly-unsaturated fatty acids(PUFAs), particularly docosahexaenoic acid (DHA), play a major role in regulating membrane fluidity in sperm. In studies of human spermatozoa, ROS has been shown to increase the membrane fluidity and rates of sperm-oocyte fusion, which occurs during the biochemical cascade of capacitation and acrosome reaction. Throughout capacitation, ROS inhibits protein tyrosine phosphatase activity and prevents dephosphorylation and deactivation of phospholipase A2 (PLA2). The PLA2 cleaves the secondary fatty acid from the triglycerol backbone of the membrane phospholipid and increases the membrane's fluidity (Khosrowbeygi and Zarghami, 2007).

2.19Pathological Roles of Reactive Oxygen Species

When the highly potent ROS overcomes the antioxidant defense systems and disrupts the intricate balance between ROS and antioxidants, pathological effects occur. Depending on the nature, amount, and duration of the ROS insult, these effects cause significant damage to biomolecules such as lipids, proteins, nucleic acids, and sugars (Agarwal and Prabakaran, 2005).

2.19.1 Process of lipid peroxidation by reactive oxygen in sperm cells

Lipids are responsible for the fluidity of membrane layers and the changes that occur during capacitation in the female reproductive tract (Sanocka and Kurpisz, 2004). The plasma membrane of mammalian spermatozoa is markedly different from mammalian somatic cells in terms of its lipid composition. The plasma membrane contains high levels of lipids in the form of

PUFAs. These lipids contain unconjugated double bonds separated by methylene groups. The placement of a double bond adjacent to a methylene group weakens the methyl carbon-hydrogen bond, consequently making hydrogen extremely susceptible to abstraction and oxidative damage. When the levels of ROS within the cell are high, ROS will attack PUFA, causing a cascade of chemical reactions oflipid peroxidation (LPO); Makker *et al*., 2009. Approximately 50% of the fatty acids in human spermatozoa are composed of DHA with 22-carbon chains and six *cis* double bonds. DHA is thought to play a major role in regulating spermatogenesis and membrane fluidity (Aitken *et al*., 2010). As the LPO cascade proceeds in the sperm, almost 60% of the fatty acid is lost from the membrane, hence affecting its function by decreasing its fluidity, increasing non-specific permeability to ions, and inactivating membrane-bound receptors and enzymes. Since LPO is an autocatalytic self-propagating reaction associated with abnormal fertilization, it is critical to understand the mechanism behind this process, which can be conveniently separated into three main steps viz: initiation, propagation, and termination (Tremellen, 2008).

Initiation involves the abstraction of hydrogen atoms associated with carbon-carbon double bonds, which results in free radical formation. These free radicals react with fatty acid chains and form lipid radicals, which then react with oxygen to form the peroxyl radicals. These peroxyl radicals, which can abstract hydrogen from lipid molecules, particularly in the presence of metals such as copper and iron, cause an autocatalytic chain reaction. The radicals eventually react with hydrogen to form lipid peroxides (Saalu, 2010). This reaction characterizes the propagation stage. These radicals act on additional lipids, forming cytotoxic aldehydes due to hydroperoxide degradation. Peroxyl and alkyl radicals are regenerated in a cyclical fashion in the propagation step until they react with another radical to form a stable end product called malondialdehyde (MDA) during the third step of termination. Thus, MDA is used in biochemical assays to

monitor the degree of peroxidative damage to spermatozoa (Sanocka and Kurpisz, 2004). Another byproduct of LPO is 4-hydroxynonenal, which is formed from low-density lipoproteins. Hydroxynonenals are hydrophilic and can cause severe cell dysfunction at both genomic and proteomic levels (Hampl *et al*., 2012).

2.19.1.1 Detrimental effects of lipid peroxidation on sperm functions

Lipid peroxides are spontaneously generated in the sperm plasma membrane and are released by the action of phospholipase A_2. They are capable of inducing DNA damage and decrease in fertility during storage of semen. The peroxides are generally associated with decreased sperm functions and viability, but, also have a significant enhancing effect on the ability of spermatozoa to bind with homologous and heterologous *zona pellucida* (Argawal *et al*., 2014).

2.19.2 Sperm cell DNA damage by reactive oxygen species

Semen parameters such as concentration, motility, and morphology are commonly used to determine the fertilization potential of sperm from an ejaculate. Although this provides a general overview of the quality of sperm, it does not provide information on one of the most important components of the reproductive outcome;that is, the integrity of the sperm DNA. Single- or double-stranded DNA breaks can be a source of differences in reproductive potential between fertile and infertile men (Zribi*et al*., 2011).

It has been reported that chromatin in the sperm nucleus is vulnerable to oxidative damage, leading to base modifications and DNA fragmentation (Zribi *et al*., 2011). The chromatin of human spermatozoa has a highly condensed and organized structure. This is further packaged into nucleosomes and coiled into a solenoid. During the process of spermiogenesis, sperm chromatin undergoes a series of modifications in which histones are replaced with transition

proteins and subsequently, protamines. DNA strands are condensed by the protamines and form the basic packaging unit of sperm chromatin called toroid. Toroids are further compacted by intra- and inter-molecular disulfide cross-links. This DNA compaction and organization help protect sperm chromatin from oxidative damage, making them particularly resistant to DNA damage (Schulte *et al*., 2010). However, in some cases where poor compaction and incomplete protamination of sperm chromatin exist, DNA is more vulnerable to OS and produces base-free sites, deletions, frame-shift mutations, DNA cross-links, and chromosomal rearrangements. Damaged DNA has been observed in testicular, epididymal, and ejaculated human spermatozoa (Kemal *et al*., 2000).

Single- and double-stranded DNA breaks can be detected by using either the TUNEL or Comet Assay. Single-strand breaks are a direct result of oxidative damage on sperm DNA, while double-strand breaks may arise from exposure to 4-hydroxyl-2-nonenal- a major product of LPO (González-Marín *et al*., 2012). It was discovered that 8-hydroxy-2-deoxyguanosine and two ethenonucleosides (1, N6-ethenoadenosine and 1, N6-ethenoguanosine) are the two major DNA adducts found in human sperm DNA, both of which have been considered key biomarkers of DNA damage caused by OS (Valavanidis *et al*., 2009). Despite these findings, DNA damage is not a cause for concern during intrauterine insemination and in vitro fertilization (IVF), because the coexisting LPO damage by ROS eliminates the possibility of fertilization. However, if normal natural selection is bypassed during intracytoplasmic sperm injection (ICSI), sperm with significant amounts of DNA damage have the opportunity to fertilize the oocyte (Makker *et al*., 2009). When DNA is minimally damaged, spermatozoa can undergo self-repair and potentially regain the ability to fertilize the oocyte and proceed with development (Aitken and Koppers, 2011). In fact, the oocyte is also capable of repairing damaged sperm DNA. In cases where the

oocyte repair machinery is not sufficient to repair DNA damage, the embryo may fail to develop or implant in the uterus and can be naturally aborted. In other cases, the oocyte may successfully repair sperm DNA-strand breaks before the initiation of the first cleavage division, thereby producing normal offspring. It has been reported that 80% of the structural chromosomal aberrations are of paternal origin in humans (González-Marín *et al*., 2012). DNA damage is a contributory factor to apoptosis, poor fertilization rate, high frequency of miscarriage, and morbidity in offspring (Chen *et al*., 2013).

The following scores indicate fertility potential for natural conception and intrauterine insemination (Osman*et al*., 2014):

(i) Less than or equal to 15 percent DFI: Excellent to good fertility potential

(ii) 15 percent to 25 percent DFI: Good to fair fertility potential

(iii) Greater than 25 percent DFI: Fair to poor fertility potential

2.19.3 Apoptosis of sperm cell results from reactive oxygen species

Sperm DNA damage and impaired fertilization occur following unsuccessful apoptosis. Apoptosis, also known as programmed cell death, is a physiological phenomenon characterized by cellular morphological and biochemical modifications that cause cells to die in a controlled manner (Makker *et al*., 2009). During early development, apoptosis is important in the ontogeny of the germ line as a means of regulating the germ cell to Sertoli cell ratio. In adulthood, apoptosis plays a vital role in selectively destroying the premeiotic spermatogonia during the first round of spermatogenesis by preventing the overproduction of germ cells from seminiferous tubules in response to ROS (Tremellen, 2011). During this process, the human ejaculate expresses various apoptotic markers that initiate apoptosis, some of which include Fas,

phosphatidylserine (PS), Bcl-Xl, and p53. Fas is a type I membrane protein that belongs to the tumor necrosis factor-nerve growth factor receptor family and is secreted by the Sertoli cells located on the germ cell surface (Agarwal *et al*., 2003). To further support this theory, the same study reported that the percentage of Fas-positive spermatozoa was as high as 50% in men with abnormal sperm parameters (Agarwal *et al*., 2003). In addition, this apoptotic pathway activates the inner and outer mitochondrial membranes to cause the release of the signaling molecule cytochrome C, which triggers caspases, such as caspases 3 and 9, and annexin-V binding (annexins are calcium-dependent phospholipid-binding proteins, which bind to PS). This pathway eventually leads to sperm apoptosis. In an earlier study, it was reported that annexin-V staining was used to study the externalization of PS-a marker for early apoptosis. It was observed that mature spermatozoa from infertile patients with increased ROS levels had significantly higher levels of apoptosis than mature spermatozoa from the control group (Aitken and Baker, 2013).

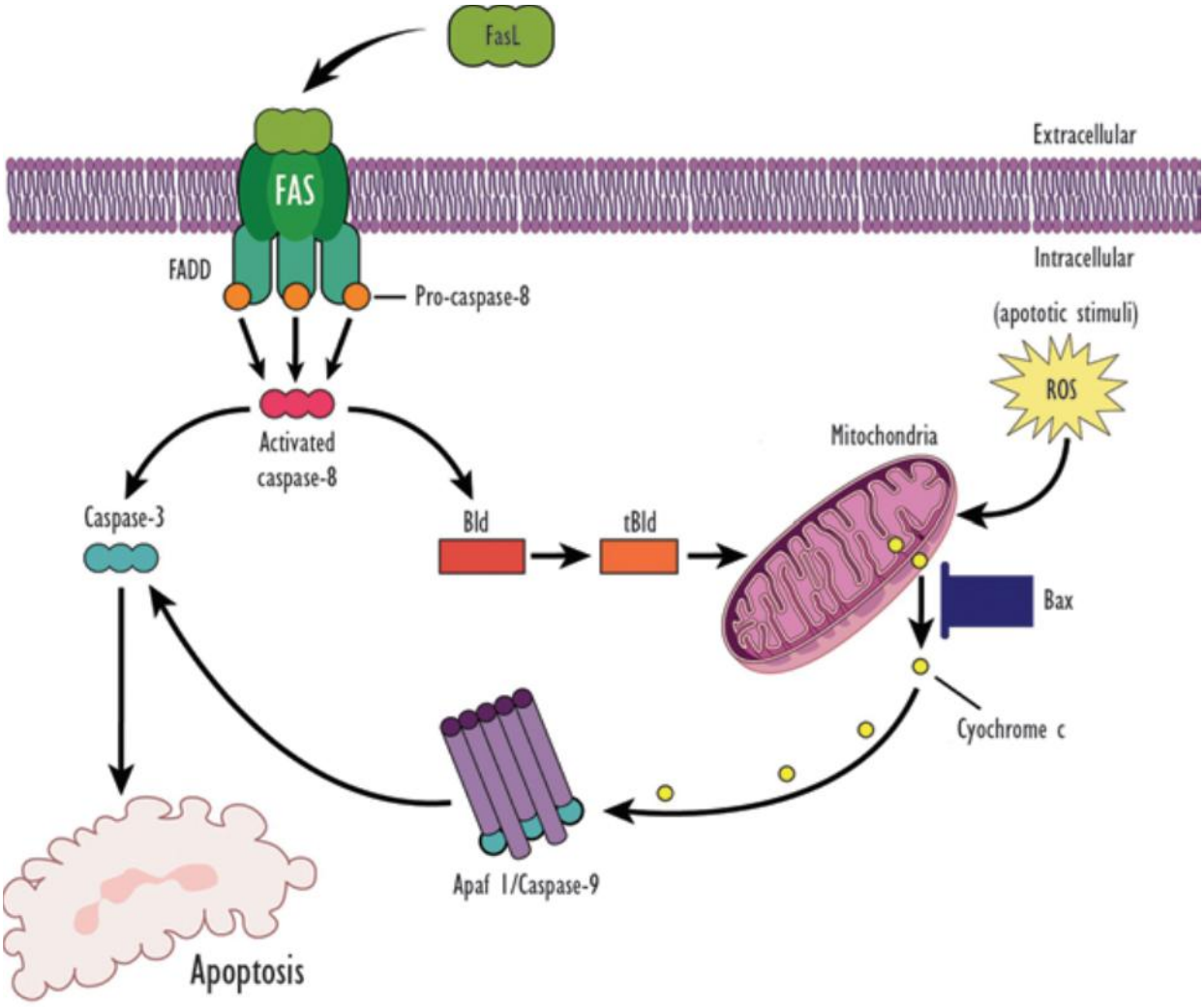


Figure 2.4: Schematic description of the release of cytochrome c protein from the mitochondria, whichactivates the caspases and induces apoptosis (Agarwal, 2005)

2.20Measurement of Oxidative Stress

Over the last decade, research has provided growing support to indicate that excess ROS production leads to abnormal semen parameters and increased sperm damage. Standard semen analysis continues to be the backbone of clinical evaluation of male infertility. Studies have shown that ROS-mediated damage to sperm is a considerable contributing pathology in 30% to 80% of unselected infertile patients (Agarwa *et al*., 2006; Makker *et al*., 2009). The detection of

the levels and sources of excess ROS production in semen is currently not included in the routine evaluation of sub-fertile men (Deepinder *et al*., 2008). Some of the reasons include mere inconvenience, cost-effective and efficient assays, and, perhaps most importantly, the lack of a universally accepted analysis method. All three contribute to the widespread limitations when measuring ROS as part of a male infertility assessment, despite its importance. At present, over 30 different assays are used to measure ROS and the presence of OS in the semen of men.

2.20.1 Indices of sperm oxidative stress from routine semen analysis

Routine semen analyses have allowed clinicians to make a fairly accurate diagnosis of OS. A reduction in any of the semen parameters (count, motility, and morphology) is more frequently seen in men with OS. Asthenozoospermia (reduced sperm motility) is most likely the best surrogate marker for OS in a routine semen analysis. The hyperviscosity of seminal plasma is also associated with increased levels of seminal plasma MDA and reduced seminal plasma antioxidant status (Aydemir *et al*., 2008), making impaired viscosity a reasonable surrogate marker of OS (Tremellen, 2008). In addition, infection of the semen with the bacterial microorganism,*Ureaplasma urealyticum* is associated with increased seminal plasma viscosity and an increase in ROS production (Wang *et al*., 2006). It is possible that this infection may damage the prostate and seminal vesicles, altering the substrates involved in maintaining normal semen viscosity. The indication and the presence of a large number of round cells imply possible OS caused by leukocytospermia. However, these round cells may be immature spermatozoa rather than leukocytes. For this reason, an accurate identification of these cells require ancillary tests such as the peroxidase test, CD45 (transmembrane glycoprotein expressed at high levels on the cell surface) antibody staining or measurement of seminal elastase (Zorn *et al*., 2003). Abnormal sperm morphology related to excess residual cytoplasm(ERC) and cytoplasmic

droplets are principal features of anomalous spermatozoa generating high levels of ROS. Also, poor sperm membrane integrity, which may be assessed by the hypo-osmotic swelling test (HOST), has been linked to the presence of OS (Menkveld, 2010).

2.20.1.1 Laboratory assessment of oxidative stress in semen

Oxidative stress results from an imbalance between reactive oxygen speciesproduction and the amount of intracellular/extracellular antioxidants present in seminal plasma. Direct assays of OS measures the net oxidative result of this imbalance by detecting and measuring the amount of oxidation in the sperm cell membrane. The MDA, which is one of the final products of membrane LPO, can be measured via the thiobarbituric acid assay, which is one of the oldest and most widely used direct assays for assessing sperm membrane oxidation. Various authors have reported that increased levels of MDA are associated with decreased sperm motility and sperm-oocyte fusion (Kefer *et al*., 2009).

Chemiluminescence assays are most commonly used for measuring seminal ROS. A luminometer is used in conjunction with a chemiluminescent probe such as luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione). Luminol is extremely sensitive and reacts with a variety of ROS at neutral pH (Aitken *et al*., 2004). It has the ability to measure both intracellular and extracellular ROS. The free radicals in the semen sample combine with luminol to generate a light signal that is converted to an electric signal (photon) by the luminometer. The number of free radicals produced is measured as relative light units/s/10^6 sperm. Normal ROS levels in washed sperm suspensions range from 0.10 to 1.03×10^6 cpm per 20×10^6 sperm (Benjamin *et al*., 2012).

Luminol can also be used to indirectly measure the total antioxidant capacity (TAC) within the seminal plasma. The TAC is then quantified against a vitamin E analog, Trolox (a water-soluble tocopherol analog). The results are expressed as an ROS-TAC score, and this gives an indication of the combined antioxidant activities of all constituents, including vitamins, proteins, and lipids. This assay appears to be the best established method for analyzing the balance between ROS and the antioxidant protection of sperm (Jamsai and O'Bryan, 2010).

An assay that has gained popularity due to its cost-effectiveness and user-friendliness is nitroblue tetrazolium (NBT). This assay provides information on the source(s) of ROS. This technique involves only a light microscope and accurately predicts whether ROS has been produced by spermatozoa or leukocytes. When NBT interacts with the O_2^- present within spermatozoa or leukocytes, it is converted into a blue pigment called diformazam. With the aid of a light microscope, the amount of diformazam can be observed, measured, and correlated to the intracellular ROS concentration (Kefer *et al*., 2009).

Both direct and indirect assays have been used to quantify the levels of ROS. However, all assays of OS in the sperm are relatively expensive and time consuming as compared to a routine semen analysis. Therefore, many clinicians continue to avoid testing their patients for OS and by default offer therapeutic plans with the hope of relieving OS and improving the overall semen quality. The OS test may precisely distinguish between fertile and infertile men as well as clinically diagnose male factor infertility. Moreover, such tests can help identify subgroups of infertile patients suffering from OS that may be treated with antioxidant supplementation (Deepinder *et al*., 2008).

2.21Prevention and Management of Oxidative Stress

There are several innate mechanisms existing in the body to prevent OS from occurring in healthy males. However, in instances where these natural defenses fail to maintain the fine balance between ROS and antioxidants, measures can be taken to alleviate OS, such as lifestyle changes and antioxidant supplementation (both enzymatic and non-enzymatic); Lampiao, 2012.

2.21.1 Prevention of oxidative stress

In healthy males, sperm DNA is protected from OS by two main mechanisms. Firstly, the DNA is tightly coiled and packaged into chromatin such that the genetic material is minimally exposed to attack by ROS (Lampiao, 2012). Secondly, natural antioxidants in the seminal plasma and spermatozoa assist in minimizing the ROS production to normal levels. Some natural antioxidants include enzymes like catalase and SOD as well as non-enzymatic compounds like vitamins C and E and carotenoids. These antioxidants react with and neutralize ROS, assisting in preventing OS onset and preserving the spermatozoa function. Spermatozoa also contain the antioxidants lactoferrin and coenzyme Q10 (Lanzafame *et al*., 2009). A third lesser-mentioned protection mechanism is that of the prostasomes from the prostate. The presence of prostasomes in the seminal plasma results in a decreased ability of neutrophils to produce superoxide radicals (Lanzafame *et al*., 2009).

Maintenance of healthy semenin males require intake of sufficient amount of antioxidants in one's diet to prevent OS from occurring. However, in some patients who suffer from infertility, there may be either an overproduction of ROS or an underproduction of antioxidants, which disrupts the intricate balance and results in OS (Lanzafame *et al*., 2009).

2.21.2 Management of oxidative stress

In the management of OS, the first step to take is to ascertain the underlying cause of the imbalance and treat it (Agarwal *et al*., 2004). For instance, chlamydia infections can be treated with antibiotics and anti-inflammatory medication, while varicocele can be corrected by surgery (Tremellen, 2008). Thereafter, antioxidant treatment may be given to supplement the natural antioxidants and increase the ability of the seminal plasma to combat OS (Agarwal *et al*., 2004).

2.21.2.1 Lifestyle changes as a way of managing oxidative stress

Modernization, affluence, and accompanying stressors in the society have resulted in an increase in negative behaviors, including, but not limited to, smoking, substance abuse, obesity, and an unbalanced diet. All these have been shown to contribute to OS, and minimizing such detrimental behavior is likely to aid in alleviating OS (Tremellen, 2008).

It is also recognized that exposure to heat, pollution, toxins, and heavy metals play a role in the development of OS. In addition, any activity that may cause the scrotum's temperature to increase, such as hot baths, saunas, extended periods of driving, and long and sedentary office hours should be avoided. Lastly, adequate protective equipment and aeration should be ensured at work places to limit exposure to any chemical or vapor that may cause OS. Undertaking these lifestyle changes can contribute to the reduction in ROS production and help correct the redox imbalance causing OS (Tremellen, 2008).

2.22Combating Oxidative Stress with Antioxidants

Antioxidants work by halting the oxidative chain reaction through eliminating, taking up, or reducing the formation of ROS (Bansal and Bilaspuri, 2010). Antioxidants can be divided into two types on the basis of their actions: (i) preventive antioxidants like metal chelators or binding proteins, such as lactoferrin and transferrin, which prevent the formation of ROS; and (ii)

scavenging antioxidants, like vitamins C and E, remove the ROS that are already present (Lampiao, 2012). There have been various studies conducted to elucidate the effectiveness of each individual antioxidant. However, results have been inconclusive as most experiments have small sample size, differ in dosage and duration of therapy, and lack controls (Zini and Al-Hathal, 2011).

Antioxidants work cooperatively, and thus, it is extremely challenging to measure the effect of any single one alone. This is supported in theory because a suitable combination of antioxidants with their different profiles will neutralize any ROS in its vicinity, hence resulting in an additive effect on the decrease in the total OS level of the body (Gharagozloo and Aitken, 2011).

2.23OxidativeStress and Contraception

Lipid peroxidation induced by H_2O_2 not only disrupts sperm motility, but, also impairs all the sperm functions which are dependent on the integrity of plasma membrane, including sperm-oocyte fusion and ability to undergo acrosomal exocytose. Such findings have raised the possibility that hydrogen peroxide or reagents producing them on contact with spermatozoa might be an effective way of contraception (Amrit and Bilaspuri, 2011).

2.24Reduction of oxidative stress strategies in sperm cells

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility (Bansal and Bilaspuri, 2008).

Antioxidants are the agents, which break the oxidative chain reaction, thereby, reducing oxidative stress. Vitamin E (antioxidant) may directly quench the free radicals such as peroxyl and alkoxyl (ROO•) radicals generated during ferrous ascorbate-induced LPO.Thus, it is proposed as a major chain breaking antioxidant (Bansal and Bilaspuri, 2009). Antioxidants, in general, are the compounds which dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Mn^{2+} enhances sperm motility, viability, capacitation and acrosome reaction by decreasing the oxidative stress in semen. Extracellular addition of Mn^{2+} ions also enhances the level of cAMP by stimulating Ca^{2+} or Mg^{2+} ATPase which leads to activation of calcium channel opening, thereby depositing more Ca^{2+}_{i} (Bansal and Bilaspuri, 2008).Thus, Mn^{2+} promotes acrosome reaction.

Thiol groups also play an important role in detoxification and antioxidation of ROS, besides maintaining the intracellular redox status. These groups serve as defense mechanisms of sperm cells to fight against oxidative stress (Bansal and Bilaspuri, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Animals

Twenty-one adult male Wistar ratsbetween the ages of 8-12 weeks, weighing from 160 -180 g were used for this research. The animals were obtained from the Department of Human Physiology, Ahmadu Bello University, Zaria. The rats were housed in the Animal House of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University,Zaria, Nigeria. The animals were kept in vivarium plasticcages (Plate V) under normal environmental temperature and fed with standard pellet diet and water given *ad libitum*except during exposure time. The rats were grouped into three groups of seven rats each and the exposure groups were exposed to *Shisha*smoke in a nose-only exposure chamber. All the animals wereweighed once weekly during the 13 weeks duration of the study to determine weight changes.



Plate V: Standard vivarium plastic cage for housing the rats during the experimental period

3.1.1 Determination of sample size

The number of animals for laboratory experiment that would yield good power of statistics is six; from resource equation method for sample size, E= (6x3)-3, E=15, E lies between 10-20 (Jaykaran and Kantharia, 2013). To avoid attrition and unforeseen death, 2 rats were added to each group. 6- number of rats in each group that would be statistically significant, 3- represent number of groups (one control group and two experimental groups).

3.2 Chemicals and Drugs

*Shisha*grapeflavour packet, ketamine sodium, diazepamand other chemicals of analytical grade were purchased from reputable chemical and pharmaceutical stores within Zaria.

3.3Experimental Protocol

3.3.1 Animal Grouping

Group I (n= 7 rats)

Group I served as control. The animals werekept in the nose-only exposure chamber for 30 minuteseach day without *Shisha*smoke administration for thirteen weeks.

Group II (n= 7 rats)

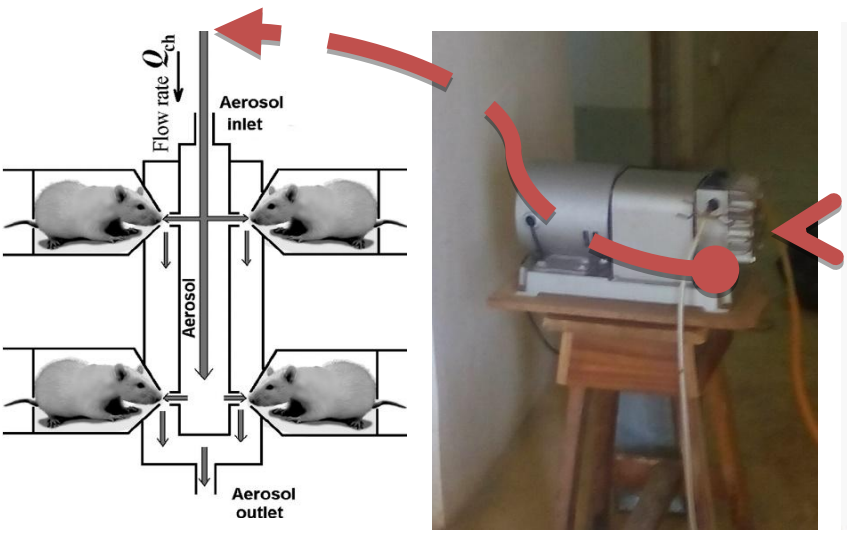
Group II wereexposed to *Shisha*smoke in the nose-only exposure chamber with water in the water-pipe smoke jar for 30 minutes daily for thirteen weeks.

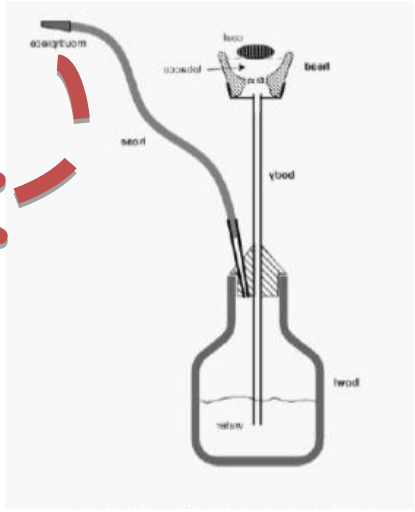
Group III (n= 7 rats)

Group III were exposed to *Shisha*smoke in the nose-only exposure chamber without water in the water jar for 30 minutes daily for thirteen weeks.

3.3.2*Shisha*Smoke Administration

Smoke produced by the *Shisha*apparatus was driven into a nose-only chamber containing the experimental rats through a vacuum compressorat a pressure of 300 KPa, as shown in Figure 3.1and Plate VIbelow for 7 seconds. Thereafter, fresh air was allowed into the chamber through the air-inlet for 53 seconds as described byForoutanjazi *et al*.(2014). This was repeated for a duration of 30 minutes daily for a period of 13 weeks.





Nose-onlyexposure chamber Vacuum compressor Scheme of *Shisha*apparatus

Figure 3.1: Scheme forprocess of *Shisha*smoke production and administration into nose-only exposure chamber



Plate VI: Photograph of the apparatuses forprocessing *Shisha*smoke production and its administration into nose-only exposure chamber during the experimental period

3.4 Sample Collection

At the end of the experiment, five animals from each group were anaesthetized with 0.4 mL/100g of combined ketamine and diazepam anaesthesia (Lijuan *et al*., 2016), 24 h after last day of administration. Blood samples were collected by cardiac puncture and placed in plain bottle (EDTA-free bottle) for serum collection using suction pipette after spontaneous sedimentation. Thereafter, the animals were dissected and the testeswere harvested. The epididymis was resected from the right testis and carefully drained of its semen. The following parameters were determined from the epididymal semen: sperm count, motility, and morphologyas described by WHO (2010).

The left testis of each rat labeled according to the groups were removed from the scrotum. The testis was homogenised in 10 ml phosphate-buffered solution (pH 7.4) using a pestle and mortar. The Homogenates were turned into test tubes labeled according the group and number of each rat. The resultant homogenates were centrifuged at 2,000 revolutions per minute for 10 minutes at 4°C. The supernatant were collected and used for MDA assays.

3.5 Determination of SerumTestosterone Level

Serum testosterone concentration was determined based on enzyme immunoassay competitive binding principle. Testosterone present in the sample competed with enzyme-labeled testosterone for binding with anti-testosterone antibody immobilised on the microwell surface. The amount of conjugate that bound to the micro-well surface wasdecreased in proportion to the concentration

of testosterone in the sample. The testosterone concentration in the sample and the control were determined from a standard curve using method of Wilkle and Utley(1987) as follows:

(i) The average absorbance value (A. 450) for each reference standard, control and test sample were calculated.

(ii) A standard curve was prepared by plotting the average absorbance (A.450) versus the corresponding concentration of the standards on a log graph paper.

(iii)The absorbance (A. 450) value for each test sample wasused to determine the corresponding concentration of testosterone in ng/ml from the standard curve (Wilkle and Utley, 1987).

3.6Analysis of Sperm Parameters

The testes from each rat were carefully exposed and removed. They weretrimmed free of the epididymal and adjoining tissues. From each separated epididymis, the caudal part was removed and placed in a beaker containing 1ml physiological saline solution. Each section was quickly macerated with a pair of sharp scissors and was left for a few minutes to liberate its spermatozoa into the saline solution (Saleem *et al.*, 2013). Subsequently, the following parameters were determined.

3.6.1 Spermmotility

After sacrificing the animals, the caudae epididymes were removed and placed in a Petri-plate containing 1 ml of physiological saline solution (PSS) at room temperature. The epididymis were homogenised to allow the sperms to swim out. Sample of the preparation was placed on a glass slide and covered with cover-slip. The motility of epididymal sperm was evaluated microscopically within 2–4 min of their isolation from the cauda epididymis and the data

expressed as percentages of progressively motile, none-progressively motile and non-motile spermatozoa. The percentage of motility was evaluated visually at x40 magnification. Motility estimations were performed from 3 different fields in each sample (Sonmez *et al.*,2007).

3.6.2 Sperm count

The spermatozoa were counted by haemocytometer using the improved Neubauer ($0.0025mm^2$, Marienfeld, Germany) chamber. The total epididymal sperm per ml was calculated as follows(Saleem *et al*, 2013):

[Total epididymal sperm /ml = *(Average number of sperm per chamber) x* 10^3 *x (Dilution Factor)]*.

3.6.3 Sperm morphology assessment by World Health Organisation criteria

The Epididymis were minced in 1 ml physiological saline solution (PSS) to make a suspension. Sperm morphology was determined by the method of WHO (1992). Following liquefaction, 10 µL of semen were spread onto a glass slide and allowed to air-dry at room temperature. The smears were stained with Giemsa stain and sperm morphology were assessed according to WHO criteria. 200 cells *per* smear were counted using brightfield illumination at final magnification of 1000X and oil immersion. According to WHO criteria, a morphologically normal spermatozoon has an oval head and an acrosome covering 40%–70% of the head area. A normal spermatozoon has no neck, midpiece, tail abnormalities nor cytoplasmic droplets larger than 50% of the sperm head.The abnormal spermatozoa were classified into two categories: (i) spermatozoa with

defective heads (amorphous, hook less, banana shaped, double head, microcephaly and cephalocaudal junction defects), and (ii) spermatozoa with defective tails (two-tailed, coiled/bent tails); Vijay *et al.*, 2013.

3.7 Determination of Sperm DNA Fragmentation

The assessment of DNA damage was measured using an improved version of the sperm chromatin dispersion test (Halosperm kit; Halotech DNA S.L., Madrid, Spain). Samples were prepared for analysis according to the protocol described by Fernandez *et al*. (2003).

Exactly 60 μL of a prepared semen with phosphate-buffered solution (pH 6.88) was added into a thawed agarose solution in a tube at room temperature for five minutes. 15 μL of the aliquot was dropped on a glass slide and covered gently a 22 x 22 mm^2cover slip and kept inside DNA warmer at 8^0C for 8 minutes. A staining step was conducted to prepare the slides for counting processes. The samples were stained with Diff-Quik solution, each slide was immersed in 8 mL Diff-Quik solution I (eosinophilic) and Diff-Quik solution II (basophilic) for 20 minutes each in a DNA immersion staining tray.Thereafter, the slide was immersed in 10 mL of distilled water. The slide was placed into a tray and covered with 70% ethanol for two minutes, followed by 90% ethanol for two minutes, and, finally, 100% ethanol for two minutes. The slide was left to dry at room temperature and stained by Wright stain solution.

Laboratory scored for 400 spermatozoa for each slide under the ×100 objective of the bright-field microscope according to the patterns established by Fernandez *et al*. (2005). Conserved spermatozoa with integrity of intact DNA, a peripheral halo of DNA loops around a central core

was observed. Spermatozoa with fragmented DNA produced very small halos or no halos at all.The classification of DNA fragmentation index (%DFI: i.e., % sperm cells containing damaged DNA) wascalculated as follows(Osman*et al*., 2014):

(i) ≤ 15% DFI = Excellent to good sperm DNA integrity

(ii) > 15 to < 25% DFI = Good to fair sperm DNA integrity

(iii) > 25 to < 50% DFI = Fair to poor sperm DNA integrity

(iv) ≥ 50% DFI = Very poor sperm DNA integrity

3.8 Determination of TesticularMalondialdehyde Concentration

Testicular hydroperoxide level wasevaluated using an analytical system. The test is a colorimetric test that takes advantage of the ability of hydroperoxide to generate free radicals after reacting with transitional metals. When buffered chromogenic substance was added to the reaction, a colored complex appeared. This complex was measured spectrophotometrically. Lipid peroxidation level in the testis were measured by method of Ohkawa*et al*. (1979) as thiobarbituric acid reactive substances (TBARS). Testis was homogenised in ice cold 0.15 M KCl (10%) and the concentration of TBARS was expressed as μmol of MDA per mg tissue using 1,1,3,3-tetramethoxypropane as standard. The absorbance was read at 532 nm using spectrophotometer.

3.9 Determination of serum prostate specific antigen

This was determined using the method of Barbosa *et al*. (2014). Theprinciple of the test is as follows: TheELISA Kit was based on two-site sandwich ELISA method. Samples and diluent are added to the wells coated with monoclonal antibody against PSA. The PSA in the serum bound to the antibody coated on the well. Unbound proteins were washed off. Horseradish peroxidase (HRP)labeled anti-PSA antibody was then added to the mixture. Unbound protein and HRP conjugate are washed off. Upon the addition of the substrate, the intensity of colour was proportional to the concentration of PSA in the samples. A standard curve was prepared by plotting color intensity and the concentration of the PSA.

3.10 Phytochemical Screening of Grape-Flavoured *Shisha* Tobacco

Phytochemical screening of the grape-flavoured *Shisha*was conducted in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The method of Trease and Evans (1983) for phytochemical screening was adopted.

3.10.1 Test for carbohydrates

Molisch's test

A pinch of the *Shisha*leaves was placed in a clean test tube then a few drops of Molisch's reagent were added. Thereafter, a small quantity of concentrated sulphuric acid was allowed to run down the side of the test tube. A purple colour at the interphase was formed indicating the presence of carbohydrates (Trease and Evans, 1983; Harborne, 1998).

3.10.2 Test for cardiac glycosides

Kella-Killani's test

A pinch of the *Shisha*leaveswas placed in a clean test tube; the *Shisha*portion was dissolved in glacial acetic acid. A few drops of ferric chloride were added. Then, 1 ml of concentrated sulphuric acid was run down the side of the test tube. A purple ring colour was seen at the interface, indicating the presence of cardiac glycosides (Trease and Evans, 1983).

3.10.3 Test for anthraquinone derivatives

Borntrager's test

A small pinch of the *Shisha* leaves was put in a clean test tube soaked in 10 ml of benzene and filtered. Then 5 ml of 10% ammonia solution was added to the filtrate and stirred. There was no pink-red or violet colour formation, indicating the absence of anthraquinone derivatives in the shisha (Trease and Evans, 1983; Harborne, 1998).

3.10.4 Test for saponins

Frothing test

A small quantity of *Shisha* leaves was placed in a clean test tube and dissolved in 10ml of distilled water. The test tube was shaken vigorously for 30 minutes and allowed to stand for 30 minutes. A honey comb was formed for more than 30 minutes indicating the presence of saponins in the shisha (Trease and Evans, 1983; Harborne, 1998).

3.10.5 Test for steroids and triterpenes

Liberman-Burchard's test

A pinch of the*Shisha* leaves was put in a test tube and an equal volume of acetic anhydrite was added to the shisha. Then 1 ml of concentrated sulphuric acid was allowed to flow down the side of the test tube. A purple colour was observed immediately indicating the present of triterpenes (Trease and Evans, 1983; Harborne, 1998).

3.10.6 Test for flavonoids

Shinoda's Test

A small pinch of the*Shisha*was placed in a test tube and mixed with 50% methanol. It was heated in a water bath. Metallic magnesium was added. Then four drops of concentrated hydrochloric acid were added. A red colour was formed indicating the presence of flavonoids (Trease and Evans, 1983; Harborne, 1998).

3.10.7 Test for tannins

Lead sub-acetate test

A smallpinch of the *Shisha* leaves was placed in a test tube and then 3 drops of lead acetate solution was added. A coloured precipitate was formed indicating the presence of tannins (Trease and Evans, 1983).

3.10.8 Test for alkaloids

Dragendroff's test

Few drops of Dragendroff's reagent were added to the *Shisha* leaves in a test tube. Precipitate was formed, indicating the presence of alkaloids (Trease and Evans, 1983).

3.11 Histological Study

The testicles and prostate glands specimens were placed in 10% formol-saline and processed by embedding in paraffin (Rosaura*et al*., 2010). Sections (5 µm) of testis and prostate glandwere stained with hemaetoxylin and eosin dyes and examined under a light microscope. The histological study was carried out to determine the presence and extent of tissue damage in the experimental groups as compared to the control rats.

3.12Data Analyses

Dataobtained from the study were expressed as mean ± SEM. The differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Values of $P< 0.05$ wereconsidered statistically significant. Statistical Package for Social Sciences (SPSS) version 20 was used for the analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical analysis of the grape flavoured *Shisha*tobacco

Phytochemical analysis of the grape flavoured *Shisha*tobacco revealed the presence of the following components: alkaloids, tannins, carbohydrates, cardiac glycosides, triterpenes, and flavonoids. However, a negative reaction was obtained for anthraquinones, saponins and steroids (Table 4.1).

Table 4.1: Phytochemical constituents of the grape flavoured *Shisha*

Constituents	**Inference**
Alkaloids+	
Tannins+	
Carbohydrates+	
Cardiac glycosides +	
Steroids	_
Triterpenes	+
Anthraquinones_	
Flavonoids+	
Saponins	+

+= positive (present)

- = negative (absent)

4.2 Effect of *Shisha* Smoke Exposureon Testicular MalondialdehydeConcentration

Figure 4.1 shows the effect of *Shisha*smoke exposure on testicular malondialdehyde (MDA) levels. Malondialdehyde level was significantly ($P< 0.05$) higher in bonged *Shisha*smoke (water-filtered smoke [BSS]) group (1.61± 0.08 μmol/mg protein) than in any other group (control: 0.44 ± 0.08 μmol/mg protein; un-bonged shisha smoke group: 1.26 ± 0.04 μmol/mg protein).

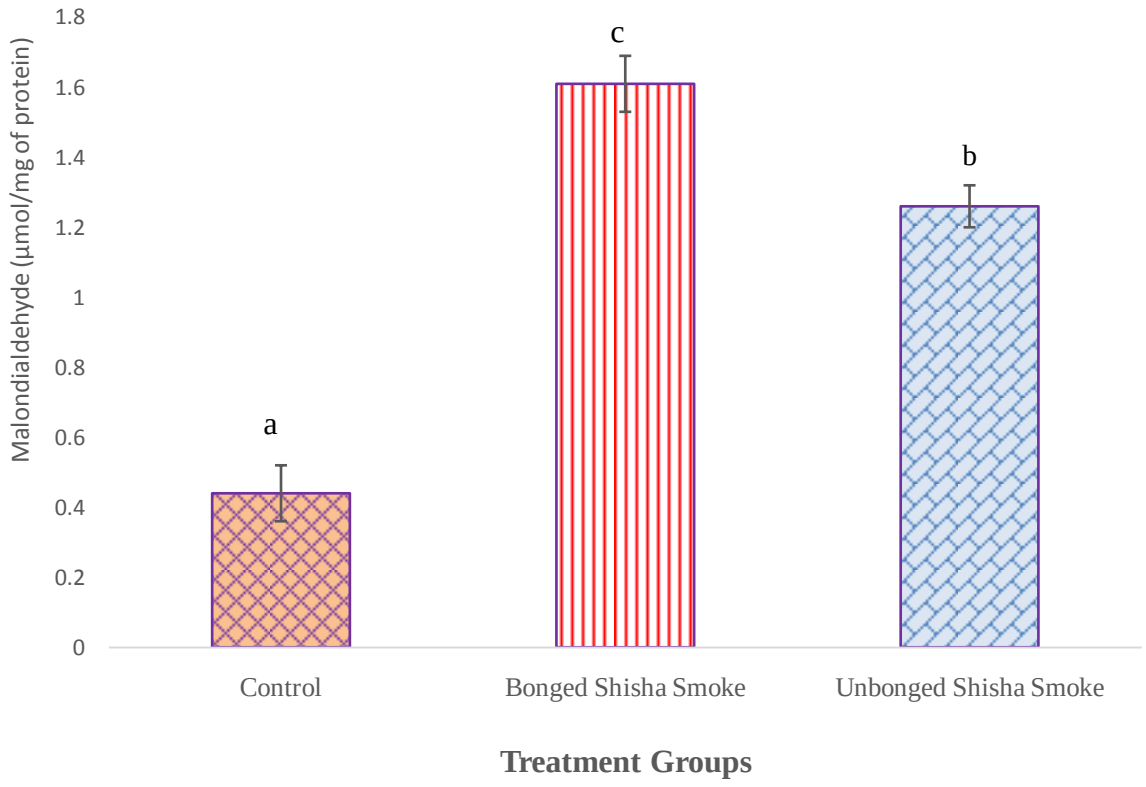


Figure 4.1: Effects of *Shisha*smoke exposure on testicular malondialdehyde level in adult male Wistar rats.
[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

4.3 Effect of *Shisha*Smoke Exposureon Prostate Specific Antigen

Figure 4.2 shows the effect of *Shisha* smoke exposure on serum prostate specific antigen. The PSA was significantly ($P < 0.05$) higher in bonged *Shisha*smoke group (3.04 ± 0.03 ng/ml) than the other groups (control: 1.30 ± 0.05 ng/ml; un-bonged *Shisha*smoke: 1.94 ± 0.06 ng/ml).

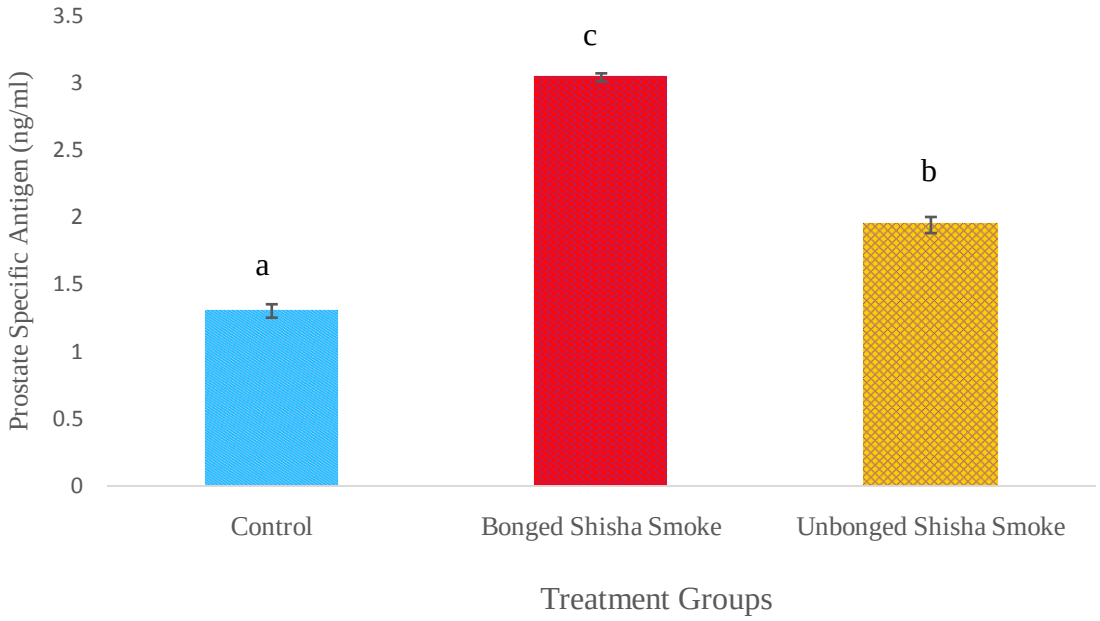
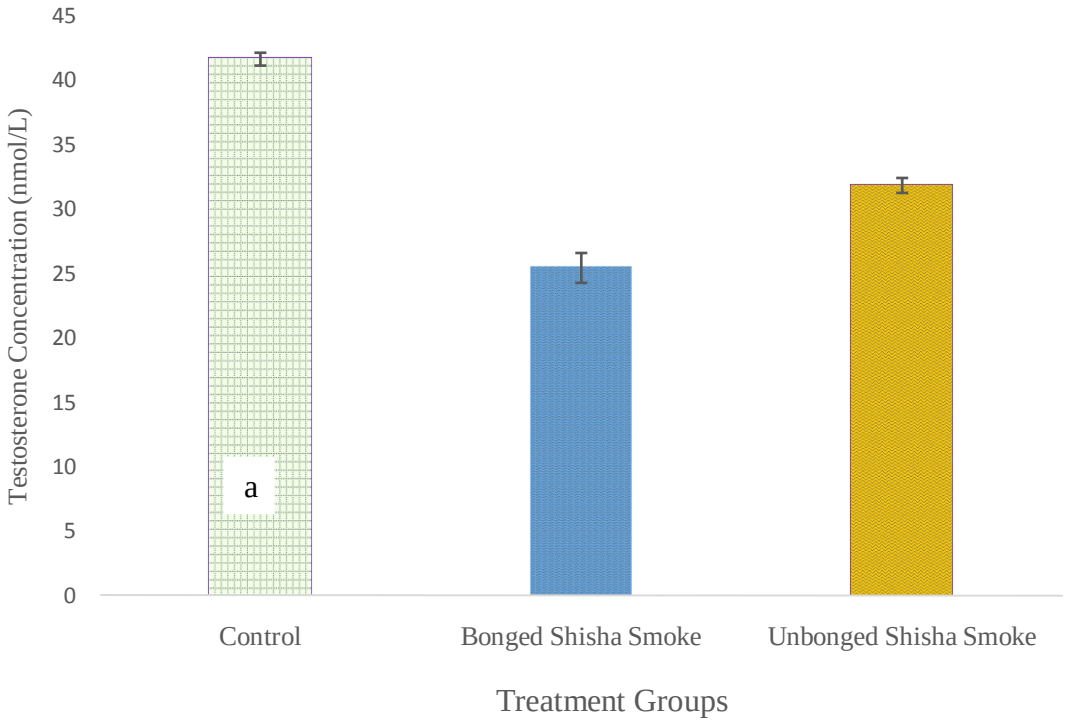


Figure 4.2: Effects of *Shisha* smoke exposure on prostate specific antigen in adult male Wistar Rat.

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

4.4 Effect of *Shisha* Smoke Exposure on Serum Testosterone Concentration

Figure 4.3 shows the effect of *Shisha* smoke exposure on serum testosterone concentration. Testosterone level was significantly ($P < 0.05$) lower in the bonged shisha smoke group (25.4 ± 1.16nmol/L) when compared to control (41.6 ± 0.50nmol/L)andun-bonged shisha smoke(31.8 ± 0.58nmol/L).



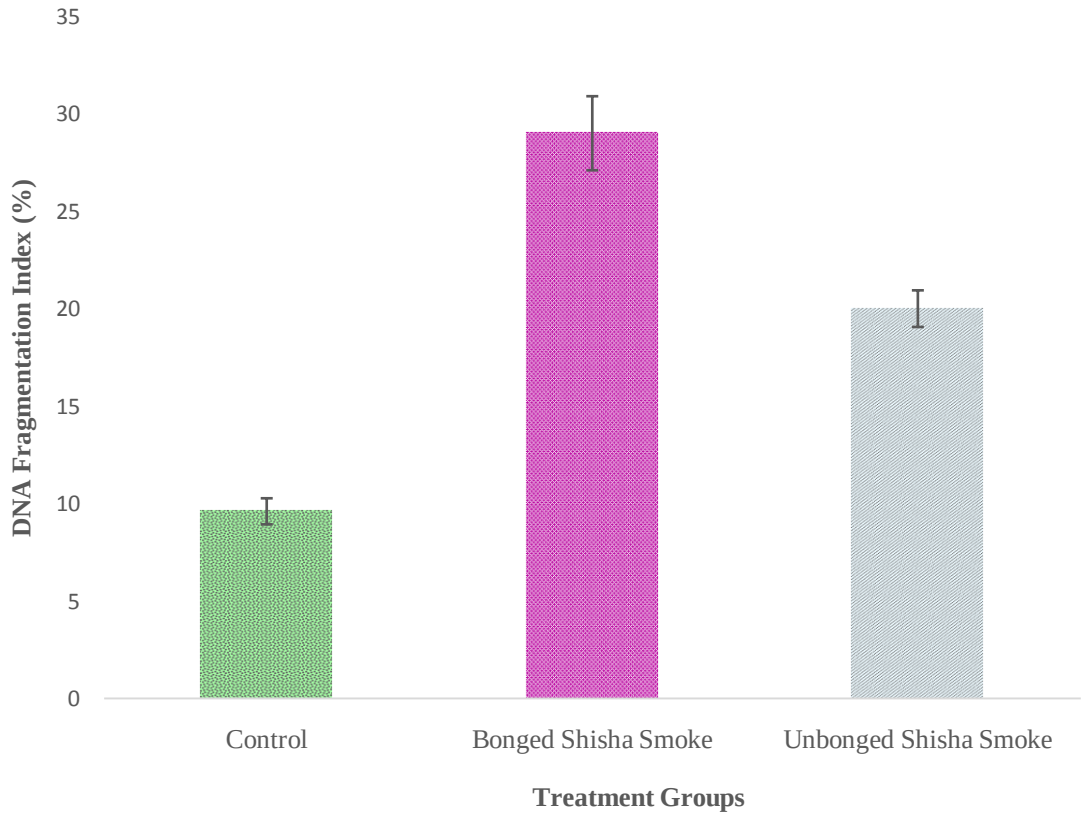
b

c

Figure 4.3: Effects of *Shisha* smoke exposure on serum testosterone concentration in adult male Wistar rats.
$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.5 Effect of *Shisha* Smoke Exposure on Sperm DNA Fragmentation Index

Figure 4.4 shows the effect of *Shisha* smoke exposure on sperm DNA fragmentation index. The SDFI was significantly ($P < 0.05$) higher in the bonged *Shisha*smoke group (29.0 ± 1.90%) than in control group(9.60 ± 0.67%) and un-bonged *Shisha*smoke(20.0 ± 0.94%), respectively.



b

a

Figure 4.4: Effects of Shisha smoke exposure on sperm DNA fragmentation index in adult male Wistar rats.
$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

4.6 Effect of *Shisha* Smoke Exposure on Epididymal Sperm Count

Figure 4.5 shows the effect of *Shisha* smoke exposure on epididymal sperm count. Epididymal sperm count was significantly ($P < 0.05$) lower in bonged *Shisha*smoke group ($3.30 \pm 0.13\ 10^7$

cell/ml) than in other groups (control: 5.62 ± 0.15 10^7 cell/ml; un-bonged shisha smoke: 4.08 ± 0.08 10^7 cell/ml, respectively).

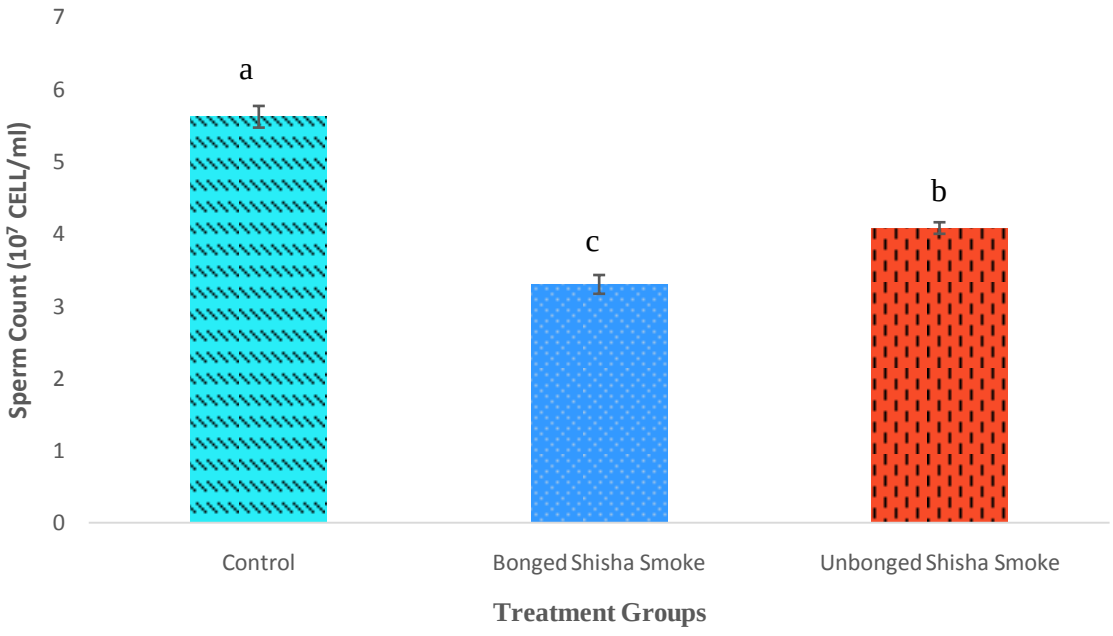


Figure 4.5: Effects of *Shisha* smoke exposure on epididymal sperm countin adult male Wistar

rats.

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

4.7 Effect of *Shisha* Smoke Exposure on Percentage Sperm Motility

Figure 4.6 shows the effect of *Shisha* smoke exposure on percentage sperm motility. Percentage progressive sperm motility was significantly ($P < 0.05$) lower in bonged shisha smoke group (32.2 ± 1.01%) than in the other groups (control: 48.0 ± 2.5%; un-bonged shisha smoke group: 40.0 ± 1.7).Percentage non-progressive motility and non-motile sperm cells were significantly ($P < 0.05$) higher in the bonged *Shisha*smoke group (47.0 ± 2.3% and 21.4 ± 2.50% for non-progressive motility and immotile sperm cells, respectively) than the other groups (control: 37.0 ± 3.00% and 15.0 ± 2.23% for non-progressive motility and immotile sperm cells, respectively);and (Un-bonged shisha smoke: 43.6 ± 0.97% and 18.0 ± 1.4% for non-progressive motility and immotile sperm cells, respectively).

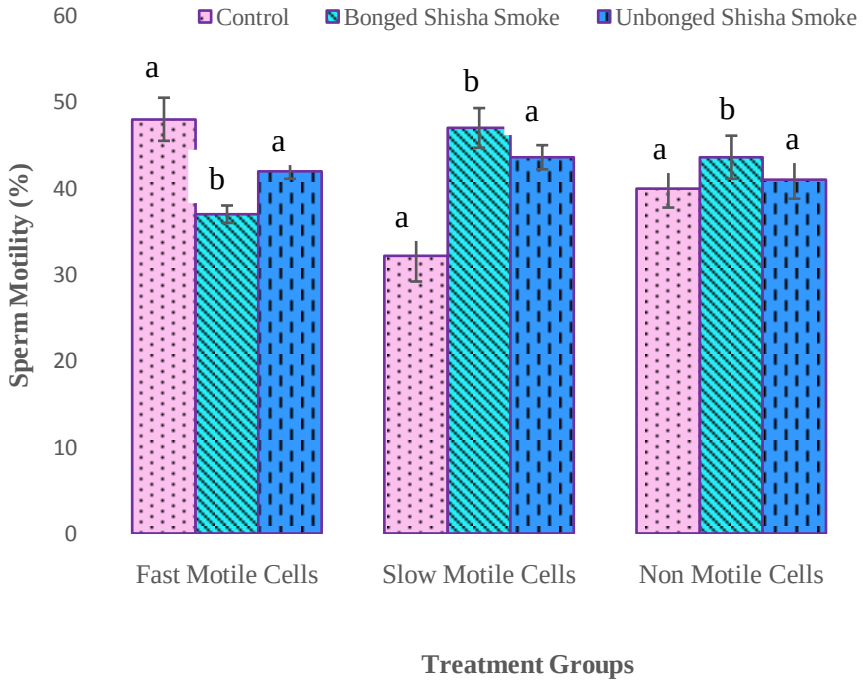


Figure 4.6: Effects of *Shisha* smoke exposure on sperm motility in adult male Wistar rats
[a, b] = Means with different superscript letters are significantly ($P < 0.05$) different

4.8 Effect of *Shisha* Smoke Exposure on Sperm Morphology

Table 4.2 shows the effect of *Shisha*smokeexposure on sperm morphology. There wasno significant difference in spermhead abnormalities between control and exposed groups. However, a significant difference wasobserved in normal sperm morphology and tail abnormalities between the experimental groups and control. Percentage normal sperm morphology count was significantly ($P < 0.05$)lower in bonged (184.0 ± 0.86) and unbonged (188 ± 2.24)*Shisha*smokegroups than in control rats (191.0 ± 1.52).

Table 4.2: Effects of Shisha smoke exposure on sperm morphology in adult male Wistar rats

Sperm Parameters	Control	Bonged Shisha Smoke	Unbonged Shisha Smoke
Normal Morphology	191.0 ± 1.52[a]	184.0± 0.86[b]	188 ± 2.24[a]
Abnormal Head	0.60 ± 0.04[a]	3.00 ± 0.14[b]	1.60 ± 0.20[b]
Abnormal Tail	8.20 ± 1.10[a]	14.8 ± 0.86[b]	7.6 ± 0.50[a]
Total number of abnormal sperm	44 ± 1.14	89 ± 1.01	56 ± 0.70
Total number of normal sperm	956± 1.52	921 ± 0.86	944 ± 2.24
% of abnormal cells	**0.044**	**0.088**	**0.056**

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

4.9Correlation matrix between *Shisha* Smoke Exposure, Serum Testosterone, Testicular Biomarkers of Oxidative Stress and Sperm Parameters

Table 4.3 shows the correlation among the MDA and testosterone and sperm DNA fragmentation index and sperm count parameters. A significant ($r = -0.904$; $P < 0.01$)negative correlation was observed between MDA and testosterone concentration, and sperm count; while a significant ($r = 0.881$; $P < 0.01$) positive correlation was observed between MDA and sperm DNA fragmentation index; serum testosterone concentration and sperm count ($r = 0.939$; $P < 0.01$); and significant ($r = -0.922$; $P < 0.01$) negative correlation between sperm DNA fragmentation index and sperm count.

Table 4.3: The correlation matrix between testicularMalondialdehyde, serum testosterone concentration, sperm DNA fragmentation index and sperm count in adult male Wistar rats exposed to *Shisha*smoke

	Malondialdehyde	**Testosterone**	**SpermDNA fragmentation Index**	**Sperm Count**
Malondialdehyde	1	-0.904**	0.881**	-0.952**
Testosterone	-0.904**	1	-0.944**	0.939**
Sperm DNA fragmentation index	0.881**	- 0.944**	1	-0.922**
Sperm Count	-0.952**	0.939**	-0.922**	1

** = Means with different asterisk are significantly (P < 0.01) different

4.10 Effect of *Shisha* Smoke Exposure on Testicular Histology

4.10.1 Control group

Testicular histology reveals normal histo-architextures of testis in the group not exposed to *Shisha*smoke (Plate VII).

4.10.2 Bonged *Shisha*smoke group

Testicular histology in the group exposed to *Shisha*smoke with water in the jar revealed moderate to intense necrosis of spermatogenic cells in some of the testicular photomicrographs (Plates VIII-XII).

4.10.3 Unbonged *Shisha*smoke group

Testicular histology in the group exposed to *Shisha*smoke without water in the jar revealed moderate necrosis of spermatogenic cells with hardening of seminiferous tubules in some of the testicular photomicrograph (Plates XIII-XVII).

4.11 Effect of *Shisha* Smoke Exposure on Prostate gland Histology

4.11.1 Control group

Prostate gland histology reveals normal histo-architexture of prostate gland in the group not exposed to *Shisha*smoke (Plates XVIII).

4.11.2 Bonged *Shisha*smoke group

Prostate gland histology reveals moderate necrosis of the prostate gland epithelia in the group exposed to *Shisha*smoke with water in the jar (Plates XIX-XXI).

4.11.3 Unbonged *Shisha*smoke group

Prostatehistology in the group exposed to *Shisha*smoke without water in the jar revealed moderate necrosis of prostate gland epithelial cells with hardening of seminiferous tubules in some of the testicular photomicrograph (Plates XXII-XXIV).

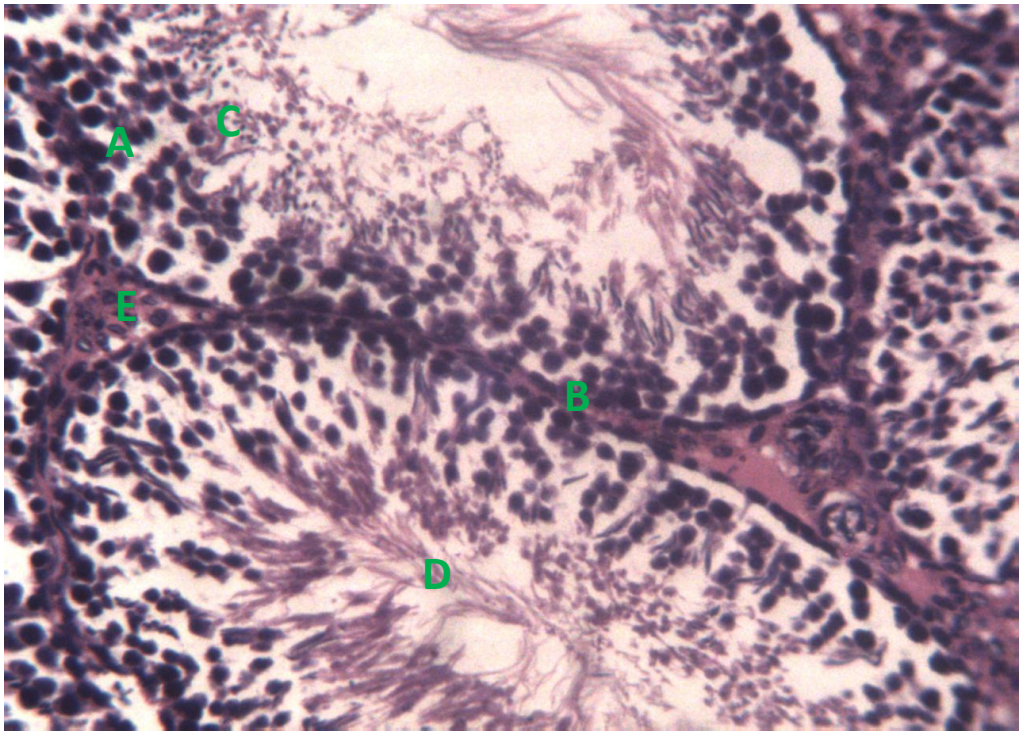


Plate VII: Photomicrograph of the testis of an adult male Wistar ratunexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X250.

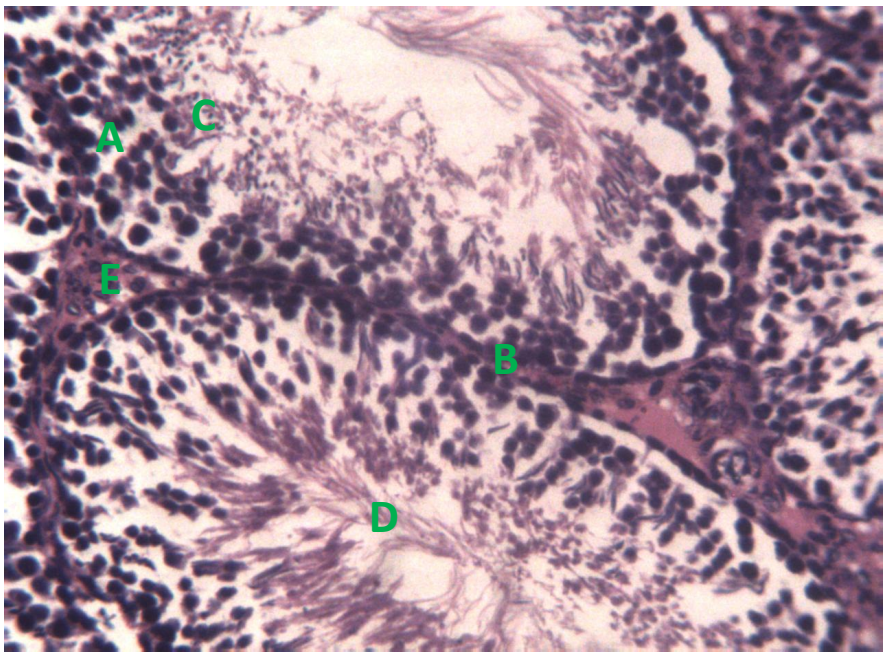


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

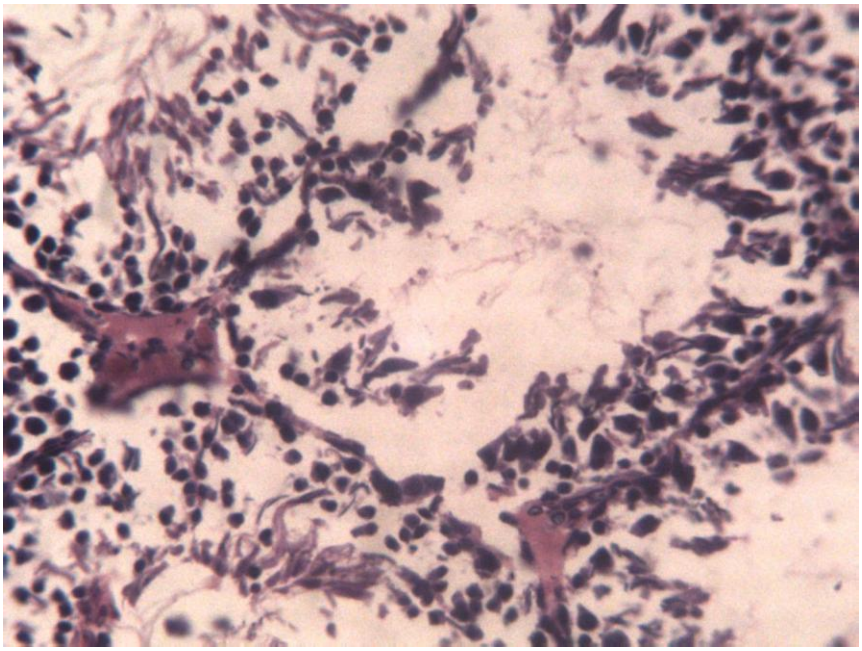


Plate VIII: Photomicrograph of the testis of an adult male Wistar ratexposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).

Note the disorganized architecture of the seminiferous tubules. H & E X250.

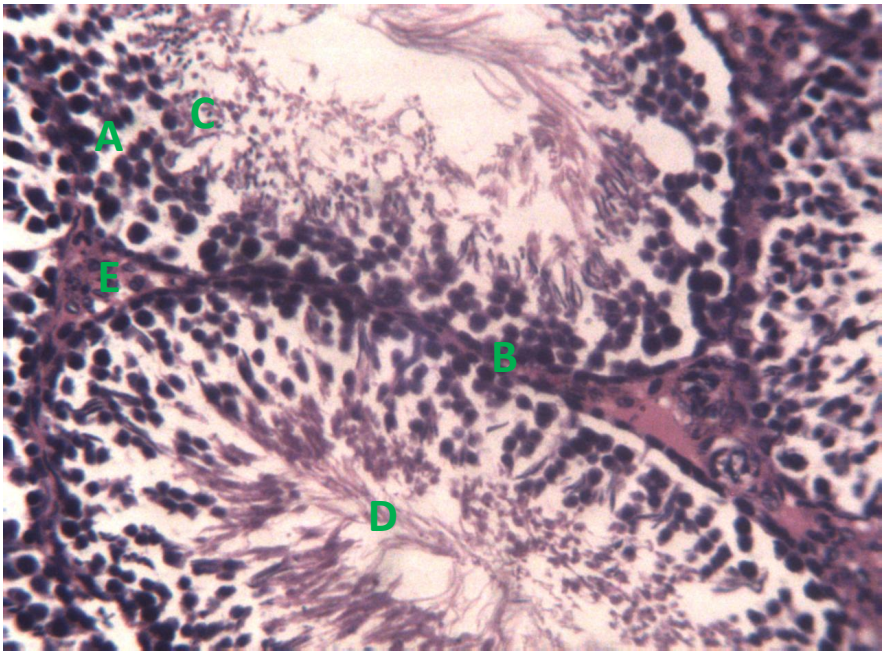


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

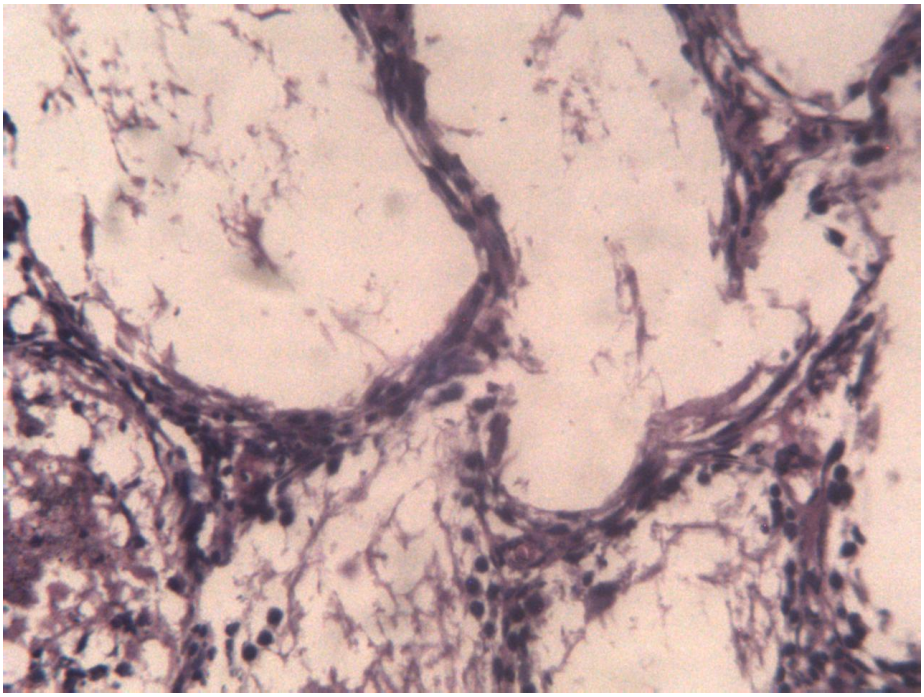


Plate IX: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).

Note intense necrosis of the spermatogonia, spermatocyte, the spermatozoa layers and the basement membrane. H & E X 250.

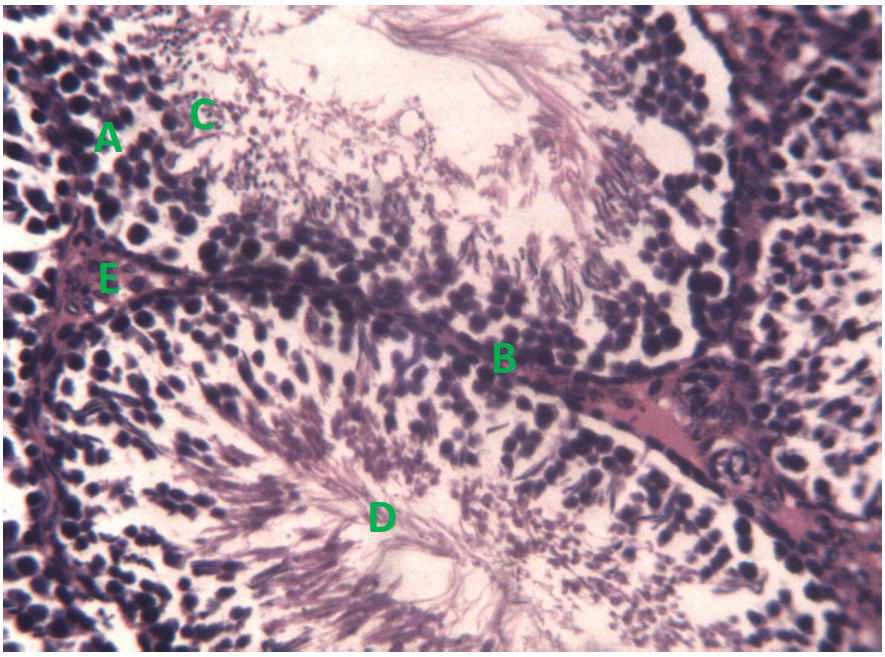


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

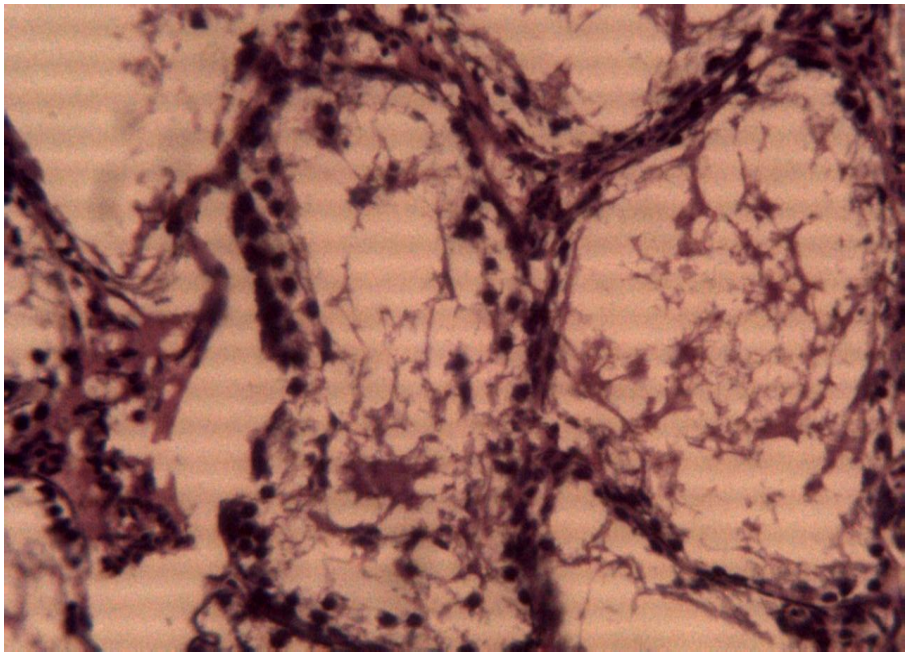


Plate X: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).

Note the degeneration of the basement membrane, the spermatogonia and the spermatocytes and the spermatozoa layers. H & E X250.

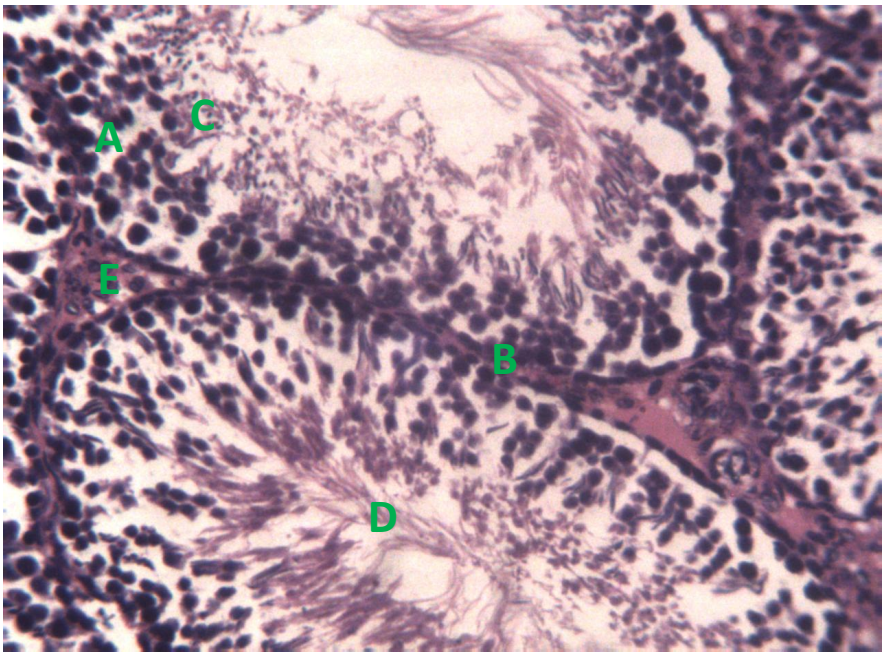


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

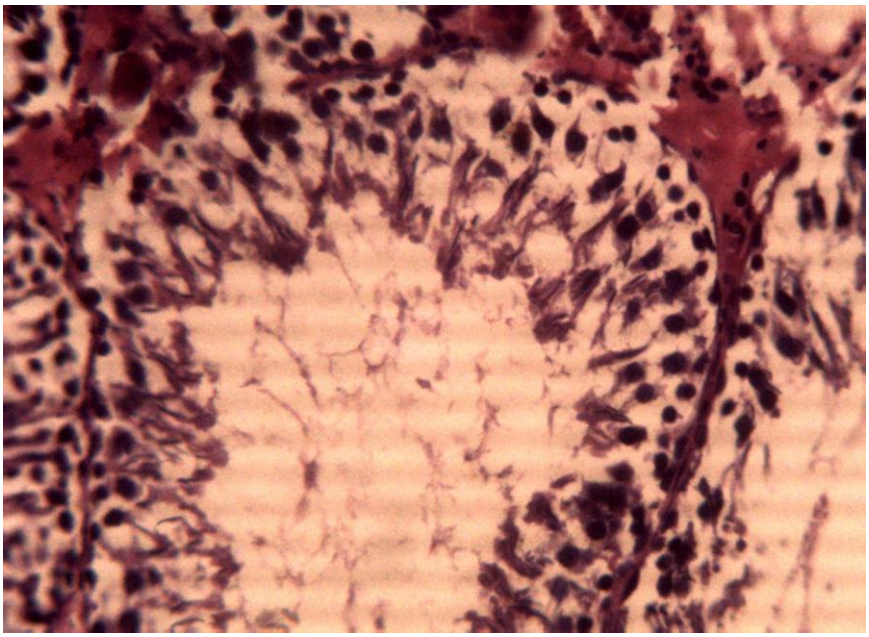


Plate XI: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).
Note the moderate generation of the developing sperm cell layers and the spermatozoa layer the central lumen. H & E X250.

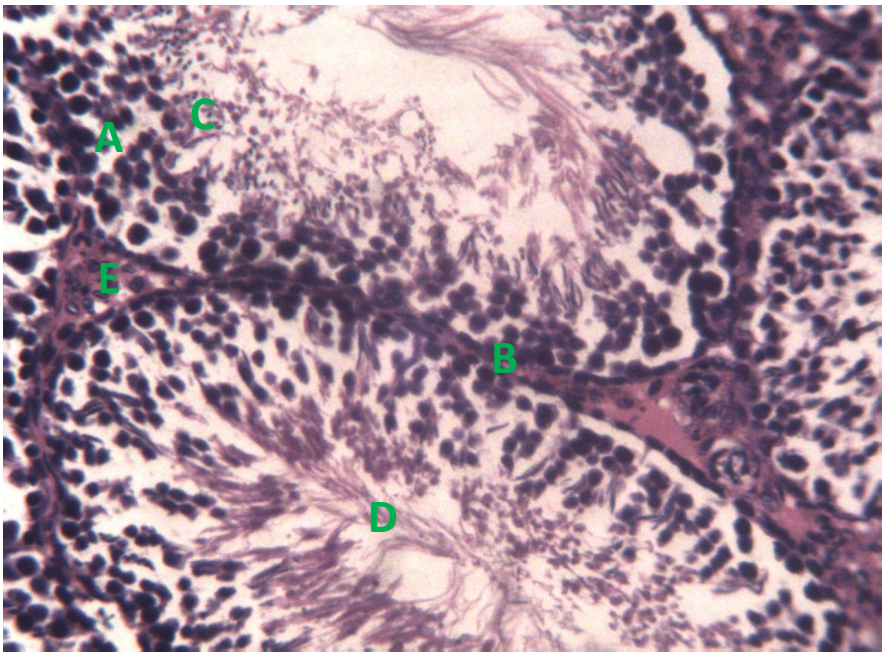


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

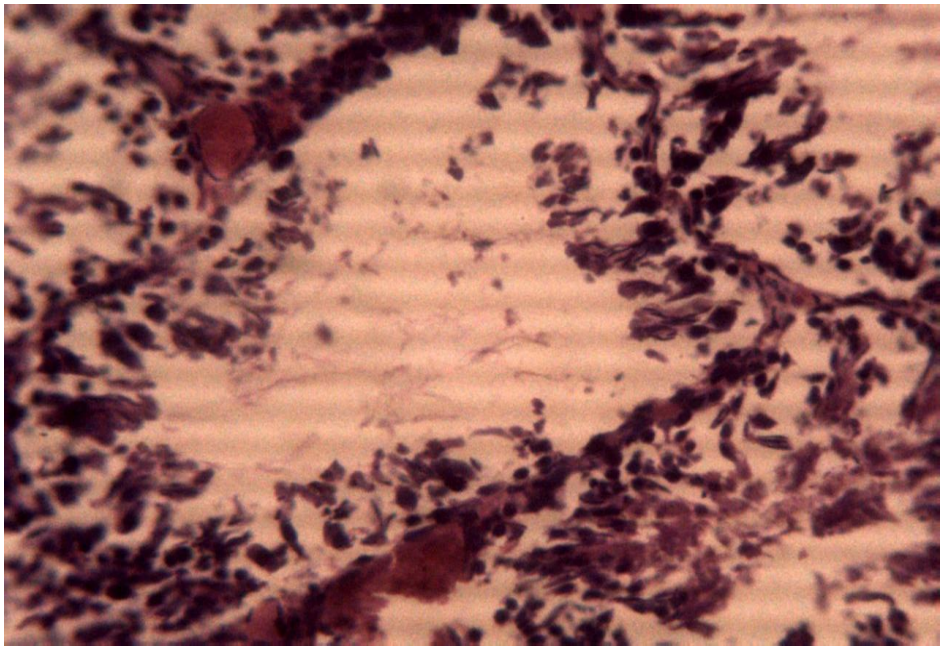


Plate XII: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group). Note the distortion in layers of the seminiferous tubules. H & E X250.

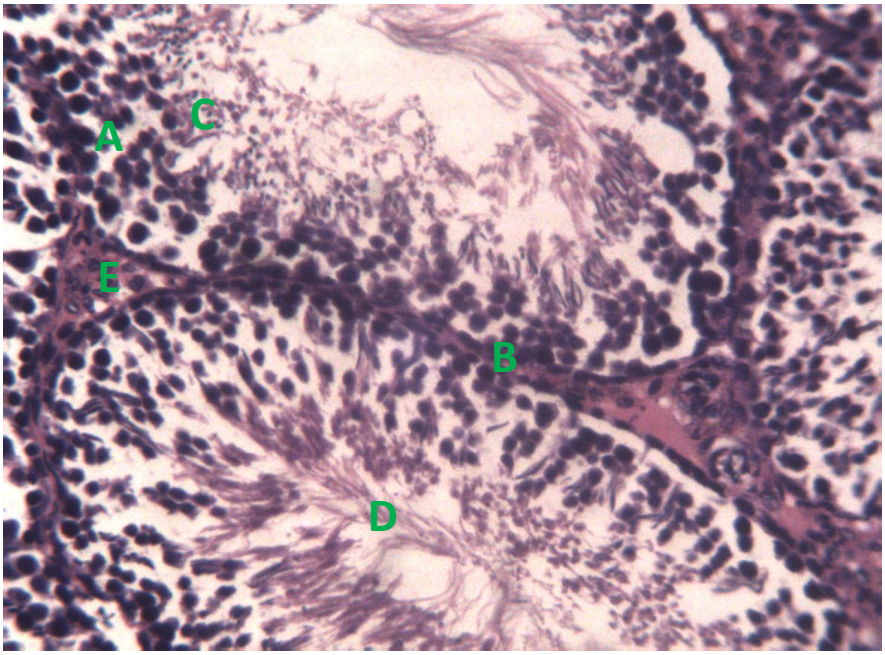


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

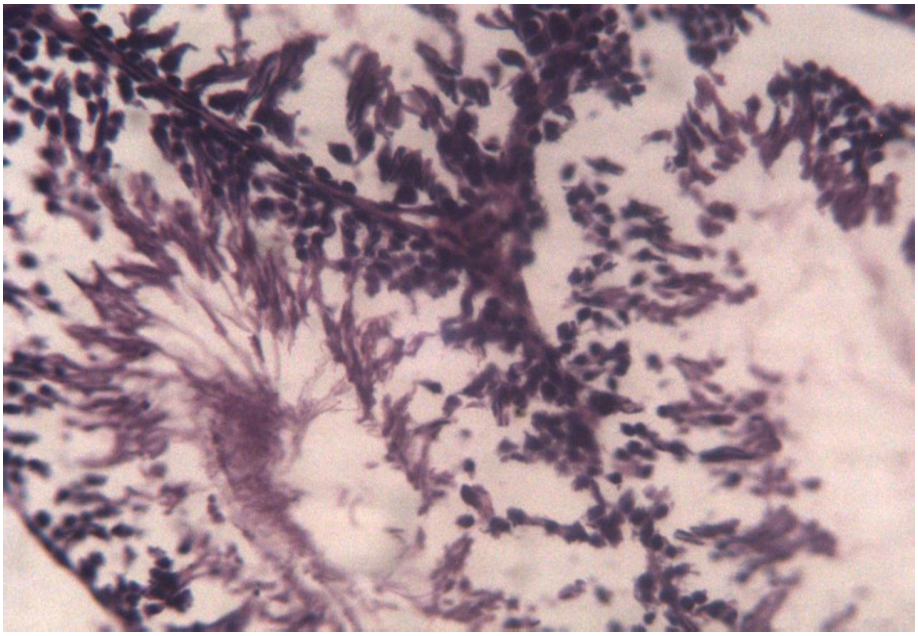


Plate XIII: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note the moderate degeneration of the seminiferous tubule layers. H & E X250.

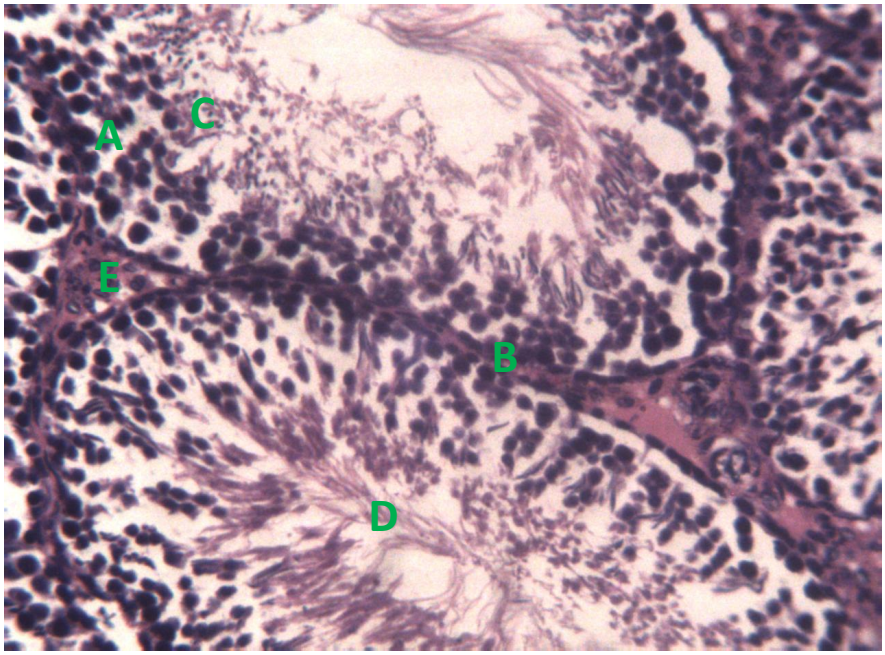


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

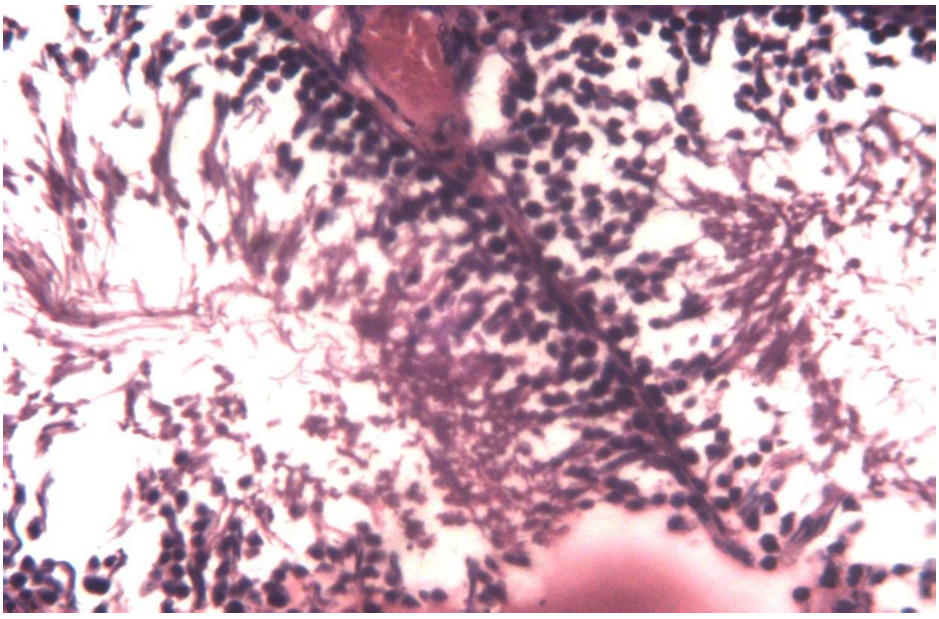


Plate XIV: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note the moderate degeneration of the seminiferous tubule layers H & E X250.

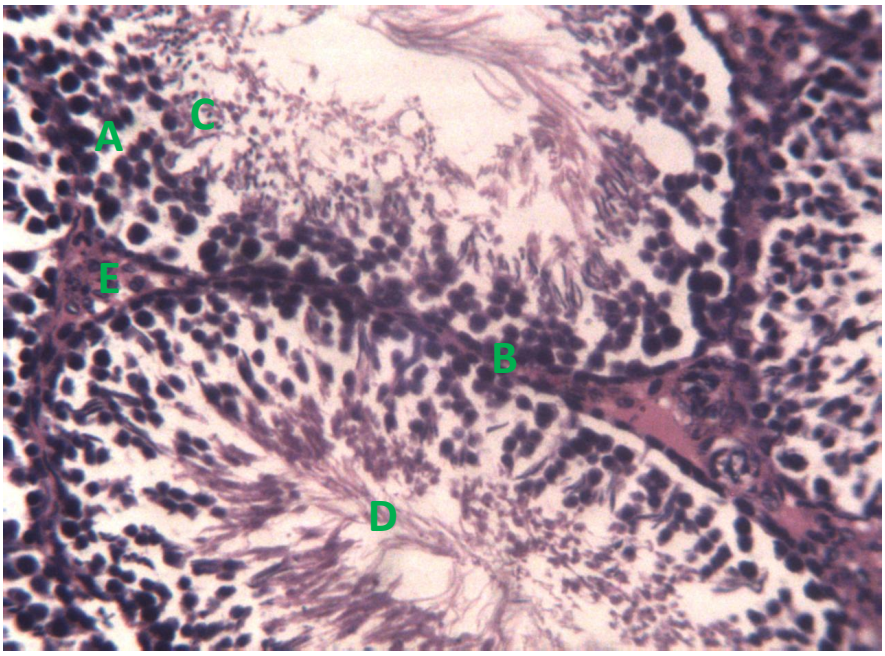


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

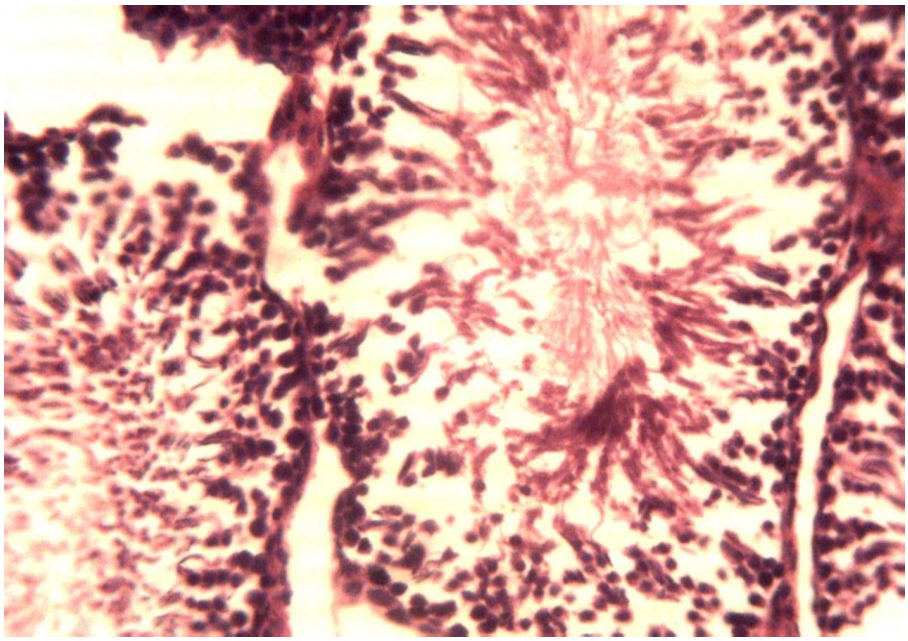


Plate XV: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note the moderate degeneration of the spermatogonia layer and the distortion of the basement membrane. H & E X250.

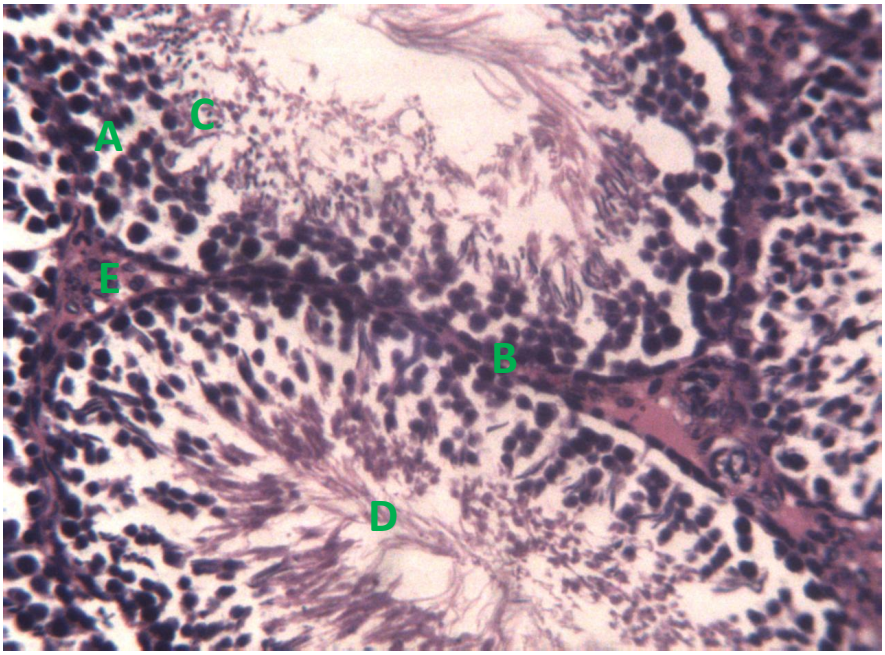


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

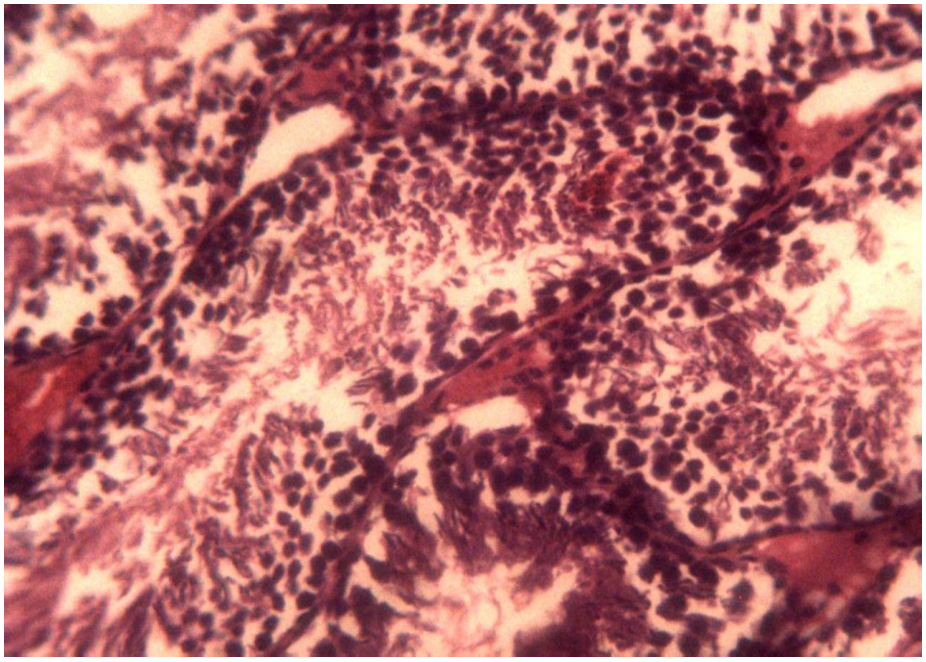


Plate XVI: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note the moderate degeneration of the seminiferous tubule layers H & E X250.

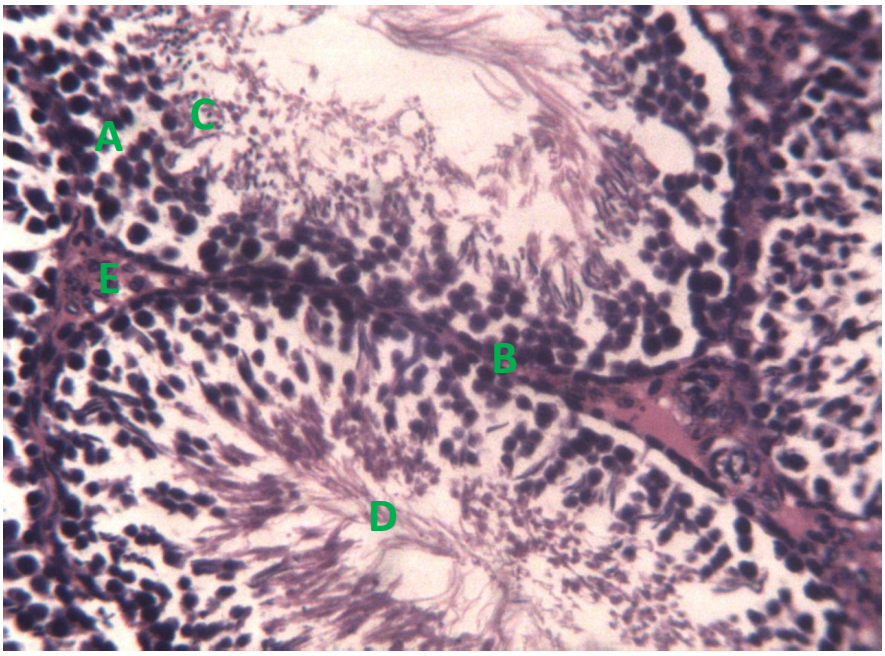


Plate VII: Photomicrograph of the testis of an adult male Wistar rat unexposed to *Shisha*smoke (control).

Note the normal spermatogonia layer (A) beneath the basement membrane (B), spermatocyte layer (C), spermatozoa (D) in the central lumen and interstitial space (E). H & E X 250.

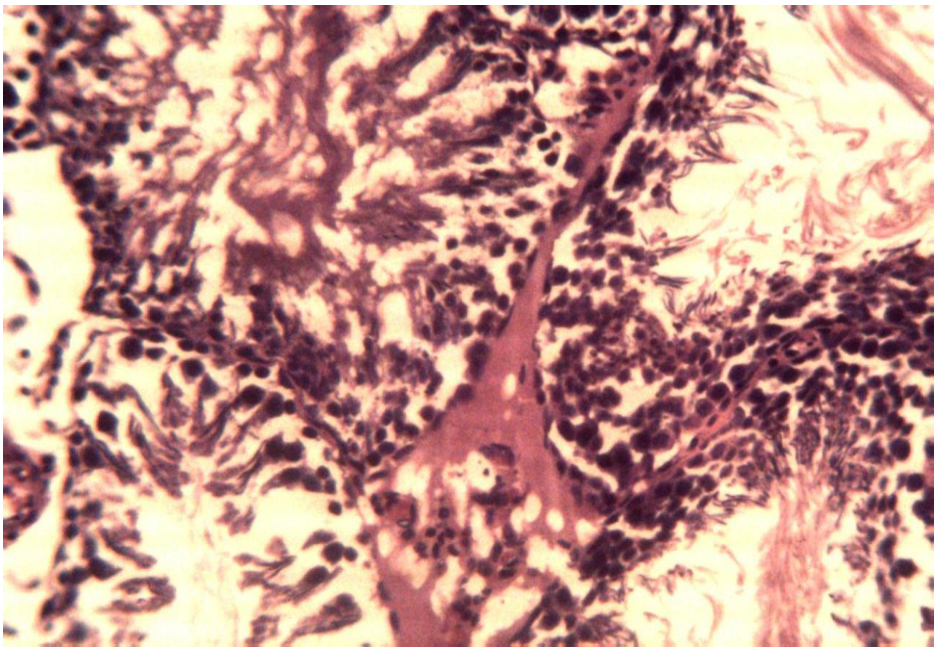


Plate XVII: Photomicrograph of the testis of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note the moderate degeneration of the seminiferous tubule layers.H & E X 250.

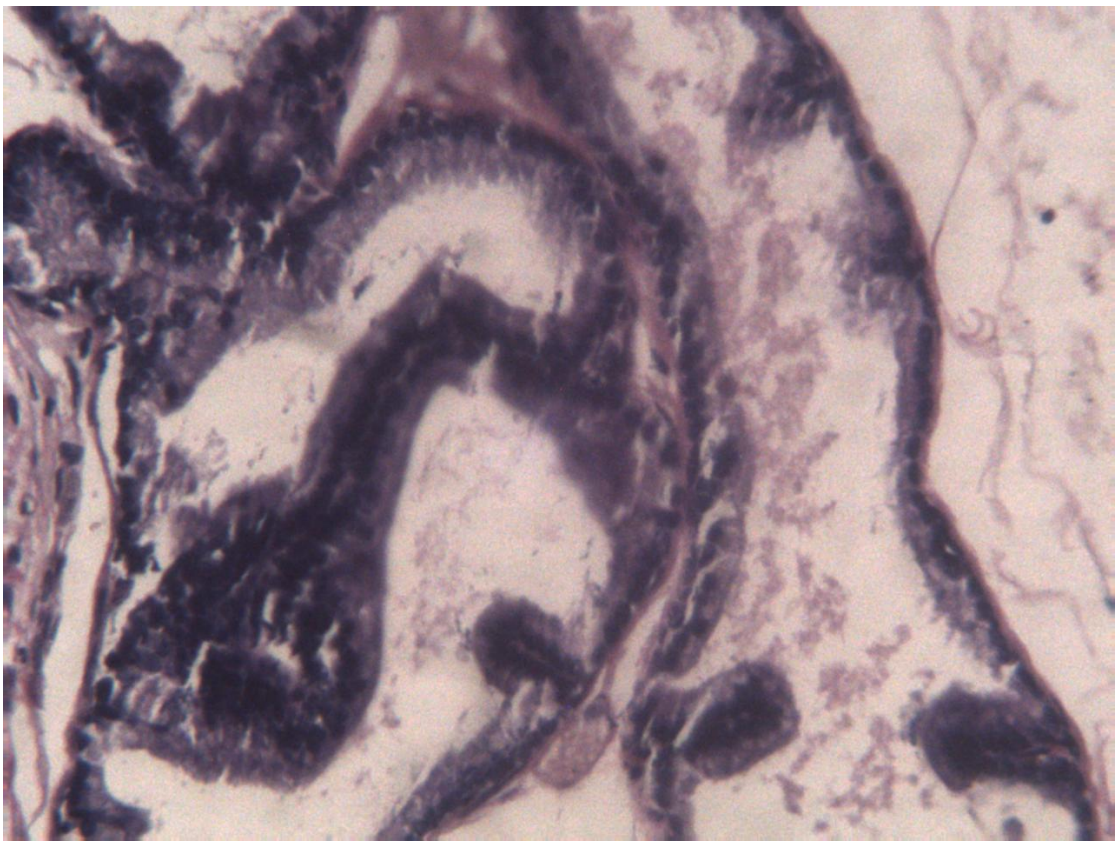


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

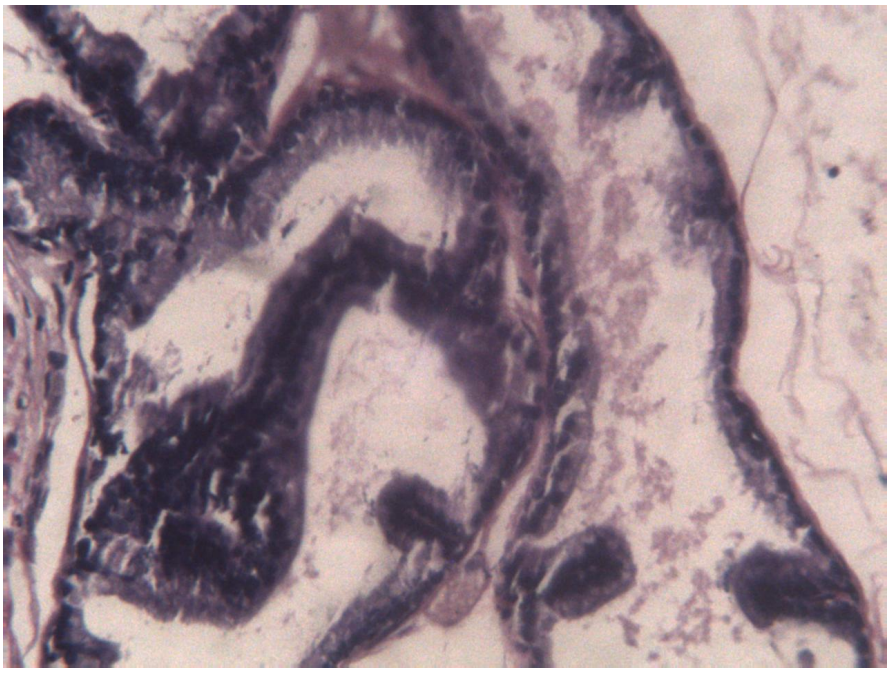


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

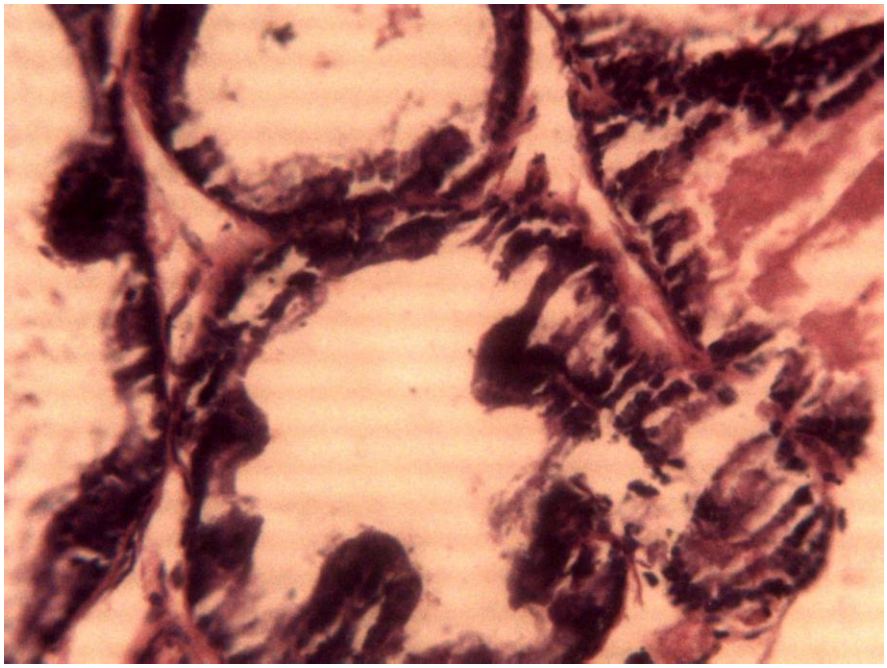


Plate XIX: Photomicrograph of the prostate gland of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).

Note themoderate necrosis of the prostate gland epithelia. H & E X250.

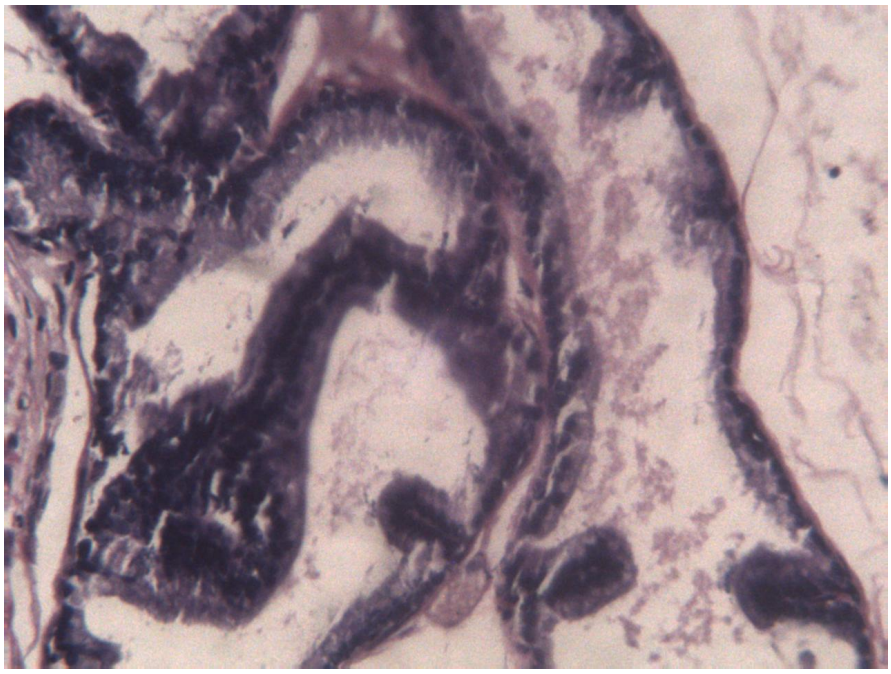


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

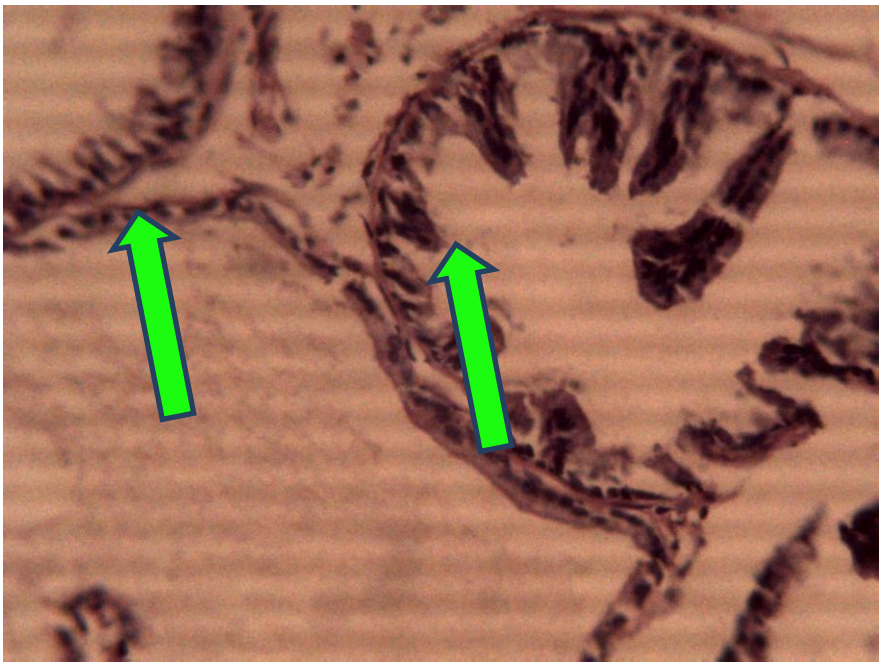


Plate XX: Photomicrograph of the prostate gland of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).

Note the decreased epithelia height in some lumen (E). H & E X250.

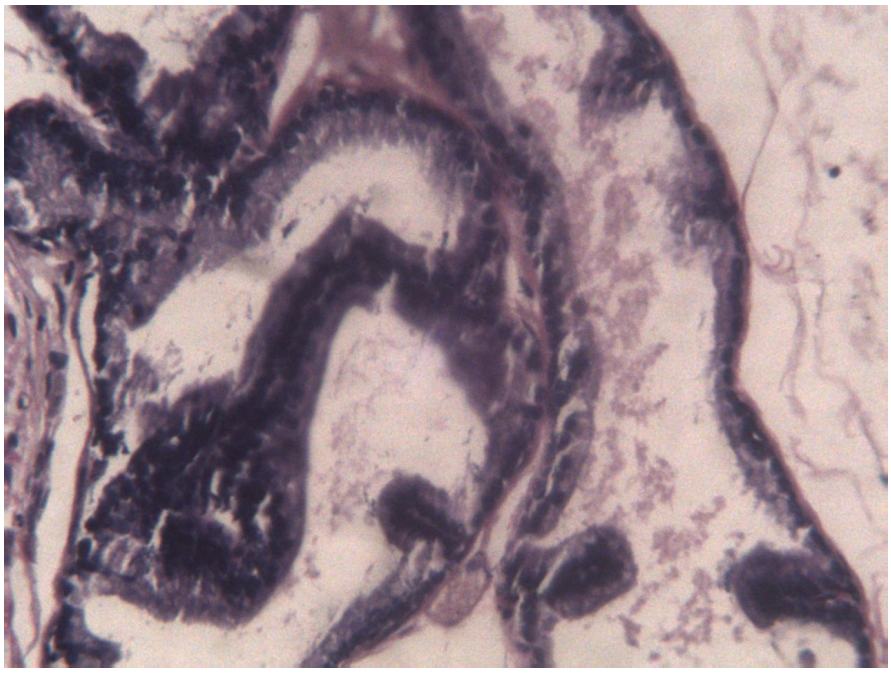


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

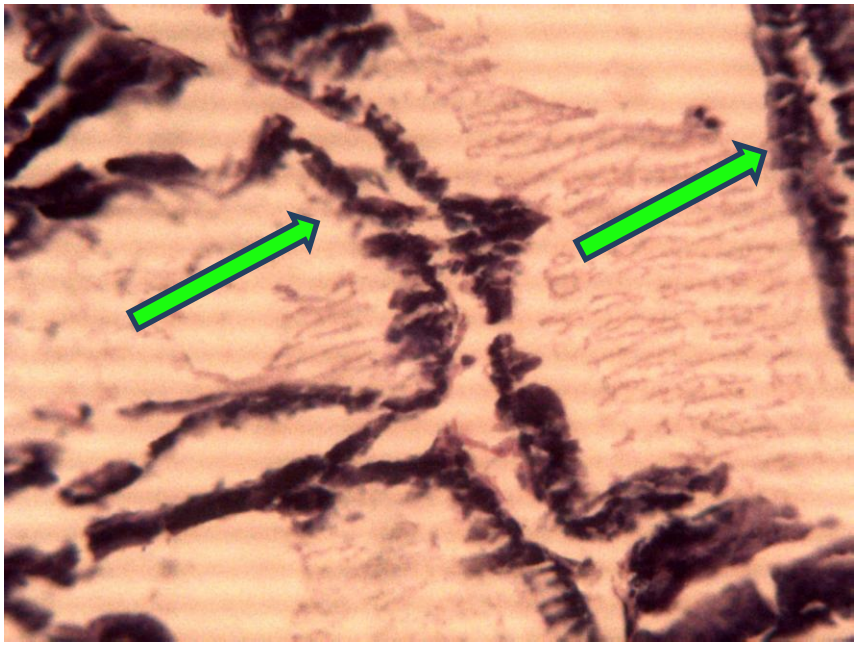


Plate XXI: Photomicrograph of the prostate gland of an adult male Wistar rat exposed to *Shisha*smoke with water in the jar (bonged *Shisha* smoke group).

Note the intense necrosis of the prostate gland epithelia. H & E X250.

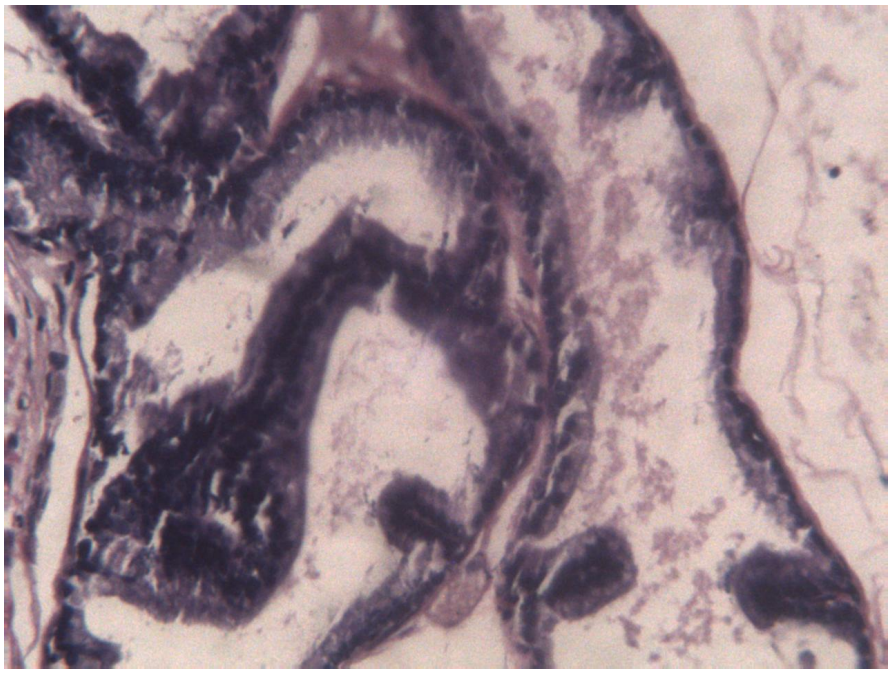


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

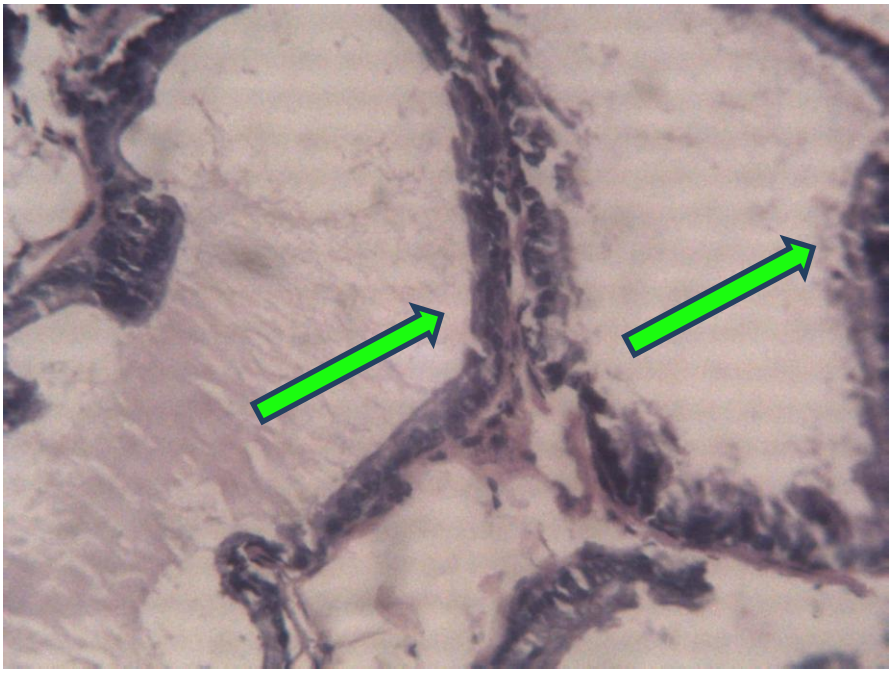


Plate XXII: Photomicrograph of theprostate gland of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note themoderate necrosis of the prostate gland epithelia. H & E X250.

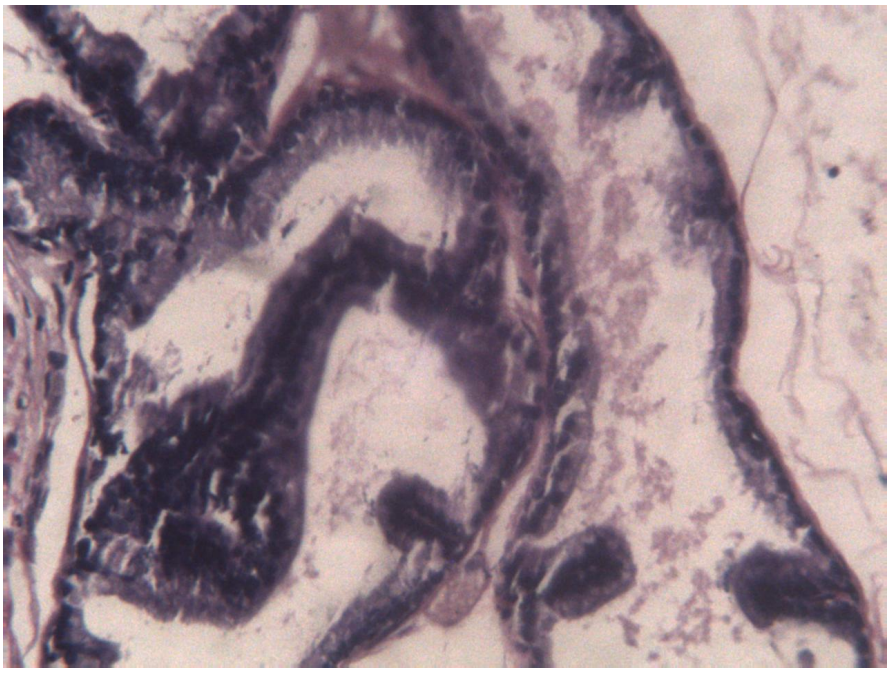


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

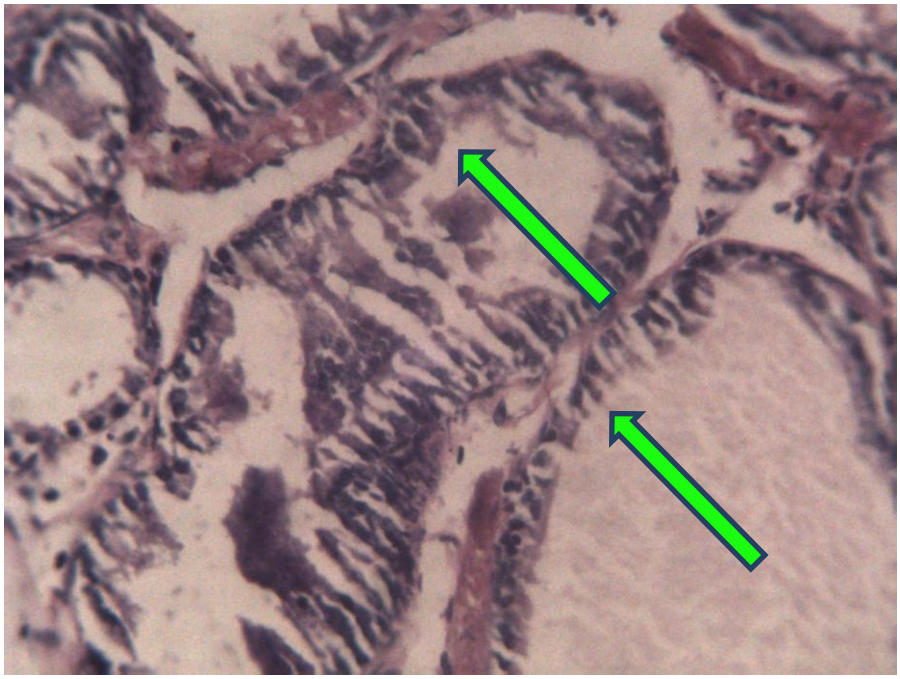


Plate XXIII: Photomicrograph of the prostate gland of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note themoderate necrosis of the prostate gland epithelia. H & E X 250.

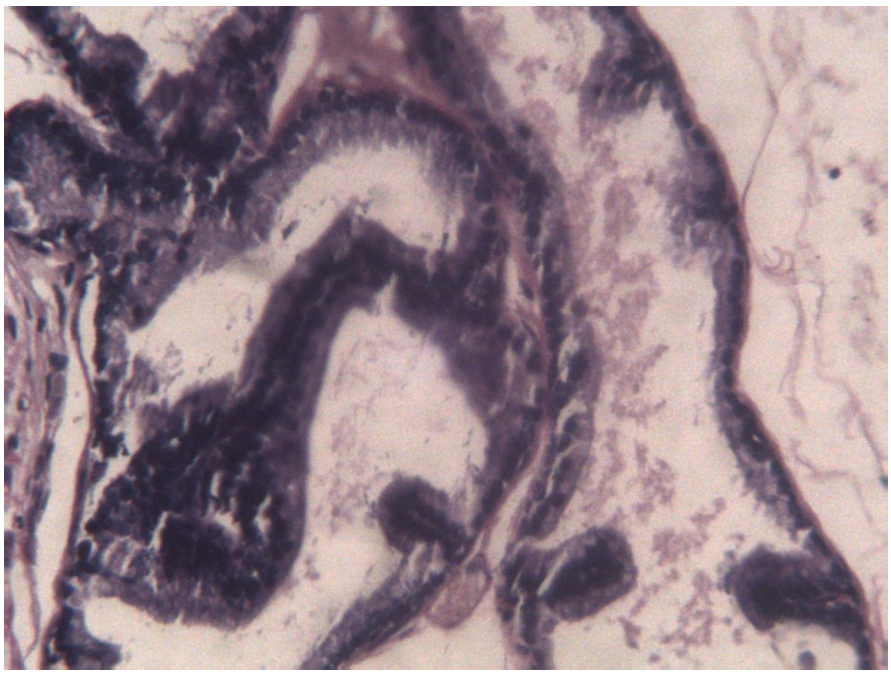


Plate XVIII: Photomicrograph of the prostate gland of an adult male Wistar rat unexposed to *Shisha*smoke (control). Normal histo-architecture of the gland with preserved epithelia cells. H & E X 250.

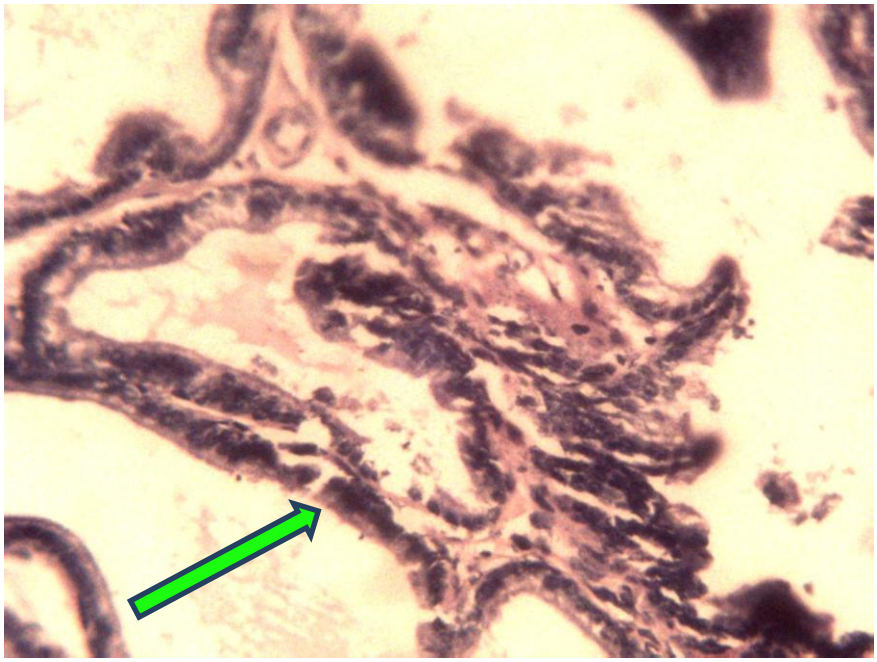


Plate XXIV: Photomicrograph of the prostate gland of an adult male Wistar rat exposed to *Shisha*smoke without water in the jar (unbonged *Shisha* smoke group).

Note themoderate necrosis of the prostate gland epithelia. H & E X250.

CHAPTER FIVE

5.1 DISCUSSION

The phytochemical screening of the grape flavoured tobacco *Shisha*revealed the presence of alkaloids, carbohydrates, cardiac glycosides, triterpenes, flavonoids, and tannins in the *Shisha*tobacco.Phytochemical compounds are known to play important roles in bioactivity of a substance. Alkaloids which are part of the phytochemical constituents of *Shisha*exhibit a wide range of reproductive toxicities. Several studies have been reported on the male reproductive toxicities due tocigarette smoking and itsactive constituent (nicotine, a liquid alkaloid) in both human andexperimental animals. They are known to cause decrease in plasma level of testosteronethrough inhibition of themultiple steps involve in testosterone biosynthesis in rats and mouse (Oyeyemi*et al*., 2014).

The present study observed significant increase in testicular MDA level in the bonged *Shisha* smoke group as compared to other groups. This finding is consistent with the recent results obtained by Safyudin and Subondrate (2016). They observed significant increase in serum MDA level of smokers as compared to non-smokers. An increase in MDA level in the exposed groups in this study indicates an increase in free radicals production and lipid peroxidation in the testes of the rats. Tobacco smoke is made up of gas and organic compounds. Nicotine is the most abundant organic particle present in tobacco smoke, which is responsible for some of the deleterious effects on various organs of tobacco smokers (Jorsaraei *et al*., 2008). Nicotine is a very toxic alkaloid (Branian and Hansen, 2002). Most of the inhaled nicotine is quickly oxidized to its major metabolite cotinine, which occurs mainly in vapourised state (Binnie *et al*., 2004). Cotinine has a longer half-life than nicotine which is about 20 hours against 2 hours for nicotine. The significantly higher in MDA level in the*Shisha*smoke exposed groups is probably due to the

ability of nicotine to freely dissolve in water and form un-protonated component that is highly volatileand increases its rate of absorption in the respiratory tract (Henningfield *et al*., 2004). Additionally, tobacco smoke contains a mixture of several harmful substances like carbon monoxide, hydrogen cyanide, ammonia, volatile hydrocarbons, alcohol, aldehydes and ketones (Kitawaki *et al*., 2001). These substances generate free radicals that affect the membrane of spermatozoa. The plasma membrane lipids of mammalian spermatozoa are greatly different from mammalian somatic, that is, speramtozoa plasma membrane contains high levels of lipids in the form of PUFAs. These lipids contain unconjugated double bonds separated by methylene groups. The placement of a double bond adjacent to a methylene group weakens the methyl carbon-hydrogen bond, consequently making hydrogen extremely susceptible to abstraction and oxidative damage. When the levels of ROS within the cell are high, ROS will attack PUFA, causing a cascade of chemical reactions of lipid peroxidation (LPO), Makker *et al*., 2009.

The present study showed that, serum testosterone level is considerably lower in the bonged *Shisha*smoke (BSS) and un-bonged *Shisha*smoke (UBSS) groups compared with control. Similar results were reported by Foroutanjazi*et al*. (2014) in rat exposed to *Shisha* smoke and in male rats exposed to cigarette and *Shisha*smokes (Heidary *et al*., 2012). The reduction in serum testosterone level observed in this study following *Shisha*smoke exposure may be attributed to its nicotine content as previously reported by Jorsaraei *et al*. (2008). Furthermore, a direct destructive effect of nicotine on reproductive system and sex hormone secretion through free radical formation, leading to extensive cellular deterioration following tobacco smoking has been reported by Heidary *et al*. (2012).

The findings of this study also showed a significant increment in the serum level of PSA in the BSS group as compared to other groups, which agreed with the findings of Gokhan *et al*. (2013). They reported increasedlevel of serum PSA in smokers when compared to non-smokers of the same age. The plausible mechanism through which *Shisha*smoke may adversely affect the prostate gland and cause high PSA production is not well-known. Paradoxically, it is expected that low serum testosterone level and moderate necrosis of the prostate gland epithelial cells in the exposed groups observed in the present study should concomitantly result in low serum PSA level. This is because the prostate gland requires testosterone and dihydrotestosterone (DHT) for its growth and secretion. In contrast, DHT formed from testosterone by the action of 5α-reductase enzyme, is more potent than testosterone, and has been reported to be higher in smokers (Gokhan *et al*., 2013). Furthermore, Kalsheker (2009) found that cigarette smoking decrease plasma level of α1- antitrypsin when compared to non-smokers. α-1-chymoantitrypsin is a plasma protein that is produced in the liver, its complexes with serine proteases like PSA cause their inhibition. Deficiency of α1- antitrypsin in smoking may indirectly cause an increase in PSA level as observed in the present study.

The results of the current study indicated that sperm DNA fragmentation index is significantly higher in BSS and UBSS groups compared to the control group. This result agrees with the findings of Taha *et al*. (2014), who reported a significant positive correlation between smoking and sperm DNA fragmentation in oligoasthenoterozoospermic men. Similarly, Sandrine *et al*. (2006) also reporteda significantly higher DNA fragmentation in smokers than non-smokers spermatozoa (32% versus 25.9%, $P < 0.01$). *Shisha* smoke has been reported to increase the amount of free radicals in the body of smokers through its CO, nicotine, tar, heavy metals,*etc*.

Free radicals consequently cause oxidative DNA and chromosomal damages (Theron *et al*., 2011).

The present study revealed a significant reduction in epididymal sperm count in*Shisha*smoke exposed groups compared to the control. This result is in accordance with the findings of Irene *et al*. (2011), who reported a significantly positive correlation between tobacco *Shisha*smoking and sperm count, and other semen parameters among Egyptian men who smoke *Shisha*. The possible mechanism for reduction in sperm count in the groups exposed to shisha smoke may be attributed to the potentiated toxicants in *Shisha*smoke, such as nicotine (a very toxic alkaloid) and its major metabolites like cotinine and trans-3-hydroxycotinine, which have been known to cross the blood-testis barrier. These metabolites have been shown byIrene *et al*. (2011) to mediate the deleterious effects of*Shisha*smoke on spermatogenesis in the seminiferous tubules. Also, the possible cause of lower sperm count as evidently showed in the reeked testicular histology of the *Shisha*smoke exposed groups. Furthermore, the concentrated and diverse toxins in *Shisha*smoke may directly interact with, and affect the seminiferous tubules and the Leydig cells that secrete testosterone which stimulates the development and maturation of the spermatogonia to spermatozoa.

The results of the present study also demonstrated significantly lower percentage values for progressive or fast sperm motility in the shisha smoke exposed groups compared to the control. In addition, there was a significantly higher percentage non-progressive motility and non-motile sperm cells in the group exposed to *Shisha*smoke with water in the bowl as compared to the other groups. These findings concur with the report of Irene *et al*. (2011). The decrease in progressive sperm motility (an index of fertility) seen in the *Shisha*smoke exposed groups may

be ascribed to the effects of *Shisha*smoke toxicant substances that impair spermatogenesis or epididymal sperm maturation process, secondary to secretory dysfunction at the level of the Leydig and Sertoli cells. Smoke toxins have detrimental effects on the various structural modifications and biochemical changes that spermatozoa naturally undergo during epididymal maturation and this may lead to decreased sperm motility (Irene *et al*., 2011). Also nicotine and other chemicals in tobacco*Shisha*smoke may possibly cause damage to mitochondrial genome and mitochondrial enzymatic activity in the epididymis or seminal vesicles, thereby affecting sperm motility (Priyadarsini *et al*., 2014).

A significant increase in sperm head and tail abnormalities were observed in the BSS group compared to any other groups in this study. This finding is in agreement with the work of Jason (2015), who reported significant increase in abnormal sperm morphology in male cigarette smokers.

Similarly, the present study showed significant positive correlation between MDA level and DFI and PSA; testosterone concentration and sperm count and a significant negative correlation between MDA and testosterone; DFI and sperm count. This implies that higher the levels of testicular MDA is associated with the lower serum testosterone concentration and also the higher DFI, the lower sperm count. This finding is consistent with the report of Abasalt *et al*. (2013), who found negative correlation between MDA production and sperm count in astheno- and oligoastheno- teratospermic men. Higher MDA level observed in the *Shisha* smoke exposed groups may be due to oxidative stress induced by *Shisha*smoke through generation of reactive oxygen species. Lipid peroxidation is one of the deleterious effects of ROS and is considered as an indicator of membrane polyunsaturated fatty acid oxidation (Tremellen, 2008). Furthermore, it has been reported that ROS, in addition, to reaction with the polyunsaturated fatty acids, also

react with DNA nucleotides leading to base modification particularly 8-hydroxy-2'-deoxyguanosine (8-oxoguanine) formation and consequently DNA fragmentation (Nassira *et al*., 2011).

The tissue degeneration and necrosis of the seminiferous tubules observed in this study in *Shisha*smoke exposed rat testes compared to the control, could be attributed to oxidative stress induced by *Shisha*smoke causing depletion of enzymatic and non-enzymatic antioxidants, and increased level of LPO and ROS production. The degree of degeneration and necrosis were pronounced in the BSS group compared to UBSS and control groups.This could be due to the fact that nicotine, a very toxic alkaloid is highly water soluble and readily absorbed in the respiratory system and metabolized to its active metabolites that are deleterious to the body. The degeneration observed in the histology of the testes of *Shisha*smoke exposed groups may be an importantcontributory factor to the lower sperm count and testosterone level in these groups compared to control rats. These findings are in concordance with the findings of Adekomi *et al*. (2011) and Oyeyemi *et al*. (2014), who separately reported the degenerative effect of nicotine and extracts of tobacco nicotiana on rat testicular histology.

In the present study, there was moderate degeneration and necrosis of the prostate gland epithelia lining in the groups exposed to *Shisha*smoke compared to the control. The findings are similar with a study conducted by Mahaneem *et al* (2012), whose results revealed atrophy of epithelial cells of the prostate in rats exposed to cigarette smoking. The distortion of epithelial cell lining of the gland seen in the present study could be partly due to direct oxidative stress effect, induced by *Shisha* smoke on the prostate gland. Oxidative stress has been postulated as one of the

plausible mechanisms that cause free radical attacked on epithelial cells, gradually degrading them and reducing their height.

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CHAPTER SIX

5.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The present study has demonstrated that exposure of Wistar rats to *Shisha*smoke for 13 weeks resulted in significant increase in testicular MDA and serum PSA levels; decreased serum testosterone concentration, sperm count, sperm progressive motility; and increase in non-progressive motility, immotile sperm cells; and higher percentage of sperm abnormalities of head and tail; and sperm DNA fragmentation. There were significant histological changes in the testes and prostate glands of *Shisha* smoked exposed ratsespecially in the BSS groups. Furthermore the present study has demonstrated significant positive correlation between increasedtesticular MDA level and the following parameters: increase insperm DNA fragmentation and PSA level; decrease in testosterone concentration and sperm count; and also negative correlation between sperm DNA fragmentation and sperm count.

6.2 Conclusion

In conclusion, the present study has revealed significantly harmful effects of *Shisha*smoking in the testis and prostate gland of the experimental animals as evidenced by the histological

damages in these organs. The study also highlighted the possible role of oxidative stress on the adverse effects of *Shisha* smoking on sperm count, motility, and morphology and the enhanced sperm DNA fragmentation in the*Shisha* exposed groups, which were higher in the bonged group.

6.3 Recommendations

Based on the results obtained from the present study, the following recommendations were made:

i. *Shisha* tobacco smoking as an alternate to the traditional cigarette smoking should be discouraged as the acclaimed less harmful method of smoking does not significantly remove the toxins in the smoke. Itrather compounds the harmful effect of tobacco as demonstrated in this study.

ii. Further studies should be conducted to quantify the amount or level of oxidative stress damages on sperm DNA imposed by *Shisha*smoke in animals through determination of 8-hydroxy-2'-deoxyguanosine (8-oxoguanine) level in sperm cells.

iii. Further studies should be conducted to determine the exact levels of some oxidative stress biomarkers such as 1α-antitrypsin in order to verify the findings of other researches that reported lower levels of this chemical in tobacco smokers; as a possible explanation for the high level of PSA observed in the study despite the marked degeneration of the prostate gland observed in this study.

6.4 Contributions to knowledge

Based on the findings of the present study, the following contributions to knowledge were drawn:

i. To the best of our knowledge this is the first research of its kind that has been reported in literature, where a study design compared reproductive parameters between bonged and un-bonged *Shisha* smoke in experimental Wistar rats.

ii. Bonged shisha smoke Inducedhigher MDA oxidative damage than unbonged *Shisha*smoke [$F(2,12)=67.8, p = 0.00$]

iii. Sperm DNA fragmentation was higher in the *Shisha* smoke exposed groups compared to control rats [$F(2,12)= 55.8, p = 0.00$]. The study alsodemonstrated higher sperm DNA fragmentation in the bonged *Shisha*smoke exposed group than unbonged *Shisha* smoke group.

iv. Serum PSA level washigher in the *Shisha*smoke exposed groups compared to control group [$F(2,12)= 19.2, p = 0.000$].

v. Bonged shisha smoke induced oxidative damage in testis and prostate gland more than un-bonged shisha smoke.

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APPENDIX I

Committee on Animal Use and Care
Directorate of Academic Planning & Monitoring
Ahmadu Bello University, Zaria
Director: Professor M. I. Sule B Pharm, MSc, PhD, (ABU), MPSN 08036184581
Chairman: Prof C. A. Kudi DVM, M.Sc, PhD (ABU), PhD (Lei.) PGDE (PLY) 08065978003 Committee Secretary: U. D. Abdullahi, 08034619772

Appl No.: ABUCAUC/2016/Human Phy./007. **Date:** 19th September, 2016

Approval No: ABUCAUC/2016/007.

Dr. Aliyu Mohammed
Department of Human Physiology,
Faculty of Medicine,
Ahmadu Bello University,
Zaria.

Sir,

APPROVAL OF RESEARCH STUDY 'EVALUATION OF CHIRONIC SHISHA INHALTION ON SOME REPRODUCTIVE PARAMETERS AND SPERM DNA FRAGAMENTATION IN ADULT WISTA RATS'

This is to convey the approval of the ABUCAUC to you for the aforestated study domiciled in the Department of Human Physiology. The approval is predicated on the assumption that you shall maintain and care for the Experimental Animals as approved after the visitation of the Committee.

Monitoring of the Research by spot checks, invitations or any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCAUC Page on the University's website.
Note upon completion of the research, ethical clearance certificate will be issued.

U.D. Abdullahi
For: Chairman, ABUCAUC.

Cc. Director, DAPM
Director, IC &ICT
Dean, Faculty of Medicine
HOD, Human Physiology
Dr. Mohammed Aliyu, Faculty Rep. Medicine



APPENDIX II

Sperm count and percentage progressive, non-progressive and non-motile sperm cellsin control and *Shisha*exposed adult male Wistar rats

Sperm Parameters	Control	BSS	UBSS
Sperm cell (x10^7/ml)	5.62 ± 0.15^a	3.30 ± 0.13^c	4.08 ± 0.08^b
Prog. Sperm cell (%)	48.0 ± 2.50^a	32.2 ±1.01^c	40.0 ± 1.70^b
Non-prog. Sperm cell (%)	37.0 ± 3.00^a	47.0± 2.30^b	43.6± 0.90^a
Non motile spermCell (%)	15.0± 2.23^a	21.4± 2.50^b	18.0± 1.40^a

Data were expressed as Mean ± SEM. N = 5

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

BSS= Bonged shisha smoke

UBSS= Un-bonged shisha smoke

APPENDIX III

Testicular MDA, Serum testosterone, sperm DNA fragmentation and serum PSA levelin control and exposed adult male Wistar rats

Sperm Parameters	Control BSS	UBSS
Testicular MDA (µmol/mg)	0.44 ± 0.08^{a}	1.61 ± 0.08^{c} 1.26 ± 0.04^{b}
Serum testosterone (nmol/L)	41.6 ± 0.50^{a}	25.4 ± 1.16^{c} 31.8 ± 0.58^{b}
Sperm DNA frag.Index (%)	9.60 ± 0.67^{a}	29.0 ± 1.90^{c} 20.0 ± 0.94^{b}
Serum PSA (ng/ml)	1.30 ± 0.05^{a}	3.04 ± 0.03^{c} 1.94 ± 0.06^{b}

Data were expressed as Mean ± SEM. N = 5

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

BSS= Bonged shisha smoke

UBSS= Un-bonged shisha smoke

APPENDIX IV

Scheme of plausible mechanism of *Shisha* smoke on reproductive system

