EFFECT OF ROSELLE EXTRACT (*HIBISCUS SABDARIFFA*) ON SPERM QUALITY AND TESTICULAR OXIDATIVE STRESS IN HIGH FAT DIET INDUCED RATS

CHANOKNAN PUCHAI

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science Degree in Physiology
July 2020

Thesis entitled "Effect of Roselle extract (*Hibiscus sabdariffa*) on sperm quality and testicular oxidative stress in high fat diet induced rats"

By Chanoknan Puchai

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Physiology of Naresuan University

Oral Defense Committee

..... Chair

(Colonel Assistant Professor Anusara Vattanajun, Ph.D.)

..... Advisor

(Assistant Professor Onrawee Khongsombat, Ph.D.)

..... Co – Advisor

(Assistant Professor Sakara Tunsophon, Ph.D.)

..... Co – Advisor

(Assistant Professor Hathairat Kruevaisayawan, Ph.D.)

..... Internal Examiner

(Assistant Professor Pornnarin Taepavarapruk, Ph.D.)

Approved

.....

(Professor Paisarn Muneesawang, Ph.D.)

Dean of the Graduate School

2 JUL 2020

ACKNOWLEDGEMENT

Firstly, I would like to express my deepest and sincere appreciation to my thesis advisor, Assistant Professor Dr. Onrawee Khongsombat, for her supervision, guidance, encouragement and continued support throughout this study.

I would like to express my sincere appreciation to my co-advisors, Assistant Professor Dr. Sakara Tunsophon and Assistant Professor Dr. Hathairat Kruevaisayawan for their valuable advice, support, guidance and encouragement throughout this study.

I would like to thank for my chair, Colonel Assistant Professor Dr. Anusara Vattanajun and my internal examiner, Assistant Professor Dr. Pornnarin Taepavarapruk for suggestions and all their help.

I would particularly like to acknowledge Dr. Wanlapa Kokmas for helpful suggestions and kindness.

Special thanks to my friends for their valuable help and technical assistance. Finally, I do extremely appreciate and deeply thanks my family for their encouragement, support and caring throughout my life.

Chanoknan Puchai

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Author	Chanoknan Puchai
Advisor	Assistant Professor Onrawee Khongsombat, Ph.D.
Co - Advisor	Assistant Professor Sakara Tunsophon, Ph.D.
	Assistant Professor Hathairat Kruevaisayawan, Ph.D.
Academic Paper	Thesis M.S. in Physiology, Naresuan University, 2019
Keywords	Obesity, *Hibiscus sabdariffa*, Sperm, Testis, Oxidative stress

ABSTRACT

Obesity, a condition as an accumulated excess amount of body fat, is a major risk factor for deleterious associated pathologies such as insulin resistance, type 2 diabetes and male infertility. Several studies have shown the positive correlation between obesity and oxidative stress and its effect on the male reproductive system. Roselle (*Hibiscus sabdariffa*) contains phenolic compounds, especially anthocyanins which act as antioxidants. The objective of this study was to evaluate the effect of roselle on sperm quality and oxidative stress in the testes of rats fed a high fat diet. The rats were divided into 4 groups (n=7): the control group fed with a normal diet, rats fed with a high fat diet (HFD), rats fed with a high fat diet treated with 250 mg/kg B.W. roselle (HFD-L), and rats fed with a high fat diet treated with 500 mg/kg B.W. roselle (HFD-H). Eight weeks after administration, sperm were collected from rat epididymis and the sperm quality and morphology were assessed. The rats' testes were evaluated for malondialdehyde (MDA) level, antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) in addition histological parameter tubular differentiation index (TDI), seminiferous tubule diameter and epithelium thickness. The sperm concentration, motility and normal morphology showed a significant increase in HFD-L and HFD-H groups when compared with the HFD group ($p < 0.05$). Sperm viability showed a significant increase in HFD-H group when compared with the HFD group ($p < 0.05$). The MDA levels in the HFD group showed a significant increase when

compared with the control group ($p < 0.05$). The MDA levels in the HFD-L and HFD-H groups were significantly decreased when compared with the HFD group ($p < 0.05$). The CAT activity in the HFD-L and HFD-H groups were significantly increased when compared with the HFD group ($p < 0.05$). The SOD activity in the HFD group showed a significant decreased when compared with the control group ($p < 0.05$). There was no significant change the SOD activity in the HFD, HFD+L and HFD+H groups. The seminiferous tubule diameter and seminiferous epithelium thickness in the HFD group showed a significant decreased when compared with the control group ($p < 0.05$). There was no significant change the seminiferous tubule diameter and seminiferous epithelium thickness in the HFD, HFD+L and HFD+H groups. The TDI were not significantly different among experimental groups. These results show that roselle reduced the oxidative stress and increased whereas, the sperm quality, which clearly demonstrate that roselle has the beneficial effects on sperm quality in high fat diet induced rats.

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ABBREVIATIONS

°C	=	Degree Celsius
•GS	=	Glutathiyl radical
•$GSSG^-$	=	Glutathione disulfide
$^•O_2^-$	=	Superoxide anion
•NO	=	Nitric oxide
•NO_2	=	Nitrogen dioxide
•R	=	Alkyl radical
•RO	=	Alkoxyl radical
•ROO	=	Alkylperoxyl radical
•RS	=	Reactive species
106	=	1 million
4-HNE	=	4-hydroxy-2-nonenal
Abs	=	Light absorbance
ANOVA	=	Analysis of Variance
ABP	=	Androgen-binding protein
ATP	=	Adenosine triphosphate
BCA	=	Bicinchoninic Acid
BMI	=	Body mass index
BSA	=	Bovine serum albumin
B.W.	=	Body weight
CAT	=	Catalase
CBZ	=	Carbamazepine
cm	=	Centimeter
cm^{-1}	=	Typically centimeters
cm^2	=	Square centimeter
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
FDA	=	Food and Drug Administration
FFA	=	Free fatty acid
FSH	=	Follicle-stimulating hormone

ABBREVIATIONS (CONT.)

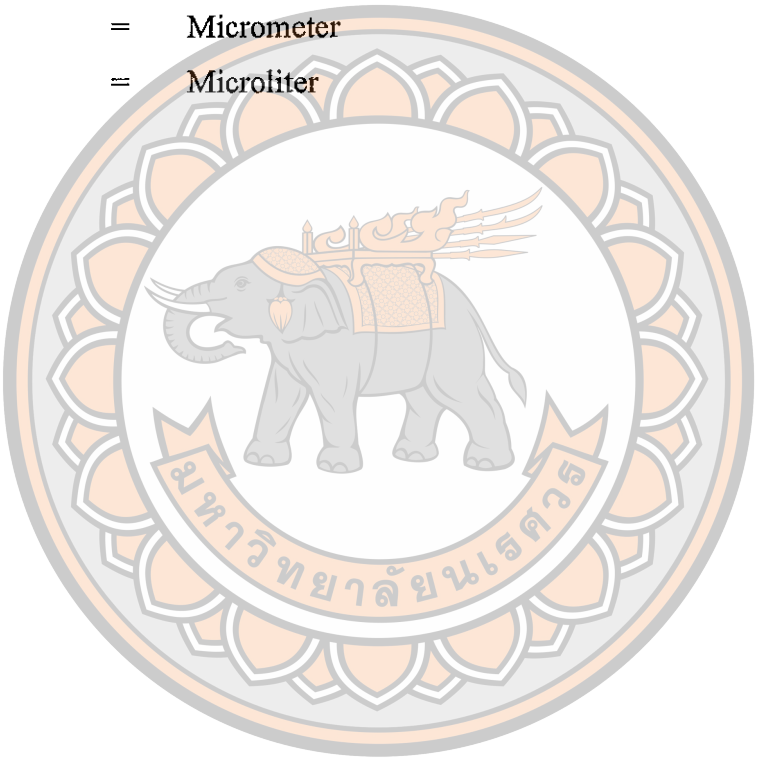
g	=	Gram
g	=	Gravity
GnRH	=	Gonadotropin-releasing hormone
GPx	=	Glutathione peroxidase
GSH	=	Reduced glutathione
GSSH	=	Glutathione disulphide
h	=	Hour
H&E	=	Hematoxylin and eosin
HCl	=	Hydrochloric acid
HDL	=	High-density lipoprotein
HFD	=	High fat diet
HFD-L	=	High fat diet with roselle 250 mg/kg B.W.
HFD-H	=	High fat diet with roselle 500 mg/kg B.W.
H_2O_2	=	Hydrogen peroxide
HOCl	=	Hypothalamic-pituitary-adrenal-gonadotropic
HPG	=	Kilogram
$^1O_2$	=	Iodite dioxidoiodate (1-)
K_2HPO_4	=	Dipotassium phosphate
Kg	=	Kilogram
Kg/m^2	=	Kilogram force per square meter
L	=	Litter
LDL	=	Low-density lipoprotein cholesterol
LH	=	Luteinizing hormone
LPO	=	Lipid peroxidation
LSD	=	Fisher's Least-Significant Difference
M	=	Molarity
m^2	=	Square meter
MDA	=	Malondialdehyde
mg	=	Milligram

ABBREVIATIONS (CONT.)

min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
n	=	Number
NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate
Na_2HPO_4	=	Sodium phosphate dibasic
nm	=	Nanometer
NO	=	Nitric oxide
NO_2^-	=	Nitrogen dioxide
O_2	=	Oxygen
O_2^-	=	Superoxide anion
OD	=	optical density
OH^-	=	Hydroxyl radicals
ONOO-	=	Peroxynitrite
PBS	=	Phosphate buffered saline
p, P value	=	Probability value
pH	=	Power of hydrogen ion concentration
PUFA	=	Polyunsaturated fatty acid
RNA	=	Ribonucleic acid
RNS	=	Reactive nitrogen species
ROO-	=	Peroxyl
ROS	=	Reactive oxygen species
SDS	=	Sodium dodecyl sulphate
SEM	=	Structural Equation Modeling
SOD	=	Superoxide dismutase
SPSS	=	Statistical Package for the Social Sciences
STs	=	Seminiferous tubules
TBA	=	Thiobarbituric acid
TBARS	=	Thiobarbituric acid reactive substances

ABBREVIATIONS (CONT.)

TDI	=	Tubular differentiation index
TMP	=	1,1,3,3- tetramethoxypropane
U	=	Unit
WC	=	Waist circumference
WHO	=	World Health Organization
x	=	Magnification microscope
µm	=	Micrometer
µl	=	Microliter



CHAPTER I

INTRODUCTION

Rational and significant of the study

Globally, obesity has nearly tripled since 1975. In 2016, more than 650 million adults were obese (WHO, 2018). Obesity caused 4 million deaths per year worldwide and estimated to increase every year (Weiderpass et al., 2019). An estimated 58% of the world's population will be overweight and obesity in 2030 (Kelly et al., 2008). Obesity becoming a global concern are highly prevalent in developed countries, obesity is quickly together with middle-income countries such as Thailand. The overall prevalence rate of obesity combined (BMI 25.0–29.9) was 4.8% in Thailand (Jitnarin et al., 2011).

Obesity occurs as an accumulated excess amount of body fat. Obesity increases risk of several medical conditions such as hypertension, cardiovascular disease (Lerman et al., 2016), type 2 diabetes and male infertility (Hammoud et al., 2008; Lavranos et al., 2012). Numerous studies confirmed that obesity is caused of various changes including oxidative stress (Fernández-Sánchez et al., 2011), inflammation (Fernandez et al., 2011), mitochondrial dysfunction (Bournat and Brown, 2010) and apoptosis (Kluth et al., 2011). These changes encourage many pathophysiological conditions in the body.

An increase of fat accumulation, especially white adipose tissue, leads to the production of adipokines (Fantuzzi, 2005) which secrete and generate free radicals (reactive oxygen species; ROS). Adipokines and ROS are important mediators for inducing oxidative stress (Fernandez et al., 2011). Lipid peroxidation is the process of oxidative composition of polyunsaturated fatty acid (PUFA) and ROS, leading to the formation of mixtures of lipid hydroperoxide and aldehydic end-products such as malondialdehyde (MDA). The reaction of lipid peroxidation occurs in several cell types, including sperm cells, particularly in the plasma membrane which contains a high content of lipid. Sperm cells are sensitive to oxidative stress. The reaction of lipid peroxidation damage sperm cells by decreasing the sperm qualities including sperm concentration, motility, viability and normal morphology (Sies, 1997; Du Plessis et al.,

2010; Lavranos et al., 2012). Furthermore, the role of antioxidant has been developed to attenuate the effects of oxidative stress from obesity (Rahal et al., 2014).

Obesity persists for a long time, the several of adipose tissue, antioxidant sources can be depleted, decreasing the activity of enzyme such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), was found to be significantly decreased (Fernandez et al., 2011). In addition, there is a study suggests that treatment with antioxidants or ROS inhibitors could restore the regulation of adipokines (Furukawa et al., 2004). Therefore, supplementation with antioxidants would reduce the risk of complications related with obesity and oxidative stress (Higdon and Frei, 2003). Antioxidants act as free radical scavengers to protect spermatozoa against ROS (Mohammadi, Salehi, & Mortazavi, 2015). These antioxidants compensate for the loss of sperm cell enzymes as the cytoplasm is removed during spermiogenesis, which in turn, diminishes endogenous repair mechanisms and enzymatic defenses (Chen et al., 2012).

Roselle (*Hibiscus sabdariffa*) is used in herbal medicines as an antibacterial agent (Abdallah, 2016), an antihypertensive treatment (Usoh, 2005), anti-diabetic (Mozaffari-Khosravi et al., 2009), anti-inflammatory (Tsuda et al., 2003) and anti-cancer effects (Wu, Yang, & Chiang, 2018) and against Alzheimer's disease (Obouayeba et al., 2014). Previous studies have reported that roselle could decrease the levels of total lipids, cholesterols and triglycerides, suggesting the anti-obesity effect of roselle (Sheba, 2016). The anthocyanin pigments have been confirmed as having a beneficial effect on various cells, such as being an antioxidant that attenuates the negative effects of oxidative stress (Ali et al., 2005; Rodrguez-Medina et al., 2009). Previous studies have shown that roselle aqueous extract ameliorates sperm defects in streptozotocin-induced diabetic rats (La Vignera et al., 2012; Shamsi et al., 2008). In addition, roselle could protect cisplatin-induced testicular damage and oxidative stress and improve sperm quality, by increasing the activities of antioxidant enzymes such as CAT and SOD (Amr Amin et al., 2006).

However, there was no research to study the effect of roselle extract on male reproductive system in obese rat induced by high fat diet. Therefore, the purpose of this study is to evaluate the effect and mechanisms of roselle extract on the sperm functions and morphology as well as antioxidant activity in high fat diet induced rats.

Purposes of the study

A general experiment objective

The purpose of this study is to investigate the effects of roselle (*Hibiscus sabdariffa*) on the sperm functions and morphology as well as antioxidant activity in the sperm and testes in high fat diet induced rats.

Specific objectives

1. To study the effect of roselle on sperm concentration motility and viability in obese rats.
2. To study the effect of roselle on sperm and testes morphology in obese rats.
3. To study the effect of roselle on the levels of MDA in the testes of obese rats.
4. To evaluate the effect of roselle on the antioxidant enzyme activity of superoxide dismutase (SOD) and catalase (CAT) in the testes of obese rats.

Scope of the study

Male Sprague Dawley rats were used in this study to investigate the effects of roselle in testis and sperm quality. The rats were fed with the high fat diet with roselle for 8-week. At the end of the 8-week period, sperm quality, the levels of MDA and the antioxidant enzyme activity of CAT and SOD in testes were tested. Sperm quality such as concentration and motility were evaluated using a Makler counting chamber, viability was evaluated by nigrosin-eosin staining and morphology was evaluated by diff-quik staining kit. The levels of MDA were evaluated by using thiobarbituric acid reactive substances (TBARS) assay. Moreover, the protective effect of roselle on rat testes histology was examined using hematoxylin and eosin staining.

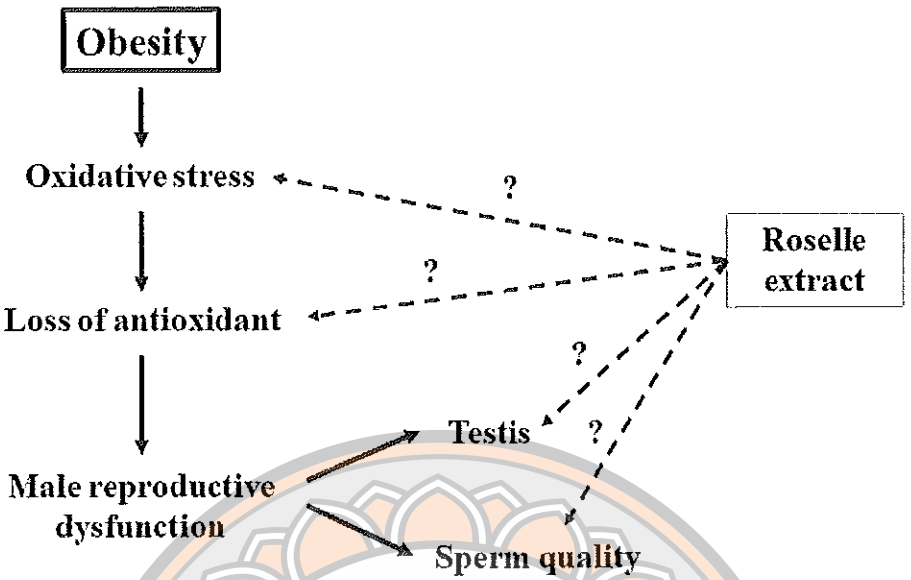


Figure 1 Conceptual research framework

Hypotheses

Roselle supplement improves sperm quality through decrease MDA level and/or increase antioxidant enzyme activity of CAT and SOD in high fat diet induced rats.

Keywords

obesity, *Hibiscus sabdariffa*, sperm quality, testis, oxidative stress

The anticipated outcomes of the study

The results from this study demonstrated the effect of roselle (*Hibiscus sabdariffa*) on oxidative stress and histology in testis as well as sperm concentration, motility, vitality and morphology in high fat diet induced rats.

CHARPTER II

LITERATURE REVIEWS

Obesity

Obesity is defined as excessive amount of body fat resulting from the body receiving an excess of energy over the body's needs. The common cause of obesity is an increased intake of energy-dense foods that are high in fat and a decrease in physical activity (WHO, 2018). The fats usually accumulate around the internal organs and often lead the occurrence of serious diseases such as heart disease, stroke, osteoarthritis and cancer (Hammoud et al., 2008; Lavranos et al., 2012; Lerman et al., 2016).

Obesity is on the rise throughout the world, and in some developed countries two-third of the adult population is either overweight or obese (WHO, 2018). In 2017–2018, the worldwide prevalence of obesity was 42.4%. It has nearly tripled between 1975 and 2016. If the consistent pattern continues, an estimated 58% of the world's population will be overweight and obesity in 2030. (Kelly et al., 2008). Obesity caused 4 million deaths per year worldwide and estimated to increase every year (Weiderpass et al., 2019).

Obesity is generally classified as abdominal obesity and generalized obesity. Abdominal obesity, also known as central obesity, occurs when excessive abdominal fat around the stomach and abdomen has built up (Yusuf et al., 2004). Visceral fat, also known as intra-abdominal fat, is located inside the peritoneal cavity, packed in between internal organs and subcutaneous fat, which is found underneath the skin (Poehlman, &Eric,1998) Generalized obesity is a characteristic of the fat accumulation in organs parts of the body (Purnell, 2018). To prevent overweight and obesity, people should eat and drink according to their nutritional need, get regular exercise, and check their weight regularly (Wirth, Wabitsch, & Hauner, 2014). The aim of obesity treatment is to reach and maintain at a healthy weight. It helps your overall health and reduces the risk of obesity-related complication. Orlistat is currently anti-obesity drug for the long-term use that is approved by the Federal Drug Administration (FDA) (Kumar, & Aronne, 2017). However, the reported that side effects of orlistat include: bowel urgency, frequent

bowel movements, oily evacuation, oily rectal leakage, flatulence with discharge, depression, malaise, lassitude, headache, forgetfulness and diabetic ketoacidosis (Filippatos et al., 2008).

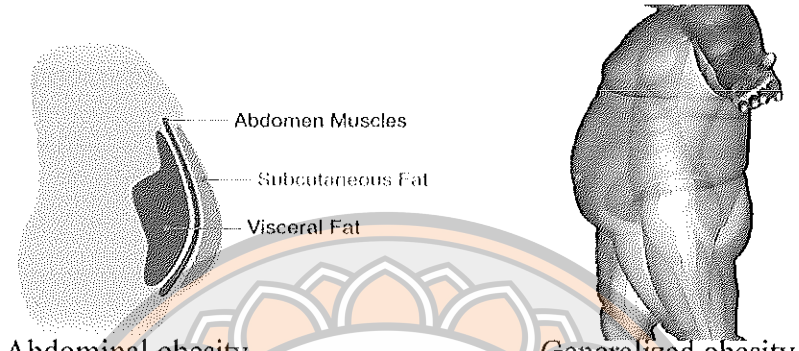


Figure 2 Types of obesity

Sources: https://theconversation.com/; http://thehaemorrhoidcentre.co.uk/

Many epidemiological studies have demonstrated that there are many different measurements for abdominal obesity such as body mass index (BMI), waist circumference (WC) and waist-hip ratio. The common approach for measuring obesity is BMI.

BMI is a simple and widely used method for estimating body fat mass. WHO categorized BMI into four categories; underweight, normal, overweight and obesity. BMI is the relationship between weight and height that is calculated by the following equation.

BMI = weight (kilograms)/height (meters^2)

Table 1 Body Mass Index (BMI) according to WHO and Asian criteria

Classification	BMI (kg/m^2) (WHO)	BMI (kg/m^2) (Asian)
Underweight	<18.5	<18.5
Normal weight	18.5-24.9	18.5-22.9
Overweight	25-29.9	23-24.9
Pre-obese	-	25-29.9
Obesity class I	30.0-34.9	30.0-34.9
Obesity class II	35.0-39.9	35.0-39.9
Obesity class III	>40.0	>40.0

WC is an accurate and simple measure of abdominal obesity. WC was measured at the end of several consecutive natural breaths, at the level parallel to the floor, midpoint between the top of the iliac crest and the lower margin of the last palpable rib in midaxillary line (Norfazilah et al., 2016). The data were analyzed using cutoffs points for Caucasians (94 cm in men and 80 cm in women) and cutoffs points for Asians (90 cm in men and 80 cm in women) (WHO, 2008).

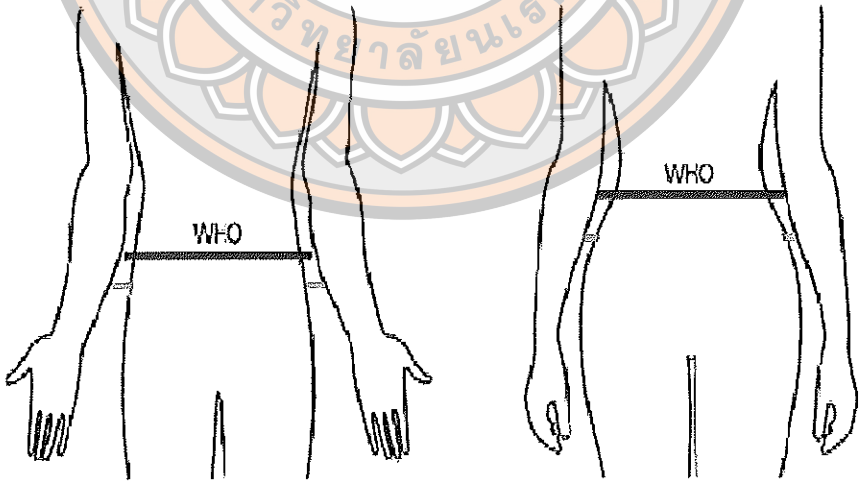


Figure 3 Waist Circumference measurement sites for men and women based on WHO

Sources: Patry-Parisien, Shields, & Bryan, 2012

The BMI of experimental animals is calculated by the following equation.

$$\text{BMI} = \frac{\text{weight (grams)}}{\text{Measure the length from the tip of the nose to the base of tail the white rat (Figure 4) (centimeters)}^2}$$

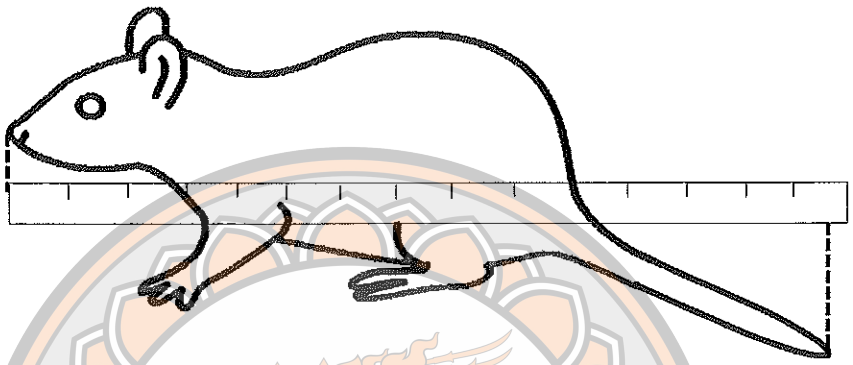


Figure 4 The length from tip of the nose to the base of tail rat

However, a guideline for obesity in rat has not been developed like BMI in human. In several studies, the degrees of obesity were measured by comparing body weight of experimental group fed a high fat diet with control animal fed chow diet. Rats that gained 10-15% more weight than those in the control group were considered obese. (Hariri, & Thibault, 2010).

Obesity has linked to various diseases, particularly metabolic diseases such as hypertension, cardiovascular disease, type 2 diabetes associated with insulin resistance and male infertility (Hammoud et al., 2008; Lavranos et al., 2012; Lerman et al., 2016). People who are overweight or obese always develop diabetes (Lerman et al., 2016). Obesity leads to abnormalities in the metabolizing of carbohydrates resulting in insulin resistance. It blocks the action of insulin hormones which are required for controlling blood sugar levels. High blood sugar and lipid could decrease the elasticity of blood vessels and causes them to narrow and obstructs blood flow leading a high risk of hypertension (Kopelman, 2000). In addition, the high levels of lipids in obese may contribute to block arteries and increase probability of cardiovascular disease or stroke (Chan, Pang, & Watts, 2015). Furthermore, obesity is associated with a group of endocrine abnormalities and reproductive dysfunction, such as obesity-related

secondary hypogonadism, erectile dysfunction and infertility, represent other abnormalities negatively affecting the quality of life of men suffering from obesity but, despite their high prevalence (Vincenzo et al., 2018).

Oxidative stress and Obesity

Obesity is abnormal or excessive fat accumulation is a result of adipocyte hypertrophy and/or hyperplasia resulting in the secretion of adipocytokines and increased lipolysis processes that lead to increased free fatty acid (FFA) (Rzheshevsky, 2013; Savini et al., 2013). FFA is a component of the cell membrane and also accumulates at the epididymal fat pad. Moreover, the increasing of oxidative stress in obesity contribute to lipid peroxidation which the process of oxidative composition of PUFAs and ROS, leading to the formation of mixtures of lipid hydroperoxide (Grattagliano et al., 2007).

Adipose tissue is a principal tissue for energy storage and regulates metabolic homeostasis (Cristancho, & Mitchell, 2011). Adipose tissue dysfunction may induce oxidative stress and develop irregular production of adipokines, which contribute to the pathological systemic. Moreover, the biomarkers of oxidative damage are higher in obese individuals and correlate directly with BMI whereas antioxidant defense markers are lower according to the amount of body fat (Chrysohoou et al., 2007).

Oxidative stress is defined as a state in which excessive oxidation occurs cannot be controlled by the antioxidant systems, creating an imbalance in the body. It not only causes hazardous events such as lipid peroxidation (Niki, 2008), protein oxidation (Dalle-Donne et al., 2003) and DNA damage (Marnett, 1999), also contributes to many pathological conditions, including cancer (Sosa et al., 2013), neurological disorders (Carvalho et al., 2017), atherosclerosis (Kattoor et al., 2017), hypertension (Harrison, & Gongora, 2009), ischemia/perfusion (Dorottya et al., 2013), diabetes (Brahm et al., 2013) and testicular damage (Nematollah et al., 2017).

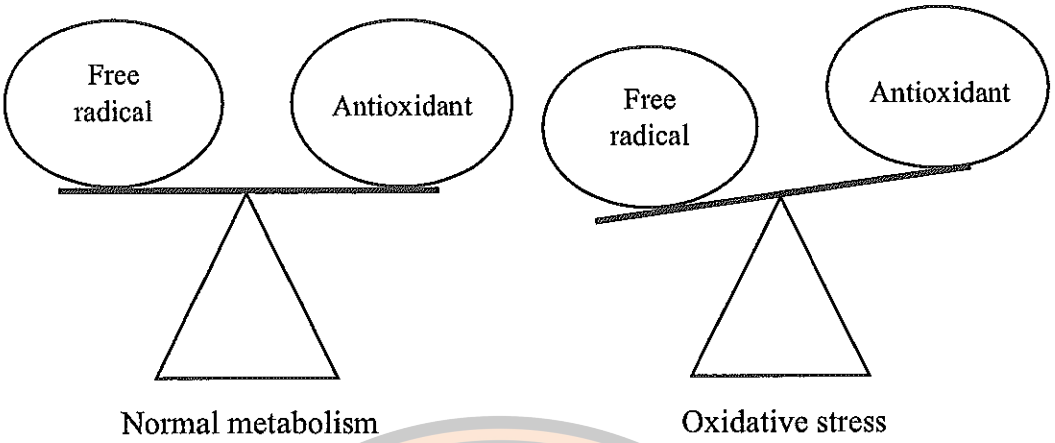


Figure 5 Schematic diagram for normal metabolism and oxidative stress

Source: Adapted from Palipoch, & Koomhin, 2015

Lipid peroxidation can disrupt fluidity and permeability of the cell membranes and damage all cells. Lipid peroxidation can be described as a process under which oxidants such as free radicals or nonradical species attack lipids (Wong-ekkabut et al., 2007). Lipids, especially PUFAs, contain carbon-carbon double bonds and are susceptible to hydrogen abstraction from a carbon, which results in oxygen insertion, creating lipid peroxyl radicals and hydroperoxides. Glycolipids, phospholipids and cholesterol are also well-known targets of damaging and potentially lethal peroxidative modification. In response to membrane lipid peroxidation and according to the specific cellular metabolic and repair capacities of the cells, cell survival may be promoted, or cell death induced (Antonio, Mario, & Sandro, 2014).

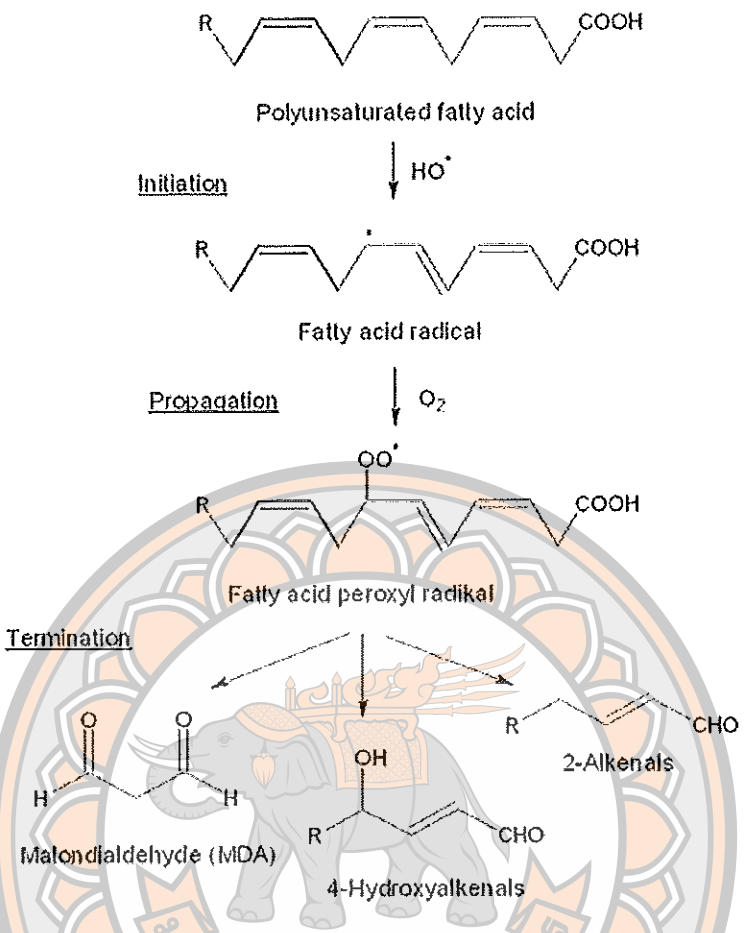


Figure 6 Steps in lipid peroxidation process

Source: https://alchetron.com/Lipid-peroxidation

The overall process of lipid peroxidation consists of three steps: initiation, propagation and termination. In the initiation phase, highly reactive hydroxyl radicals, formed in the Fenton reaction, abstracts the hydrogen atom in the α position relative to the polyunsaturated fatty acid double bond. This result in the formation of fatty acid radicals, which are highly unstable, short-lived intermediate forms that stabilize by abstracting hydrogen from other chemical species, or by reacting with triplet oxygen to generate different radical species, including fatty acid peroxyl radicals. In the termination step, peroxyl radicals transform into nonradical compounds: hydrocarbons, aldehydes, alcohols, volatile ketones and lipid polymers, some of which are harmful (Antonio, Mario, & Sandro, 2014).

Lipid peroxidation is the process of oxidative composition of PUFAs and ROS, leading to the formation of mixtures of lipid hydroperoxide and aldehydic end-products such as 4 hydroxy-2 - nonenol (4 - HNE), F2 - isoprostanes and MDA (Said et al., 2005). Lipid peroxidation in the cell membrane can disrupt fluidity and permeability of the cell membranes and damage all cells. In other words, when the cell membranes are damaged by free radicals, their protective cell is lost and thus the total cell is exposed to risk (Antonio, Mario, & Sandro, 2014).

Free radicals are molecules containing one or more unpaired electrons. When 2 free radicals share their unpaired electrons, non-radical forms are created, allowing them to target macromolecules such as DNA, RNA, lipids and proteins, which prompt cell injury and deregulation of homeostasis by depleting antioxidants and producing reactive species. Free radicals are either classified as reactive oxygen species, reactive nitrogen species, reactive sulfur species or reactive carbon species. ROS is a term that encompasses all highly reactive, oxygen-containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, singlet oxygen and hydrogen peroxide (Alugoju, Dinesh, & Latha, 2015). Most common RNS include nitric acid, nitric oxide and nitroxyl anion, all of which are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage. ROS are further divided into radical and non-radical species (Phaniendra, Jestadi, & Periyasamy, 2015)

Table 2 Type of reactive species

Reactive Species	Reactive Oxygen Species	Reactive Nitrogen Species	Reactive Sulfur Species	Reactive Carbon Species
Radicals	$^{\cdot}O_2^-$ •HO •ROO •RO •HOO	•NO $•NO_2$	•RS •GS •$GSSG^-$	•R •RO •ROO
Non-Radicals	H_2O_2 HOCl $^1O_2$	NO_2^- $ONOO^-$ NO^-		

Source: Adapted from Arjunan, Sharma, & Ptasinska, 2015

An antioxidant inhibits the oxidation reaction thereby preventing cell damage and cell death. Organisms use antioxidant defenses such as antioxidant enzymes and non-enzymes to protect themselves against ROS. These enzymes include SOD, CAT and GPx (Shahidi, 2015). Antioxidant defenses also include non-enzymatic molecules such as natural antioxidants that are found in foods or supplements. These include ascorbic acid (vitamin C) (Smirnoff, 2001), α-tocopherol (vitamin E) (Herrera, & Barbas, 2001), flavonoids (Panche, Diwan, & Chandra, 2016) and the endogenous antioxidant molecule, glutathione (GSH) (Roberta et al., 2005).

SOD catalyzes the dismutation of superoxide anion into oxygen (O_2) and hydrogen peroxide (H_2O_2). The enzyme utilizes zinc and copper as cofactors. CAT is an enzyme found in the mitochondria and peroxisomes system that converts H_2O_2, generated from SOD's catalyzation or from synthesis in the peroxisomes, into water and oxygen molecules. GPx is found in the cytoplasm of mammalian tissues and together with reduced glutathione (GSH) as a cofactor, inactivates H_2O_2 and other peroxides. The oxidized form of glutathione is glutathione disulphide (GSSG). Glutathione reductase converts GSSH to reduced GSH and in cells is predominantly present as GSH in normal physiological conditions. (Sobantu, & Pamela, 2015).

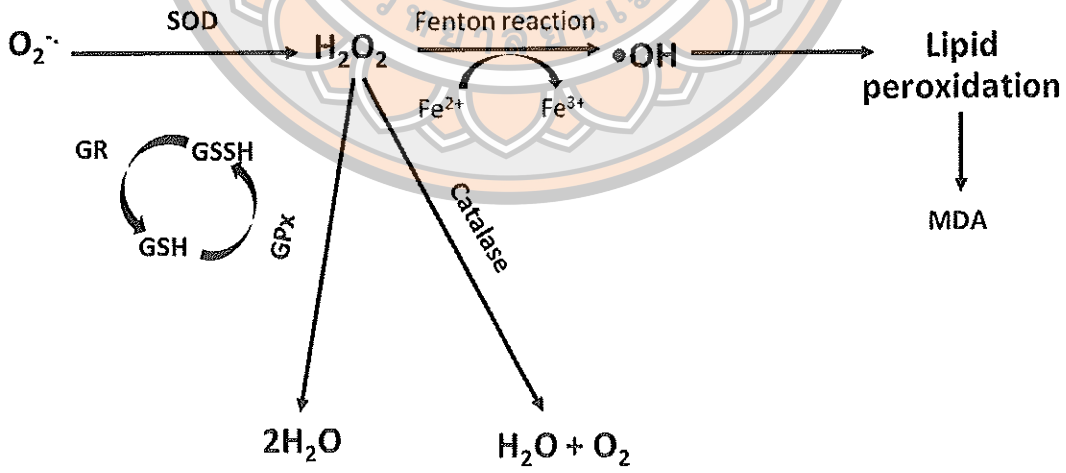


Figure 7 Antioxidant system

Source: Adapted from Sung et al., 2013

Antioxidants have been used extensively to overcome the effects of ROS in several pathologies. The common antioxidants used are vitamins E and C, coenzyme Q, α-lipoic acid, lycopene, and polyphenols. Polyphenols are a class of antioxidants which are widely available in common foods can be quite high. There are several types of polyphenols such as catechins, anthocyanins, procyanidins, resveratrol, and curcumin (Abdali, Samson, & Grover, 2015). Antioxidant have improved benefits in the antioxidant activity is the ability of a substance that contains antioxidants in reducing free radical compounds and inhibiting oxidation reaction in the body (Hasan, Damayanthi, & Anwa, 2018).

Susceptibility to oxidative damage is greater in obesity because of decrease antioxidant, including SOD, GPx, and CAT, vitamin C and flavonoids. The activity of SOD in obese is significantly lower. Moreover, it has been demonstrated that antioxidant supplementation could reduce oxidative stress and ROS decrease the risk of complications related to obesity (Marseglia et al., 2015).In addition, antioxidants have frequently been administered to male infertile in the hope of improving the quality of the semen profile and improve testicular function (Aitken, & Roman, 2013).

Specifically of interest here is that increased production of ROS induces lipid peroxidation in spermatozoa. The reaction of lipid peroxidation occurs in several cell types, including sperm cells, particularly in the plasma membrane which contains a high content of lipid. Therefore, sperm cells are sensitive to oxidative stress (Said et al., 2005). This process, the reaction of lipid peroxidation, damages sperm cells by decreasing the sperm qualities; concentration, motility, viability and normal morphology (Sies, 1997; Du Plessis et al., 2010; Lavranos et al., 2012). In addition, MDA is produced due to the degradation of the peroxides of unsaturated fatty acids and is used as a biomarker to determine the rate of oxidative damage. Testes has been one of the indicators used in previous studies of lipids peroxidation in humans and animals. It is noteworthy that the damage caused by lipid peroxidation is considered to be the most important factor for testicular dysfunction (Peltola et al., 1994).

Complications of Obesity

Obesity leads to various diseases, particularly metabolic diseases such as hypertension, cardiovascular disease, type 2 diabetes associated with insulin resistance and male infertility (Hammoud et al., 2008; Lavranos et al., 2012; Lerman et al., 2016). Obesity leads to abnormalities in the metabolizing of carbohydrates resulting in insulin resistance and blocking of the action of insulin hormones which are required for controlling blood sugar levels. People who are overweight or obese are therefore more likely to develop diabetes (Lerman et al., 2016). Hyperlipidemia occurs when excess lipids triglycerides, LDL and cholesterol levels or decreased HDL levels in the bloodstream. The high levels of lipids may contribute to block arteries and an increased probability of cardiovascular disease or stroke (Chan, Pang, & Watts, 2015). Hypertension refers to the excessive pressure that the blood applies to the inner walls of the arteries and is associated with numerous other diseases that can affect overall health and life expectancy. High blood sugar and high blood lipids can cause arteries to be inflamed or narrowed and a decrease in the elasticity of blood vessels. Obese people have a higher risk of hypertension, which can, in fact, be fatal (Kopelman, 2000).

Obesity in men has been associated with infertility. In numerous studies have identified a worldwide trend of declining semen quality parameters the increasing prevalence of obesity men (Lavranos et al., 2012). In addition, fertility among obese men may be affected by sexual dysfunction, endocrinopathy, aromatization activity and possibly the inflammatory and obstructive elements of epididymitis pathology (Atif, 2015). Impairment of the spermatogenesis by free radicals is a major cause of male infertility which can result in lipid peroxidation and the formation of products such as MDA in the semen, resulting in the reduction.

Male reproductive system

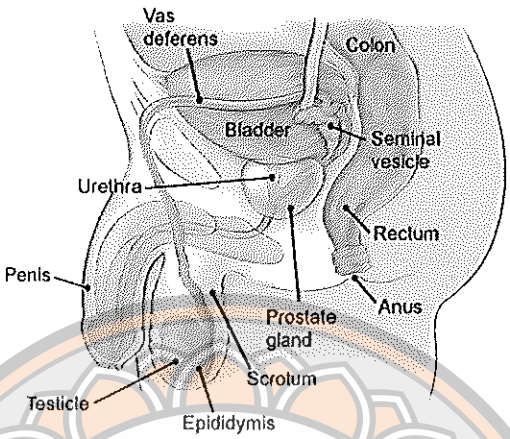


Figure 8 Male Reproductive System

Source: https://steemit.com

The male reproductive system consists of two testicles which are components of both the reproductive and the endocrine system. Therefore, the two main functions of the testicles are: producing sperm and male sex hormones (testosterone). Furthermore, a network of excretory ducts (epididymis, ductus deferens (vas deferens) and ejaculatory ducts), seminal vesicles, the prostate, the bulbourethral glands and the penis (Gerhard et al.,2010).

Testicular Function

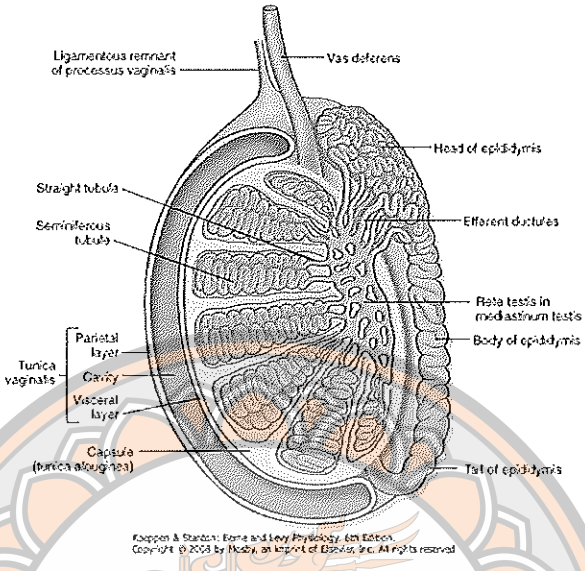


Figure 9 Essentials of anatomy of the Testis

Source: Berne, & Levy, 2008

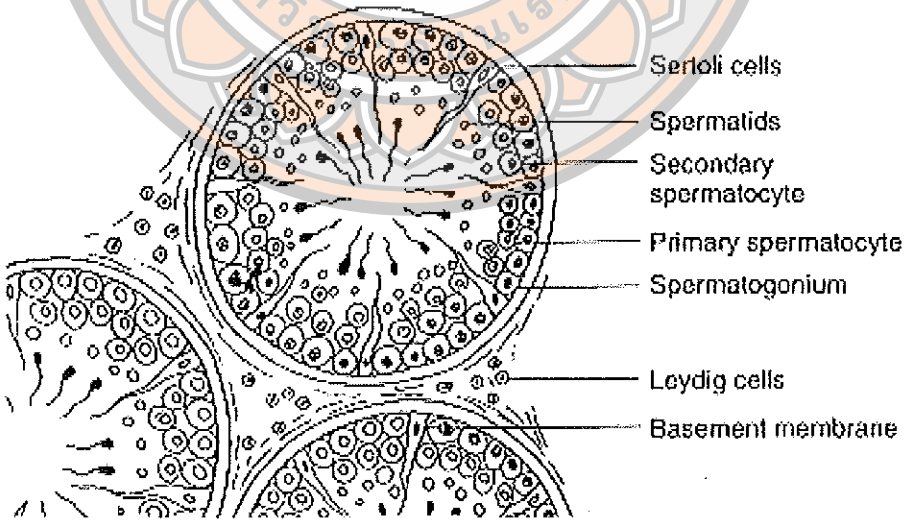


Figure 10 Cross section through the Testis

Source: https://www.ergo-log.com/cola.html

The testes produce the male gametes and the male sexual hormones (androgen). The spermatogenesis and includes all the processes involved in the production of gametes, whereas steroidogenesis refers to the enzymatic reactions leading to the production of male steroid hormones (Weinbauer et al., 2010). The integrity of both compartments is necessary for quantitative and qualitative production of sperm cells. The function of the testis compartments is regulated by the hypothalamus and the pituitary gland. These endocrine effects are mediated and modulated at the testicular by paracrine and autocrine factors control mechanisms (Basciani, et al. 2010).

The most important cells of this compartment are the Leydig cells. These cells are the source of testicular testosterone. They produce and secrete testosterone. Leydig cells are rich in smooth endoplasmic reticulum and mitochondria with tubular cristae. Other important cytoplasmic components are lipofuscin granules, the final product of endocytosis and lysosomal degradation and lipid droplets in which the preliminary stages of testosterone synthesis (Alessandro, Davide, & Antonio, 2017).

Testosterone hormone

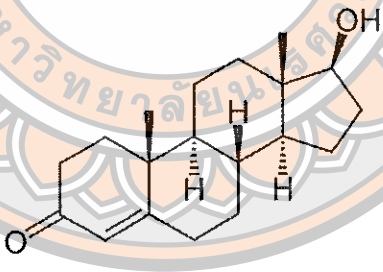


Figure 11 The chemical structure of testosterone hormone

Source: https://www.pngwave.com/png-clip-art-knhnc

Testosterone is an anabolic steroid that is the main sex hormone found in men. It controls male physical features. Testosterone, which is produced in the testes, plays a key role in the development of the male reproductive tissues, the testes and the prostate gland, as well as promoting secondary sexual characteristics such as increased muscle and bone mass and the growth of body hair. In addition, testosterone is involved in the

general health and well-being of men and contributes to the prevention of osteoporosis. Women also have testosterone but in much smaller amounts than in men. (Tuck, & Francis, 2009)

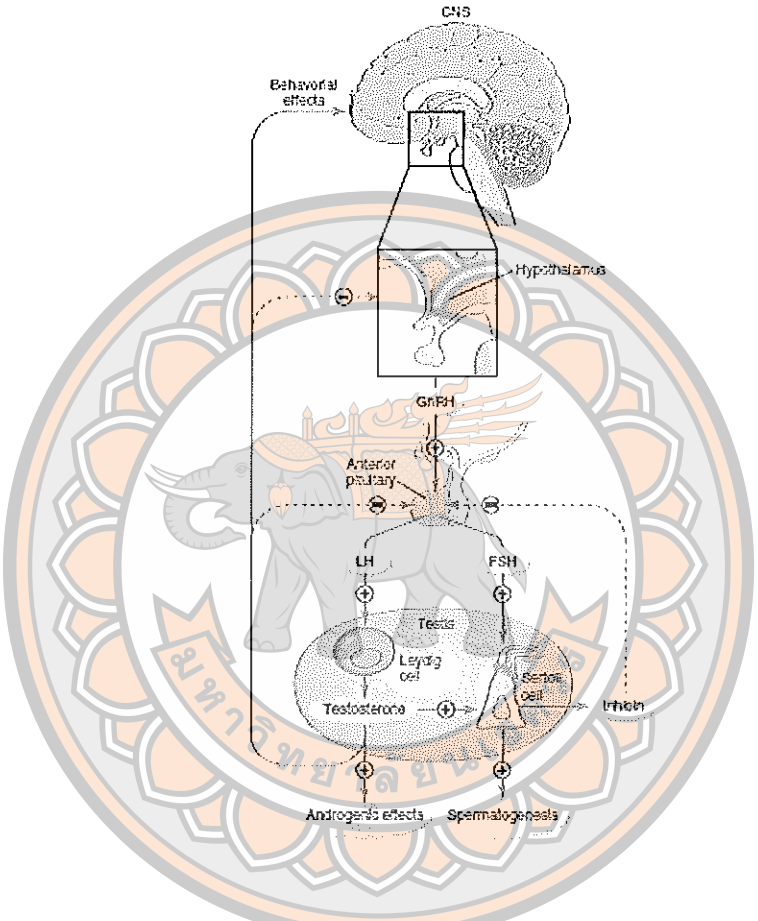


Figure 12 Hypothalamic-pituitary-testicular axis

Source: Hall, Guyton, & Arthur, 2011

The hypothalamic-pituitary-testicular axis refers to the hypothalamus, pituitary gland and testes. Gonadotropin-releasing hormone (GnRH), which is secreted from the hypothalamus, in turn stimulates the anterior portion of the pituitary gland which produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH is the primary stimulus for the secretion of testosterone by the testes and FSH mainly stimulates spermatogenesis by acts on intratubular Sertoli cells. It induces the production of androgen-binding protein (ABP) by means of which testosterone can pass

the Sertoli-Sertoli junctional complexes, as well as the production of activin and inhibin by Sertoli cells which both influences hormone release in hypothalamus and pituitary. While the seminiferous tubules fail to produce sperm, secretion of FSH by the anterior pituitary gland increases. Conversely, when spermatogenesis proceeds too rapidly, pituitary secretion of FSH decreases. The cause of this negative feedback is the Inhibin hormone which is secreted from the Sertoli cells. This hormone has a strong direct effect on the anterior pituitary gland to inhibit the secretion of FSH and provides an important negative feedback mechanism for control of spermatogenesis in parallel to the negative feedback mechanism for control of testosterone secretion (Gerhard et al., 2010).

The HPG axis responds to fluctuations in hormones causing effects on the body and aspects of reproduction. Excess fat accumulation can impair the feedback regulation of the HPG axis and be a contributing factor to decrease sperm quality. The HPG axis imbalance may in turn affect spermatogenesis and male reproductive function. Therefore, irregular hormonal profile and adipose-derived hormone levels, such as with aromatase, leptin, resistin, inhibin B, cytokines, as well as many genetic factors and physical factors. The connection between the enlarge, frequency of global obesity and subfertility (Anthony et al., 2012).

White adipose tissue exhibits elevated aromatase activity and secretion of adipose-derived hormones in abdominal and visceral fat. The Aromatase is an important cytochrome P450 enzyme involved in sexual development and the biosynthesis of estrogens from its precursor androgens, such as testosterone and dehydroepiandrosterone. Obese men show signs of elevated estrogen levels as well as low levels of testosterone and FSH (Cabler et al., 2010). Aromatase overactivity are depleted levels of free and total testosterone. Result in low levels of testosterone and sex hormones are related to a reduction in spermatogenesis and lowered sperm concentrations (Fejes et al., 2006). An inhibitory effect on androgen biosynthesis is observed, to a regulatory role of the HPG axis to cause detrimental effects on spermatogenesis and, in turn, further increasing the possibility of subfertility in obese men (Anthony et al., 2012).

Spermatogenesis

Spermatogenesis is the process of creating mature male gametes that occurs within the seminiferous epithelium on the surface of the somatic Sertoli cells. Functional Sertoli cells are required for normal spermatogenic progression resulting in the continuous production of numerous fertile spermatozoa (Michael, 2016).

Stage 1: The Diploid spermatogonia is situated in the seminiferous tubules which include twice the total number of chromosomes. These replicate mitotically in interphase before the method of meiosis 1 to create 46 pairs of sister chromatids.

Stage 2: the chromatids allow the exchange of genetic information through the synapsis process, which occurs before the division into haploid spermatocytes through meiosis, which is Stage 3.

Stage 3: In this process of division, two new daughter cells will further divide into 4 spermatids, each having unique chromosomes that are approximately half in number to the original spermatogonium.

Stage 4: In this stage, the cells move from the lumen of the testes to the epididymis. They mature and develop into four sperm cells with the growth of microtubules on the centrioles to develop an axoneme. The remaining centrioles elongate and develop into the sperm tail (Mäkelä, & Toppari, 2017).

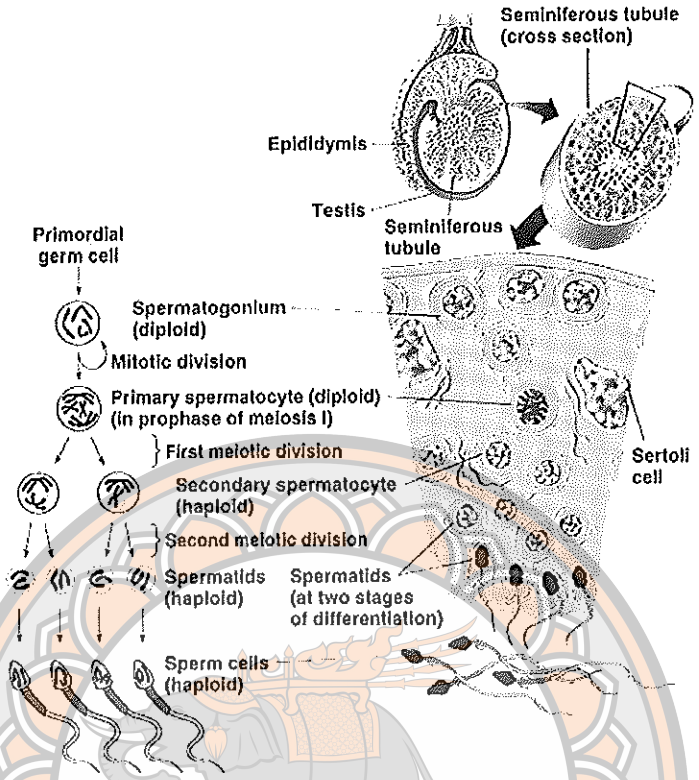


Figure 13 Spermatogenesis

Source: Benjamin Cummings, 1977

Structure of sperm

1. Head: is spherical in shape and consists of a large nucleus and a dome shaped acrosome present on the nucleus.

Function: The nucleus contains genetic information and half the number of chromosomes. The acrosome releases a hyaluronidase enzyme which destroys the hyaluronic acid of the ovum and enters the ovum.

2. Neck: contains the proximal centriole and distal centriole.

Function: The distal centriole gives rise to the axial filament of the sperm which runs to the end of the tail.

3. Middle piece: is a tubular structure in which the mitochondria are spirally arranged.

Function: The middle piece is called the powerhouse of the sperm because it gives energy to the sperm to swim in the female genital tract.

4. Tail: arises from the middle piece and is the end part of the sperm. It contains axial filaments.

Function: The tail is the main moving part of the sperm and propels the sperm along the female genital tract.

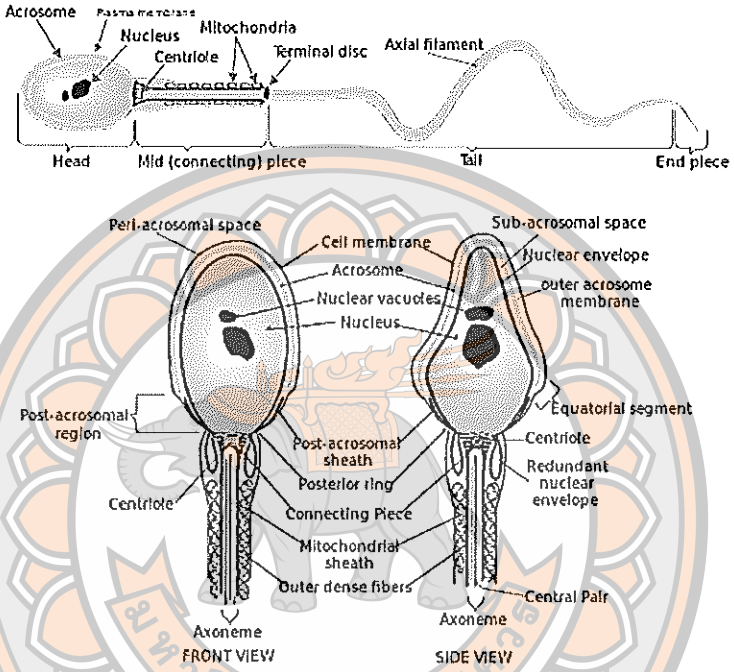


Figure 14 Structure of human sperm

Source: Adapt from Elgeti, Winkler, & Gompper, 2015

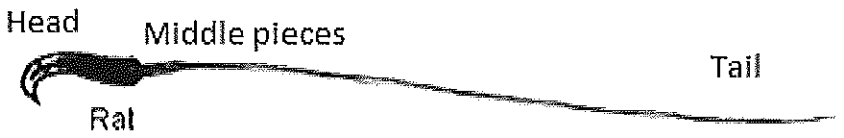


Figure 15 Structure of rat sperm

Source: Adapt from https://cit.vfu.cz/frvs2011

Then the female gametes will be fertilized to create a zygote, which is a single-celled organism. Immediately on fertilization, cell division and multiplication occur to create a fetus (Michael, 2016).

Obesity and the male reproductive system

A decline in fertility rates in obesity is becoming an increasingly prevalent issue worldwide (Lavranos et al., 2012). Obesity also affects male infertility through reducing the sperm quantity and motility, thus decreasing fertilization rate (Bakos et al., 2011; Hammoud et al., 2012.) Several studies have shown that obesity has a relevant negative effect not only on sex hormones levels but also on the overall sexual function. In addition, according to some studies, adipose tissue enlargement appears to influence also gonadal activity and spermatogenesis, leading to the decreasing of fertility in obese males. Obesity associated with hypogonadism with a lower testosterone level, poorer sperm quality, and reduced fertility thus ameliorating reproductive and sexual health (Vincenzo et al., 2018). Adipose tissue plays a primary role in the metabolism of hormones secreted by other glands, including sex hormones in both men and women (Kirschner, & Samojlik, 1991). Furthermore, a number of studies have suggested that increase in sperm DNA damage and sperm intracellular reactive oxygen species (ROS), which induces oxidative stress, is rationally linked to diet-induced obese male (Bakos et al., 2011; Fernandez et al., 2011). A high BMI and fat accumulation develop the oxidative stress. Although ROS is needed in the normal sperm function for such as capacitation and acrosomal reaction, the excessive levels of ROS can decrease sperm quality (Fernandez et al., 2011). Impairment of the spermatogenesis by free radicals is a major cause of male infertility which can result in lipid peroxidation and the formation of products such as MDA in the semen, resulting in the reduction of the sperm function.

Previous studies found that sperm concentration and sperm motility are fundamental variables that affect the viability of the sperm. Reduction of sperm motility, due to many possible factors, has been associated with male infertility. One important factor is oxidative stress caused by ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl (ROO-) and hydroxyl radicals (OH^-). ROS plays an important role in processes such as maturation, capacitation, acrosome reactions and fertilization. ROS pathology is caused by external sources such as leukocytes and immature or

abnormal sperm as well as environmental factors such as smoking and alcohol. The effect of PUFAs within the sperm cell membrane is increased ROS, which is also associated with decreased sperm motility (Colagar, Karimi, & Jorsaraei, 2013). However, the low levels of ROS are necessary in maintaining cellular homeostasis with their counteracting scavenging species, antioxidants, as well as in processes sperm maturation (Agarwal, 2006). Subsequently, this elevated production of ROS defense has been shown to have adverse effects on sperm function. Furthermore, ROS decreased motility and fertilization capacity from a lack of antioxidant-defense mechanisms in the testis and epididymis, rendering sperm extremely susceptible (Anthony et al., 2012). Sperm defect ultimately results in increased ROS production through activation of the NADPH system, providing electrons for free radicals to initiate a series of events, eventually activating NADPH oxidase (Anthony et al., 2012). Moreover, superoxide, was enhanced enzymatic activity and NO production appears to increase ROS, subsequently impairing sperm function (Anthony et al., 2012). Previous study has shown that the elevated levels of ROS in their semen, serving as biomarkers of oxidative stress from ROS-induced LPO and DNA damage, both of which decrease semen function and contribute to male infertility (Agarwal, 2006; Saleh et al., 2003). Recent studies, such as that by the World Health Organization (WHO), have found that male obesity affects sperm quality parameters such as sperm concentration, sperm motility and sperm morphology (Palmer et al., 2012). They found that a high fat diet can lead lower sperm concentration. Leisegang, et al. reported the relationship of BMI to sperm quality and strongly suggested adverse effects on sperm concentration, sperm motility, sperm viability and sperm morphology in morbidly obese men (Leisegang et al., 2014). An increase of ROS is triggered via high metabolic rates in order to maintain homeostasis in obese men. In localized areas near the testes, it can disturb spermatogenesis and result in a possible failure to discard the residual body into the Sertoli cell. As testicular spermatozoa with proximal cytoplasmic retention lack enough cytoplasmic reductive enzymes to control free radicals, a decline in antioxidant scavenging would allow for a higher susceptibility to ROS impairment. The resulting imbalance of oxidative stress is linked to sperm dysfunction (Anthony et al., 2012).

From the above evidence, it seems certainly obvious to suggest that ROS induced oxidative stress has an enormous impact on male fertility, as well as on the detrimental obesity-implicated consequences. It is plausible to suggest that an increased level of ROS in the testicular may be due to an accumulation of white adipose tissue. This collection of lipocytes would lead to an augmented ROS production in the testis's environment. ROS generation in the testes may denature enzymes involved in spermatogenesis, providing further evidence for a link between obesity and male infertility (Cabler et al., 2010).

Roselle (*Hibiscus sabdariffa*)

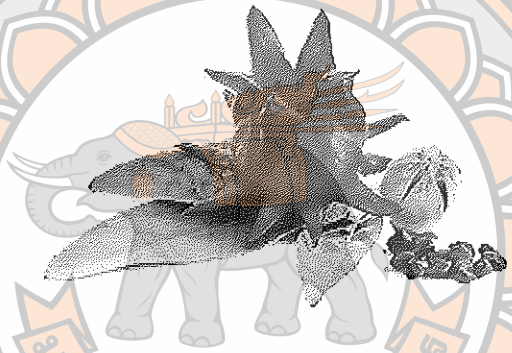


Figure 16 Roselle (***Hibiscus sabdariffa***)

Source: https://www.allwinfoodthailand.com

Hibiscus sabdariffa, also known as 'roselle' in English and 'Karkadee' in Arabic, is a native plant of China and Thailand and can be found in many tropical and subtropical areas of the northern hemisphere. It is widely used in Thailand, Mexico, China, Egypt and Western countries (Badreldin et al., 2011). Roselle is an annual crop used in food, nutraceuticals, cosmeceuticals and pharmaceuticals. The calyces, stems and leaves are benefit. The juice from roselle is claimed to be a health-enhancing drink due to its high content of anthocyanins, vitamin C and other antioxidants. Traditionally, roselle is used as folk medicine for many conditions such as management of hypertension, liver disease, nerve disorder, cancer, anti-constipation and circulation promotion. Furthermore, the use of roselle has been reported to anti-obesity in several studies by regulation of various metabolic pathways, including lipid absorption, intake

and expenditure of energy, increase of lipolysis and decrease proliferation of adipocyte cells. The bioactive components of roselle comprise of anthocyanins, polyphenolic acids and organic acids (Ali et al., 2005; Da-Costa-Rocha et al., 2014).

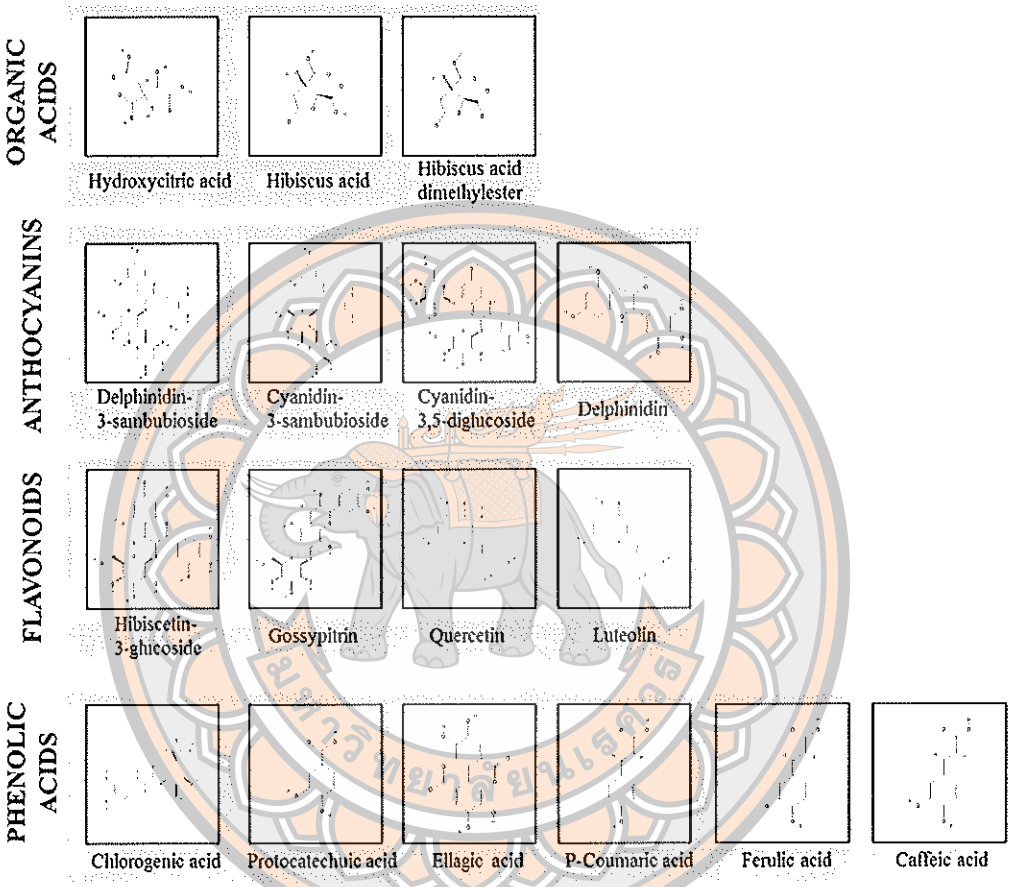


Figure 17 Chemical structural formula of major bioactive compounds in roselle

Source: Ojulari, Lee, & Nam, 2019

Table 3 Various phytochemicals in the petals extract of *Hibiscus sabdariffa*

Compounds	Contents
Anthocyanins	60.26 %
Flavonoids	26.98 %
Polyphenols	12.76 %

Source: Pacome et al., 2014

Roselle is rich in anthocyanins which confer its antioxidant activity (Ajiboye et al., 2011). Anthocyanins are a very large group of red plant pigments that occur in all higher plants, mostly in flowers and fruits (Mazza, 1995). Additionally, anthocyanins exhibit potential effects such as protection of DNA damage, boosting immunity, inhibition of lipid peroxidation, anti-inflammatory activity and decreasing capillary permeability (Lefevre et al., 2004).

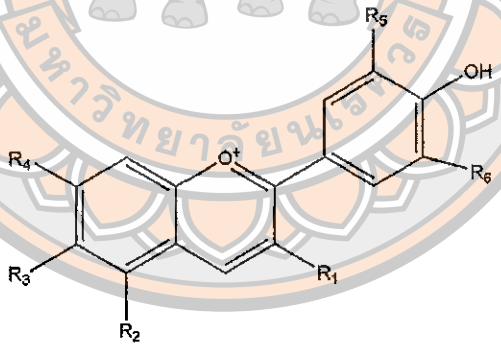


Figure 18 The chemical structure of anthocyanin

Source: https://www.pathway27.eu

Previous studies have reported that roselle decreased the levels of total lipids, cholesterols and triglycerides, suggesting the possibility that roselle functions as an agent for the prevention of obesity (Sheba, 2016). The anthocyanin pigments have been confirmed as having a beneficial effect on various cells, such as being an antioxidative substance that attenuates the negative effects of oxidative stress (Ali et al., 2005; Rodriguez-Medina et al., 2009). Previous studies have shown that roselle aqueous extract was administered orally 100 mg/kg B.W. for 28 consecutive days can be ameliorated sperm defects in streptozotocin-induced diabetic rats (Md Idris et al., 2012). Aqueous extract of roselle was treated with 200 mg/kg/day orally for 8 weeks can be ameliorated carbamazepine-induced oxidative stress responses in the testes of Wistar rats (Suleiman et al., 2015). In addition, the ethanol extract of roselle 1 g/kg B.W./day for 26 consecutive days using an oral tube can be used as a treatment against cisplatin-induced testicular damage and oxidative stress in rats. It can improve sperm quality; roselle increases the activities of antioxidant enzymes such as CAT and SOD by decreasing the MDA level of the sperm cells (Amr Amin et al., 2006). The treatment of roselle extract at the dose of 300 and 600 mg / kg B.W. for 30 days can be optimized for the management in the attenuation of Carbon tetrachloride (CCl_4) mediated oxidative stress (Victor, Ugorji, & Adeyinka, 2014).

CHAPTER III

RESEARCH METHODOLOGY

Instrument and materials

1. Instrument

1.1 Makler counting chamber (Irvine Scientific, USA)

1.2 Centrifuge (TOMY digital, Japanese)

1.3 Analytical Balances (Mettler Toledo, USA)

1.4 Hotplate stirrer (Daihan Labtech Co.Ltd., Korea)

1.5 Vortex (Labnet International, Inc., USA)

1.6 Shaker (Fisher Biotec, Australia)

1.7 Heat box (Labnet International, Inc., USA)

1.8 Microplate reader (Labsystems, Finland)

1.9 pH meter (Mettler Toledo, USA)

1.10 Microscope (Olympus, Japan)

1.11 Microscope slides (Sail brand, China)

1.12 Cover glasses (Menzel-Glaser, Germany)

1.13 Spectrophotometer (Bara Scientific Co., Ltd., Thailand)

1.14 Microtome (Leica, Germany)

1.15 Tissue processor (Leica, Germany)

1.16 Water bath (Medax, Italy)

1.17 Tissue Embedding Center (Leica, Germany)

2. Materials

2.1 Phosphate buffered saline (PBS) (SIGMA-ALDRICH®, USA)

2.2 Sodium dodecyl sulphate (SDS) (SIGMA-ALDRICH®, USA)

2.3 Acetic acid solution (SIGMA-ALDRICH®, USA)

2.4 Thiobarbituric acid (TBA) (SIGMA-ALDRICH®, USA)

2.5 1,1,3,3- tetramethoxypropane (SIGMA-ALDRICH®, USA)

2.6 Pierce™ BCA protein assay kit (Thermo fisher scientific inc)

2.7 Permount (Thermo Fisher®)

2.8 Diff-quik staining kit (RVL supply, Thailand)

2.9 Catalase from bovine liver (SIGMA-ALDRICH®, USA)

2.10 Hydrogen peroxide solution (SIGMA-ALDRICH®, USA)

2.11 Disodium hydrogen phosphate (EMSURE®, Germany)

2.12 Potassium dihydrogen phosphate (SIGMA-ALDRICH®, USA)

2.13 Pyrogallol (SIGMA-ALDRICH®, USA)

2.14 Ethylenediaminetetraacetic acid (EDTA) (EMSURE®, Germany)

2.15 Tris base (EMSURE®, Germany)

2.16 Formaldehyde (RCI Labscan, Australia)

2.17 Absolute Ethanol solution (RCI Labscan, Australia)

2.18 95% Ethanol solution (TTK science, Thailand)

2.19 Paraplast (Leica, Germany)

2.20 Xylene (RCI Labscan, Australia)

2.21 Lithium carbonate (EMSURE®, Germany)

2.22 Hematoxylin (C.V. LaboratoriesCO., LTD., Thailand)

2.23 Eosin (C.V. LaboratoriesCO., LTD., Thailand)

2.24 Nigrosin (SIGMA-ALDRICH®, USA)

Plant material and preparation of extract

The water extraction of roselle powder was purchased from Specialty natural products Co., Ltd. (Chonburi, Thailand). Two hundred milligrams of roselle powder was mixed with 1 ml of distilled water and freshly prepared before use.

Animals

Five-week-old male Sprague Dawley rat were obtained from National Laboratory Animal Center, Salaya, Mahidol University, Nakhon Prathom, Thailand.

Rats were housed in the animal room at the Center for Animal Research, Naresuan University. They were housed in group of 2 animals under a standard light-dark cycle, constant humidity and controlled temperature (22±1°C). Control rats fed with standard chow diet and ad libitum water. Obese rats were induced by high fat diet. All animal protocols were ethically approved by the institutional animal care and committee of Naresuan University, Thailand ethic number: NU-AE 580713.

Methods

1. Experimental procedure

Twenty-eight Sprague Dawley rats were used in this experiment. The rats were acclimated to housing for 7 days before the beginning of the experiment. They were housed in the cages, under controlled 12 h light-dark cycle and temperature condition at 22±1°C. They were divided into four groups of seven animals (n=7/group). The first group was used as the control and fed a normal diet, the second group was fed a high fat diet (HFD), the third group was fed a high fat diet with roselle 250 mg/kg B.W. (HFD+L). for oral administration and the fourth group was fed a high fat diet with roselle 500 mg/kg B.W. (HFD+H). for oral administration. A guideline for obesity in rat, the degrees of obesity has been measured by comparing body weight of experimental group fed a high fat diet with control animal fed chow diet. Rats that gained 10-15% more weight than those in the control group were considered obese. (Hariri and Thibault, 2010). This process took place over an 8-week period and weighed once a week. At the end of the 8-week experimental period, the rats were sacrificed using pentobarbital sodium 50 mg/kg. Their testes, epididymis and vas deferens were removed, weighed and dissected. Sperm obtained from epididymis and vas deferens were used for sperm quality analysis.

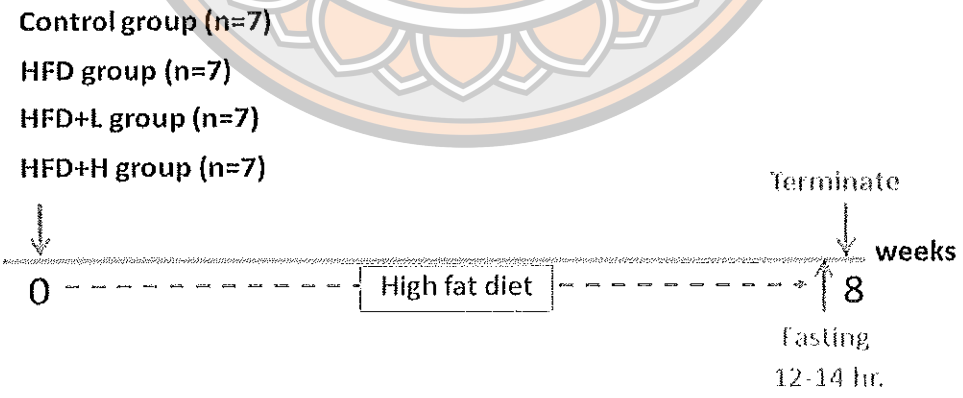


Figure 19 Experimental design

2. Sample collection and preparation

For sperm collection, the epididymis and vas deferens were squeezed using forceps in a 4-well plate containing 1 milliliter of PBS at 37°C. For biochemical analysis, the testicular tissues were homogenized in 1 milliliter of PBS at 37°C and collected in microcentrifuge tube. The samples were centrifuged at 3500g at 4°C for 20 minutes and the supernatants were collected and stored at -80°C until analysis.

3. Sperm concentration and motility

Sperm concentration and motility were examined by loading ten microliters of the sperm suspension on the Makler counting chamber and observed under light microscope. The sperm concentration and motility were analyzed under 20x bright-field objective microscope. They were conventionally investigated in duplicate by a double-blind experiment operator using video offline. The number of spermatozoa counted in any strip of 10 squares in millions per milliliter indicates their average of sperm concentration. The total of spermatozoa of all squares were counted for motility and categorized as motile refers to sperm that are swimming in a mostly straight line or in very large circles or swim in very tight circles and non-motile refers to sperm that have completely lost their ability to rhythmically swing its tail and move constantly in a forward direction and reported as the percentage of sperm motility. The sperm concentration and the motility were evaluated according to the method described by the World Health Organization (WHO 2010).

$$\text{Sperm concentration level average} = \frac{\text{The number of sperms in 3 rows}}{3}$$

$$\text{The percentage of sperm motility} = \frac{\text{Motile sperm number}}{\text{Total sperm number}} \text{x} 100$$

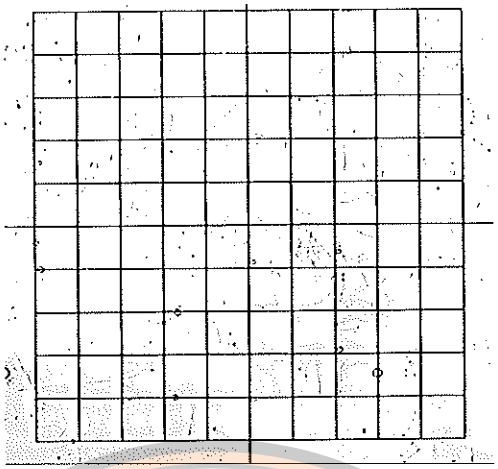


Figure 20 Sperm in Makler counting chamber

4. Sperm viability

Sperm viability was evaluated by using the eosin and nigrosin staining technique and was carried out according to WHO standards, 1% eosin and 10% nigrosin was prepared in distilled water. Ten microliters of sperm suspension were mixed with 1% eosin. After 30 seconds, an equal volume of nigrosin was added to this mixture and then smears were prepared and observed by light microscopy at 400x magnification. The viable sperm was calculated, viable sperm remained colorless (unstained heads) while nonviable sperm stained pink or red head (Fig. 21). The viability of at least 200 spermatozoa was assessed in duplicate by a double-blind experiment operator, and the percentage of viable sperm was calculated.

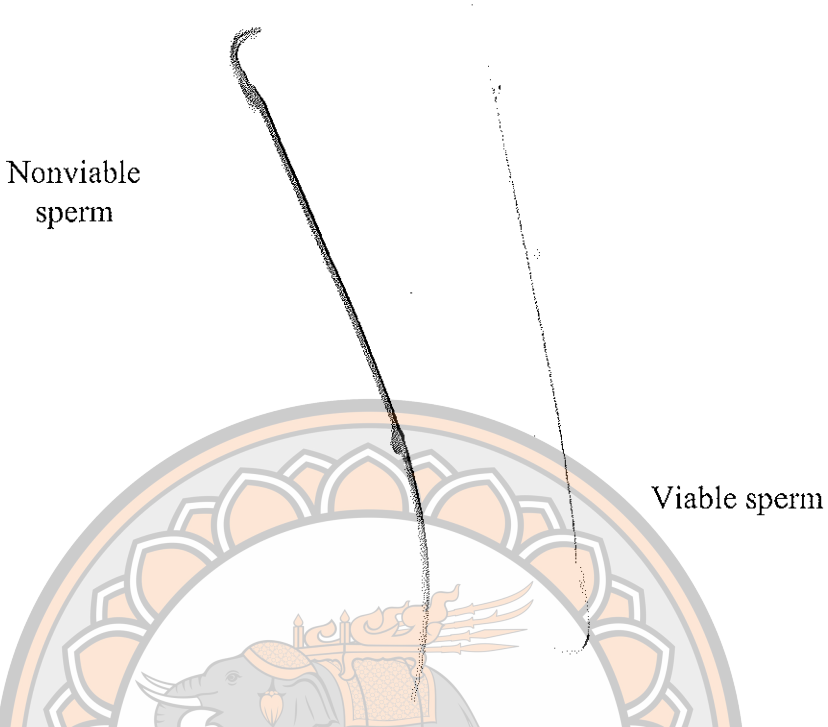


Figure 21 Evaluation of rat sperm viability using eosin- nigrosin staining

5. Sperm morphology

To study sperm morphology, the ten microliters of sperm suspension was spread onto a glass slide and allowed to air-dry at room temperature. The smears were then stained with Diff- Quik staining kit. The normal and abnormal sperm morphology of at least 200 spermatozoa was assessed in duplicate by a double-blind experiment operator. The percentage of normal and abnormal sperm morphology were calculated and evaluated at magnification of 400x. An abnormal sperm morphology shows the defects of head, midpiece or tail (Fig.22).

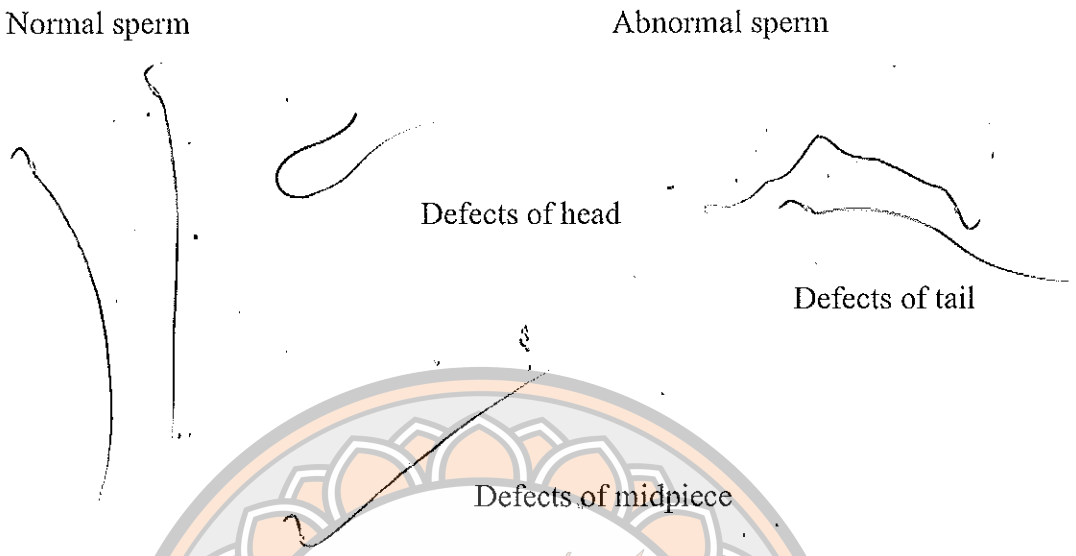


Figure 22 Types of sperm morphology alterations

6. Thiobarbituric acid reactive substance (TBARS) assay

TBARS was expressed in terms of MDA equivalents. The testes were placed into an ice-cold PBS. A mixture of standard or sample, 8.1 % SDS, 20% acetic acid solution and 1 % TBA were placed in a centrifuge tube and vortexed. The mixtures were incubated at 95°C for 1 hour and were then added into each well of a 96-well plate. All these reactions were done in duplicate. Absorbance was read at 532 nm on a microplate reader and MDA levels were calculated using a standard curve (Figure 23). TBARS results were expressed in nmoles/mg protein.

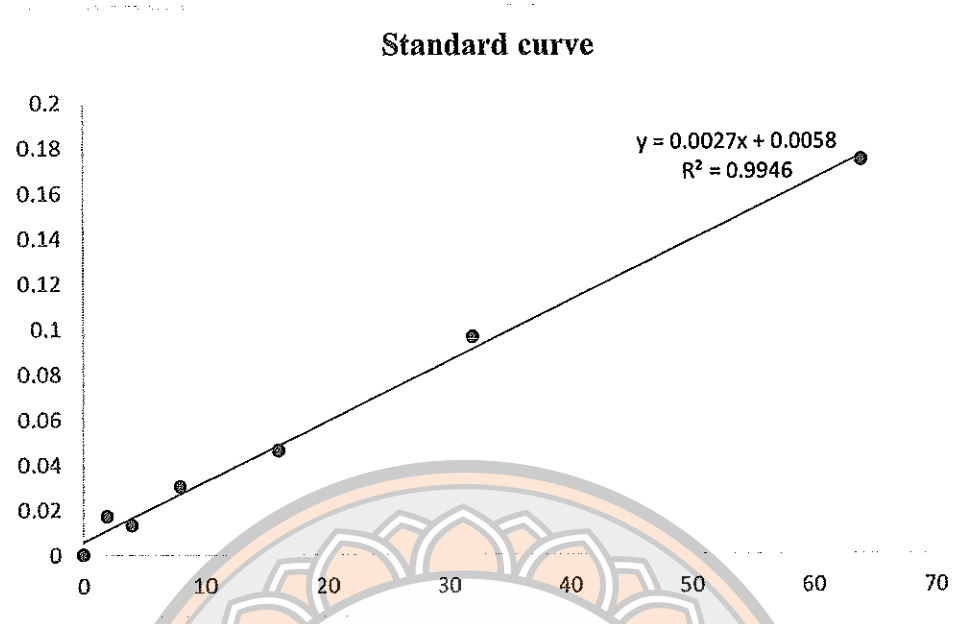


Figure 23 MDA standard curve

7. Determination of catalase (CAT) activity

CAT activity was determined according to the method of Beer and Sizer (1952). CAT is an antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen. The method was based on the decomposition of the hydrogen peroxide by the CAT which shows a continual increase in absorption with a decreasing wavelength. A mixture of 2 ml of catalase assay buffer (0.5 M, pH 7) and 1 ml of hydrogen peroxide working solution were mixed with a 1 ml volume of blank (distilled water), standard, or sample. The absorbance was read at 240 nm for 3 minutes at 30-second intervals using a spectrophotometer. The catalase assay buffer was prepared with disodium hydrogen phosphate and dipotassium hydrogen phosphate and the hydrogen peroxide working solution were prepared with 0.34 ml of hydrogen peroxide together with 100 ml of catalase assay buffer. The activity of the CAT activity was calculated using U/mg protein extinction coefficient 40 cm^{-1}.

CAT activity calculation:

$$\frac{Abs_{240}\text{ (Blank)} - Abs_{240}\text{ (Reaction)/min} \times 1000 \times 3}{40 \times \text{mg protein in sample}}$$

8. Determination of superoxide dismutase (SOD) activity

SOD activity was determined according to the method of Marklund and Marklund (1974) by measuring the inhibition of pyrogallol auto-oxidation by SOD. The reaction mixtures contained 100 µl volume of blank (distilled water), standard, or sample, together with 1000 µl of Tris-EDTA buffer and 1000 µl of 0.2 mM pyrogallol. The Tris-EDTA buffer was made up of 50 mM Tris-HCL with 1 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.2. The absorbance will be read at 420 nm at 30-second intervals for 3 minutes using a spectrophotometer. The activity of the SOD activity was calculated as U/mg of protein.

SOD activity calculation:

$$\text{SOD activity (Units)} = \frac{(\Delta\text{OD of pyrogallol} - \Delta\text{OD of sample})}{(\Delta\text{OD of pyrogallol})} \text{x}100$$

One unit of enzyme corresponds to 50% inhibition of the ΔO/D.

$$\text{SOD unit activity (Units)} = \frac{\text{SOD activity}}{50} \text{xsample dilution}$$

$$\text{Specific activity of SOD (Units/mg of proteins)} = \frac{\text{SOD unit activity}}{\text{mg of proteins}} \text{xsample dilution}$$

9. Determination of protein concentration

Protein concentration was done using the Bicinchoninic Acid (BCA) method using bovine serum albumin (BSA) as a standard. Then 25 µl of sample or standard and 200 µl of BCA working reagent were added into each well of a 96-well plate. All these reactions were done in duplicate. The 96-well plate then was covered, shaken for 30 seconds and incubated at 37°C for 30 minutes. Absorbances were read at 562 nm using a microplate reader and protein concentrations were calculated using a standard curve (Figure 24).

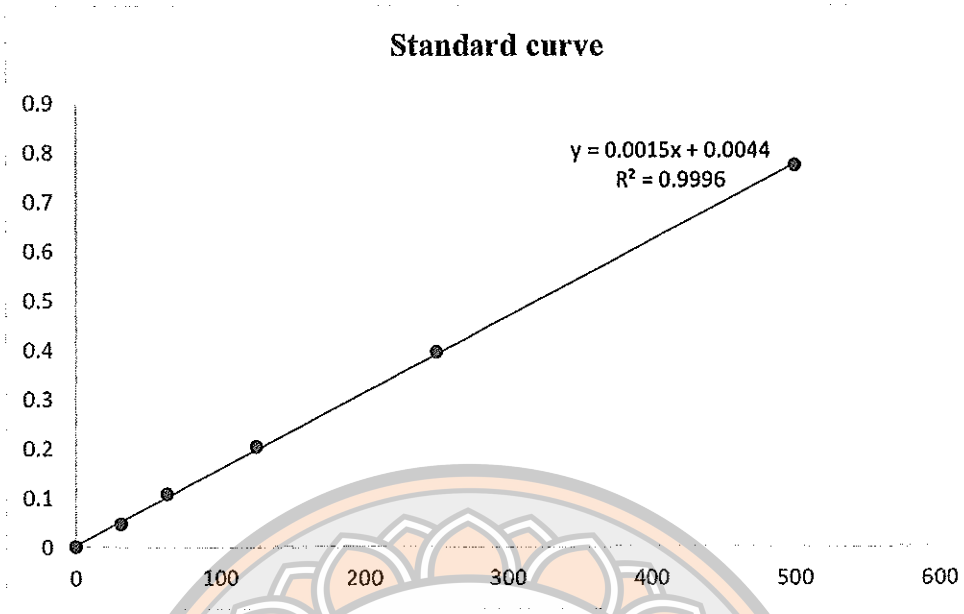


Figure 24 Protein standard curve

10. Histological study of testes

For the histological study, testes were fixed in 10% neutral buffered formalin for at least 24 hours. After that, the tissues were processed according to the routine method including tissue dehydration in ascending grades of alcohol, then cleared in xylene and embedded in paraffin to form blocks of tissue. The tissue block was sectioned at a thickness of 5 μm, using a microtome. The tissue sections were stained with hematoxylin and eosin (H&E) for histological observation under a light microscope.

Table 4 Tissue processing procedure

Number	Chemicals	Time (min.)
1	70% Ethanol	30
2	80% Ethanol	30
3	85% Ethanol	30
4	90% Ethanol	30
5	95% Ethanol (I)	30
6	95% Ethanol (II)	30
7	100% Ethanol (I)	30
8	100% Ethanol (II)	30
9	Xylene (I)	60
10	Xylene (II)	120
11	Paraplast	120
12	Paraplast	120
	Total time	660 min. (11 h)

Table 5 Hematoxylin and eosin (H&E) staining procedure

Number	Chemicals	Time
1	Xylene I	5 min.
2	Xylene II	5 min.
3	Absolute Ethanol	3 min.
4	Absolute Ethanol	3 min.
5	95% Ethanol	1 min.
6	95% Ethanol	1 min.
7	Tap water	5 min.
8	Hematoxylin	10 min.
9	Tap water	10 min.

Table 5 (cont.)

Number	Chemicals	Time
10	1% Lithium carbonate	10 dip
11	Tap water	1 min.
12	95% Ethanol	1 min.
13	Eosin	12 s.
14	Tap water	2 min.
15	95% Ethanol	10 dip
16	95% Ethanol	10 dip
17	Absolute Ethanol	20 dip
18	Absolute Ethanol	20 dip
19	Xylene	5 min.
20	Xylene	5 min.
21	Mounting with permount medium	-

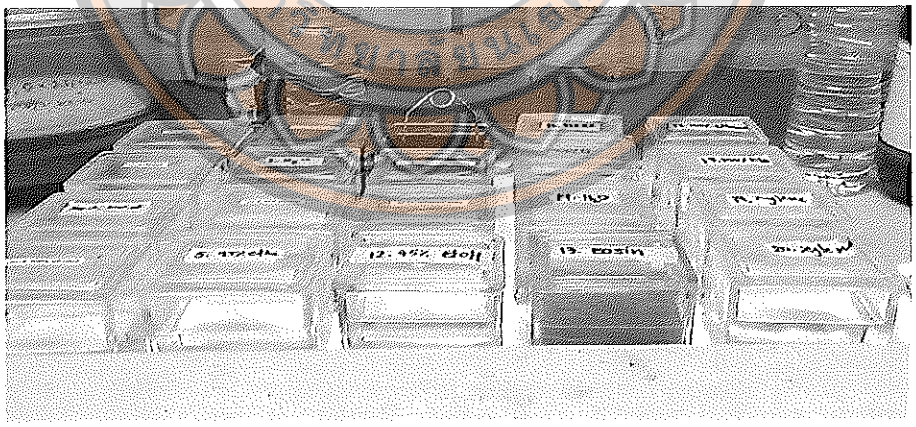


Figure 25 Hematoxylin and eosin (H&E) staining

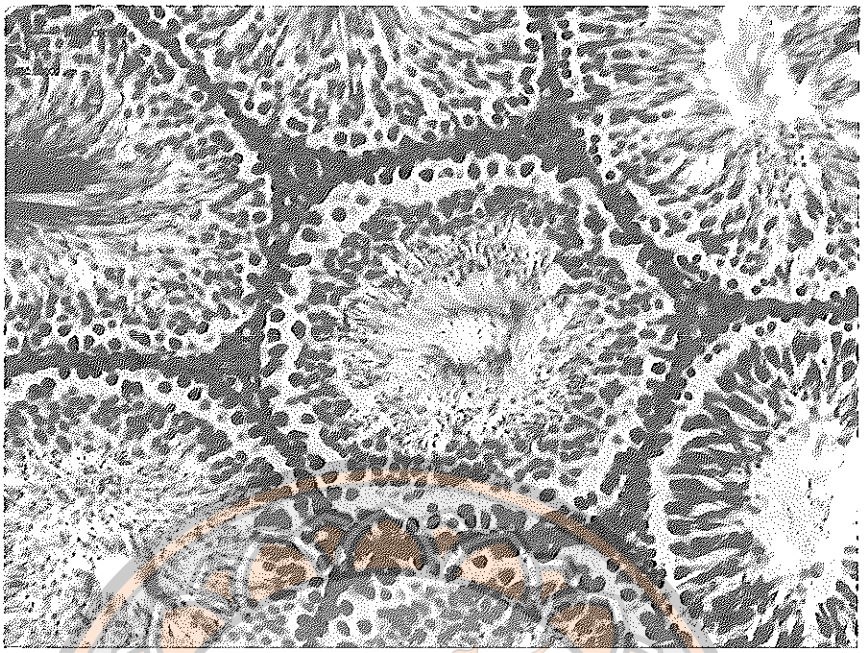


Figure 26 Histology of the testes

10.1 The diameter of seminiferous tubules and seminiferous epithelium height

The mean seminiferous tubule diameter and seminiferous epithelium height were determined in 20 cross sections, made as circular of each animal, using a light microscope in combination with the "SXView program" tool. These morphometric analyses were performed with a 10X objective. The height of the seminiferous epithelium was measured from the basal membrane to the luminal edge (Figure 27 and 28).

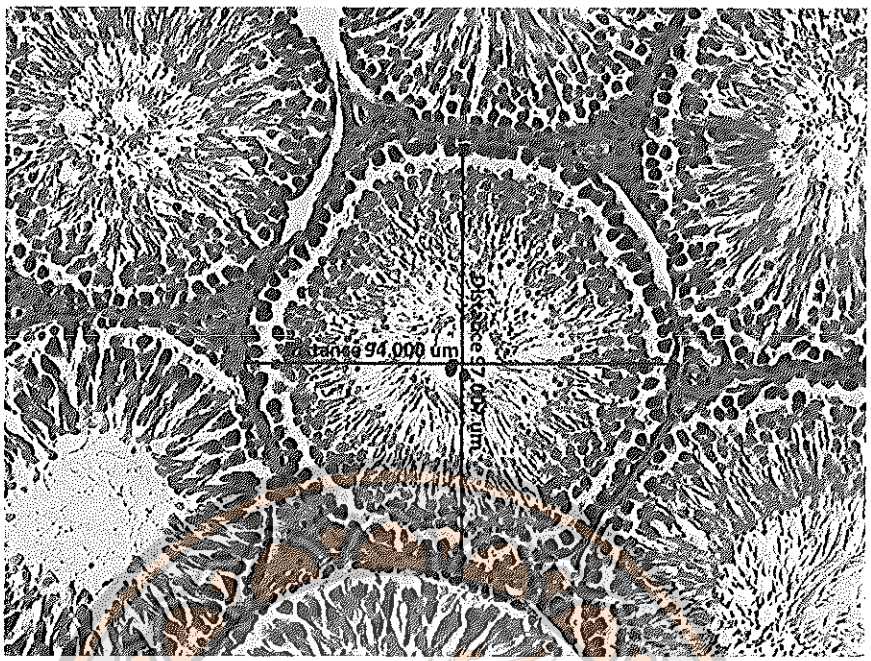


Figure 27 The seminiferous tubule diameter

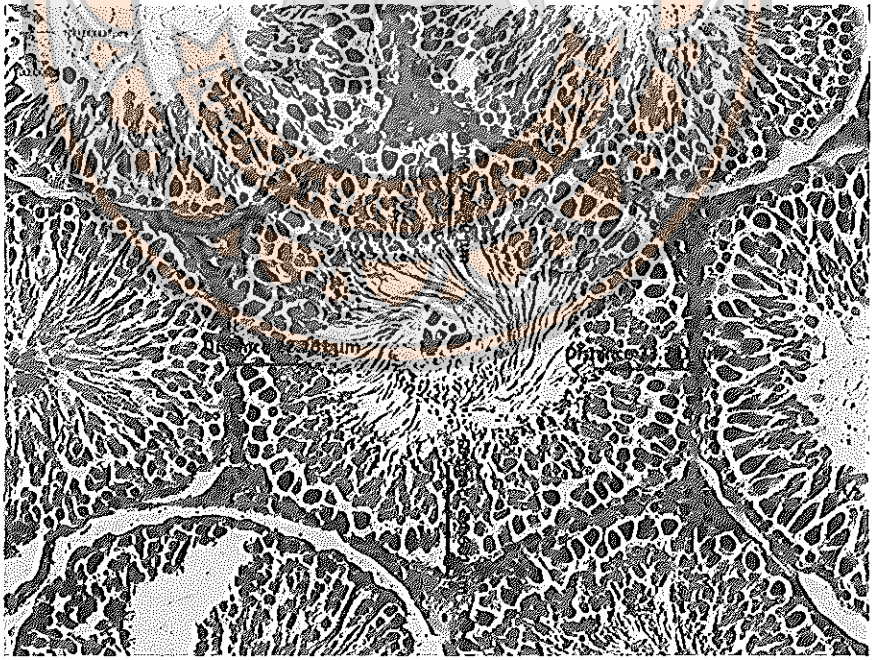


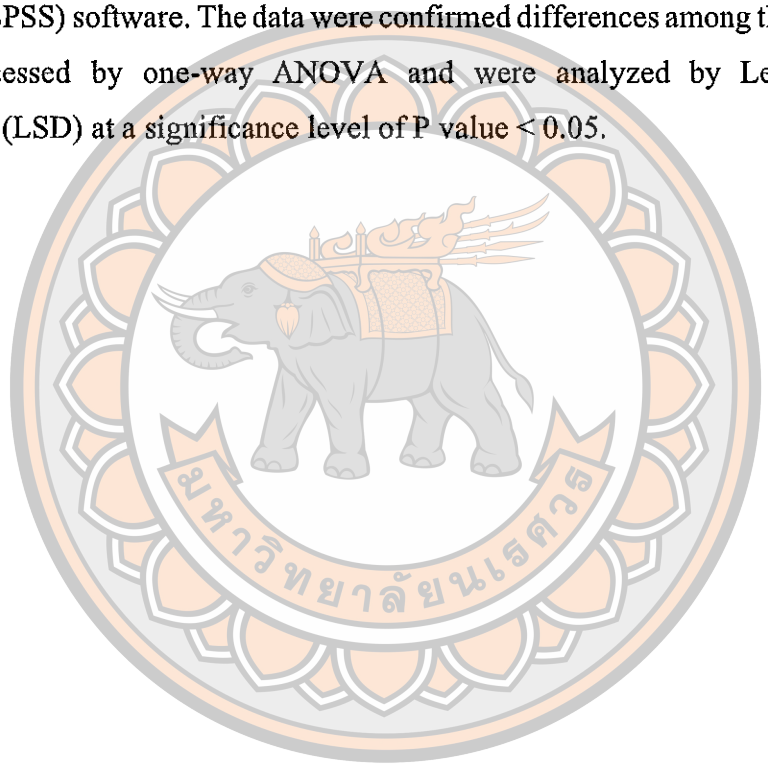
Figure 28 The seminiferous epithelium height

10.2 Tubular differentiation index (TDI) determination

Tubular differentiation index (TDI) used for estimation of spermatogenesis in testicular tissue. To determine the TDI, the 100 seminiferous tubules was inspected to ascertain the percentage of the seminiferous tubules (STs) that show more than three layers of differentiated germinal cells from spermatogonia. The STs which show more than three layers will be considered as TDI positive.

11. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software. The data were confirmed differences among the experimental groups assessed by one-way ANOVA and were analyzed by Least Significant Difference (LSD) at a significance level of P value < 0.05.



CHAPTER IV

RESULTS

Table 6 Effect of Roselle on relative testes weight Effects of *Hibiscus sabdariffa* extracts on the relative testes weights in rats fed with high fat diet

Group	Control	HFD	HFD+L	HFD+H
Absolute testes weight (g/100 g B.W.)	1.93±0.03	2.01±0.02	1.94±0.02	1.92±0.05
Relative testes weight (g/100 g B.W.)	0.48±0.01	0.41±0.01*	0.44±0.01#	0.45±0.01#

Note: Data were expressed as mean ± SEM, n = 7

* $p < 0.05$ as compared to control group.

\# $p < 0.05$ as compared to HFD group.

The absolute testes weight was not significantly different among the experimental groups. The relative testes weight was calculated as the ratio of testes to BW. The relative testes weight was significantly decreased in the HFD group (0.41 ± 0.003 g/100 g B.W.) compared with the control group (0.48 ± 0.009 g/100 g B.W.) ($p < 0.05$). In comparison with HFD group, the relative testes weight of HFD+L (0.44 ± 0.013 g/100 g B.W.) and HFD+H groups (0.45 ± 0.012 g/100 g B.W.) were significantly increased ($p < 0.05$).

Effect of Roselle on sperm concentration

Sperm concentration in the HFD group (13.77 ± 0.79 $\times 10^6$/milliliter) was significantly decreased compared with the control group (19.92 ± 0.98$\times 10^6$/milliliter) ($p < 0.05$). Sperm concentration in HFD group was significantly lower than those of HFD+L (17.13 ± 1.05$\times 10^6$/milliliter) and HFD+H groups (18.9 ± 1.41$\times 10^6$/milliliter) ($p < 0.05$).

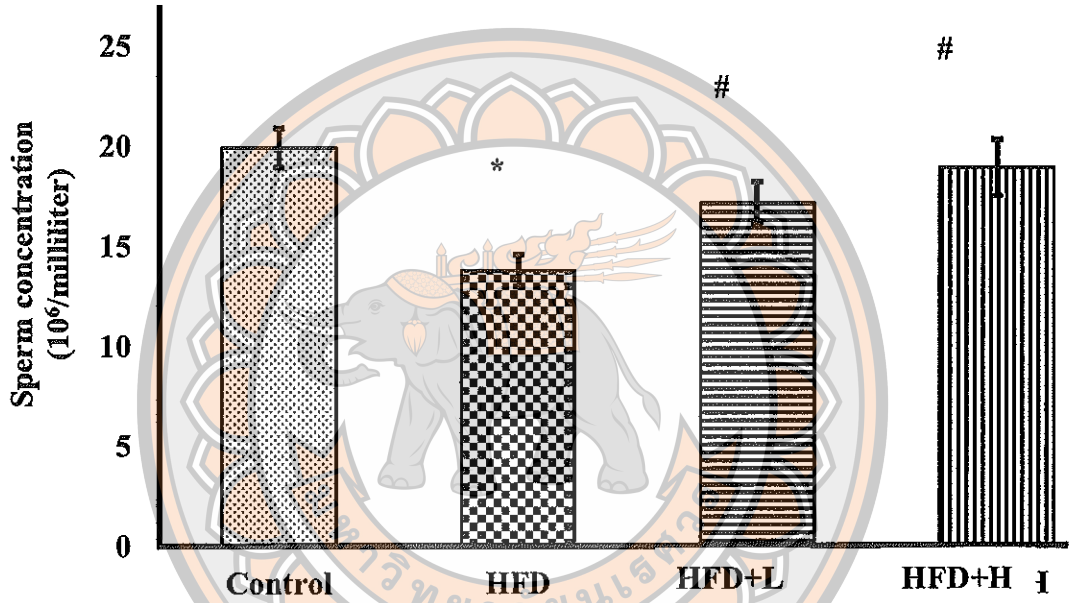


Figure 29 Effects of *Hibiscus sabdariffa* extracts on sperm concentration in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 5

Note: * $p < 0.05$ as compared to control group.

$^{\#}$ $p < 0.05$ as compared to HFD group.

Effect of Roselle on sperm motility

The percentage of sperm motility in the HFD group (37.40 ± 0.79 %) was significantly decreased compared with the control group (51.77 ± 0.98 %) ($p < 0.05$). The percentage of sperm motility were significantly increased in HFD+L and HFD+H groups (47.87 ± 1.05 % and 46.36 ± 1.41 %, respectively) compared with the HFD group ($p < 0.05$).

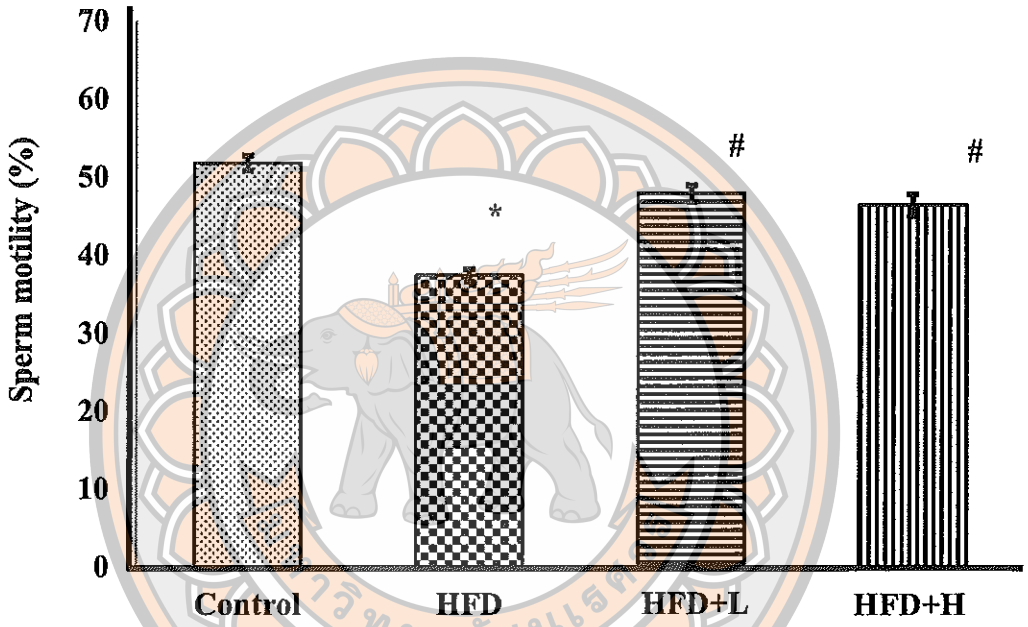


Figure 30 Effects of *Hibiscus sabdariffa* extracts on the percentage of sperm motility in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 6

Note: * $p < 0.05$ as compared to control group.

# $p < 0.05$ as compared to HFD group.

Effect of Roselle on sperm viability

The percentage of sperm viability in the HFD group (55.08 ± 6.35 %) was significantly decreased compared with the control group (82.47 ± 2.67 %) ($p < 0.05$). In comparison with HFD group, administration of roselle 500 mg/kg B.W. (75.39 ± 6.07 %) was significantly increased the percentage of sperm viability ($p < 0.05$). The percentage of sperm viability was increased in HFD-L group (71.55 ± 8.33 %) whereas it was not significantly changed from HFD group.

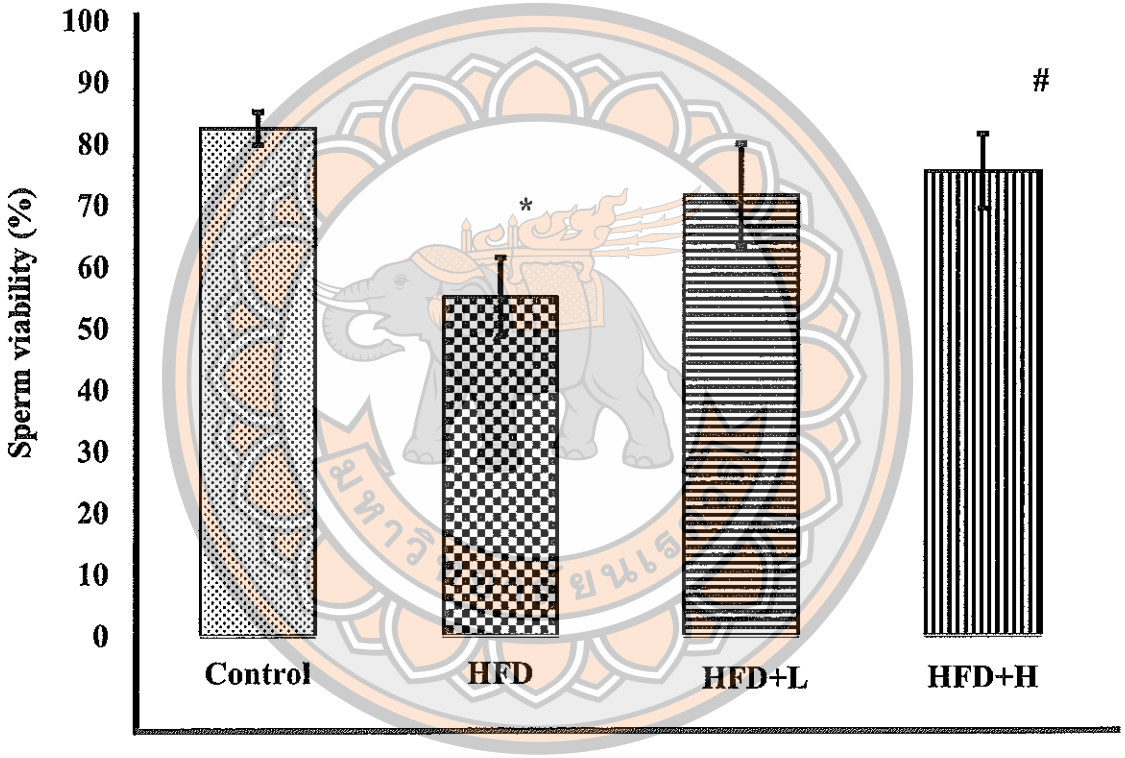


Figure 31 Effects of *Hibiscus sabdariffa* extracts on the percentage of sperm viability in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 5

Note: * $p < 0.05$ as compared to control group.

# $p < 0.05$ as compared to HFD group.

Effect of Roselle on sperm morphology

Table 7 Effects of *Hibiscus sabdariffa* extracts on the percentage of sperm morphology in rats fed with high fat diet.

Groups	Control	HFD	HFD+L	HFD+H
Normal (%)	88.48±1.0	77.80±5.1*	86.76±0.5#	86.18±2.2#
Abnormal				
Head defect (%)	3.16±0.5	5.83±1.3	3.45±0.6	4.82±1.7
Middle piece defect (%)	1.02±0.3	0.96±0.3	0.46±0.1	0.46±0.2
Tail defect (%)	7.34±0.7	15.21±3.7*	9.33±0.4#	8.54±0.5#

Note: Data were expressed as mean ± SEM, n = 7

* $p < 0.05$ as compared to control group.

# $p < 0.05$ as compared to HFD group.

The percentage of normal sperm morphology in the HFD group (77.80 ± 5.1 %) was significantly decreased when compare with the control group (88.48 ± 1.0 %) ($p < 0.05$). In comparison with HFD group, the percentage of normal sperm in HFD-L and HFD-H groups (86.76 ± 0.5 % and 86.18 ± 2.2 %, respectively) were significantly increased ($p < 0.05$).

The percentage of head and middle piece defects were not significantly different among experimental groups. In addition, the percentage of tail defects in the HFD group (15.21 ± 3.7 %) was significantly increased compared with the control group (7.34 ± 0.7 %) ($p < 0.05$), whereas in the HFD-L and HFD-H groups (9.33 ± 0.4 % and 8.54 ± 0.5 %, respectively) were significantly decreased compared with the HFD group ($p < 0.05$). Our present study showed the presence of various morphological defects in obese rat. Interestingly, the roselle treatment can partially diminish these defects induced by high fat diet.

Effect of Roselle on testicular MDA levels

The testicular MDA levels in the HFD group (3.18 ± 0.54 nmols/mg protein) was significantly increased compared with the control group (0.71 ± 0.16 nmols/mg protein) ($p < 0.05$). In comparison with the HFD group, the HFD+L and HFD+H groups (1.19 ± 0.07, 0.97 ± 0.09 nmols/mg protein) were significantly decreased the MDA levels ($p < 0.01$). These results demonstrated the antioxidative activity of roselle.

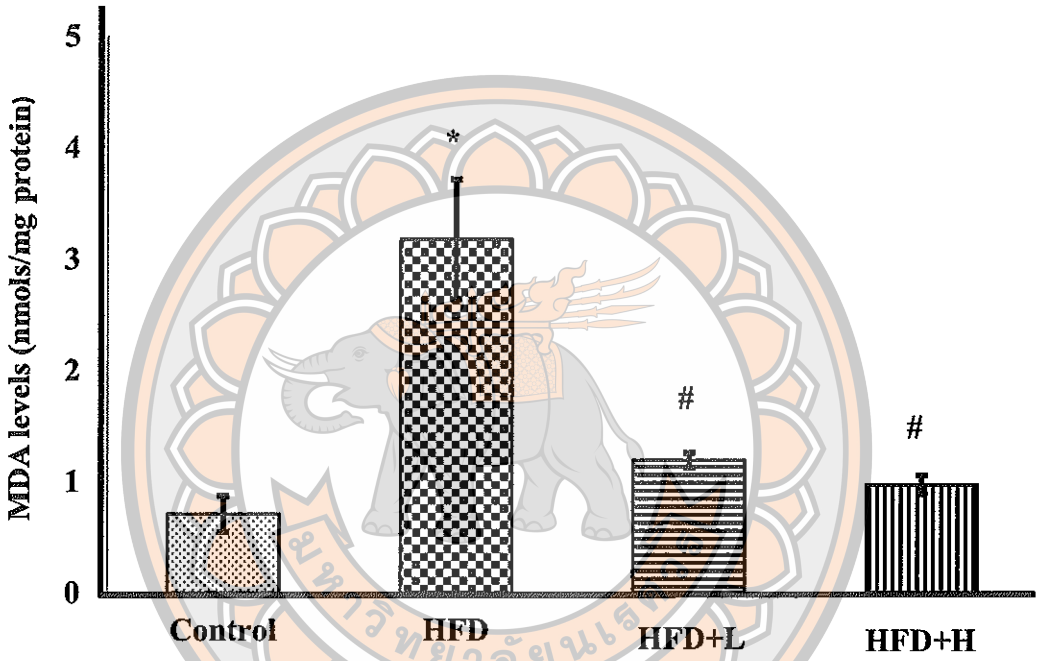


Figure 32 Effects of *Hibiscus sabdariffa* extracts on MDA levels in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 6

Note: * $p < 0.05$ as compared to control group.

# $p < 0.01$ as compared to HFD group.

Effect of Roselle on testicular CAT activity

There was no significant change in the CAT activity between the control and HFD groups. However, the CAT activity in control group (2.09 ± 0.20 U/mg protein) was higher than HFD group (1.59 ± 0.3 U/mg protein). The CAT activity in the HFD+L and HFD+H groups (3.13 ± 0.52 and 2.65 ± 0.32 U/mg protein, respectively) were significantly increased compared with the HFD group ($p < 0.05$).

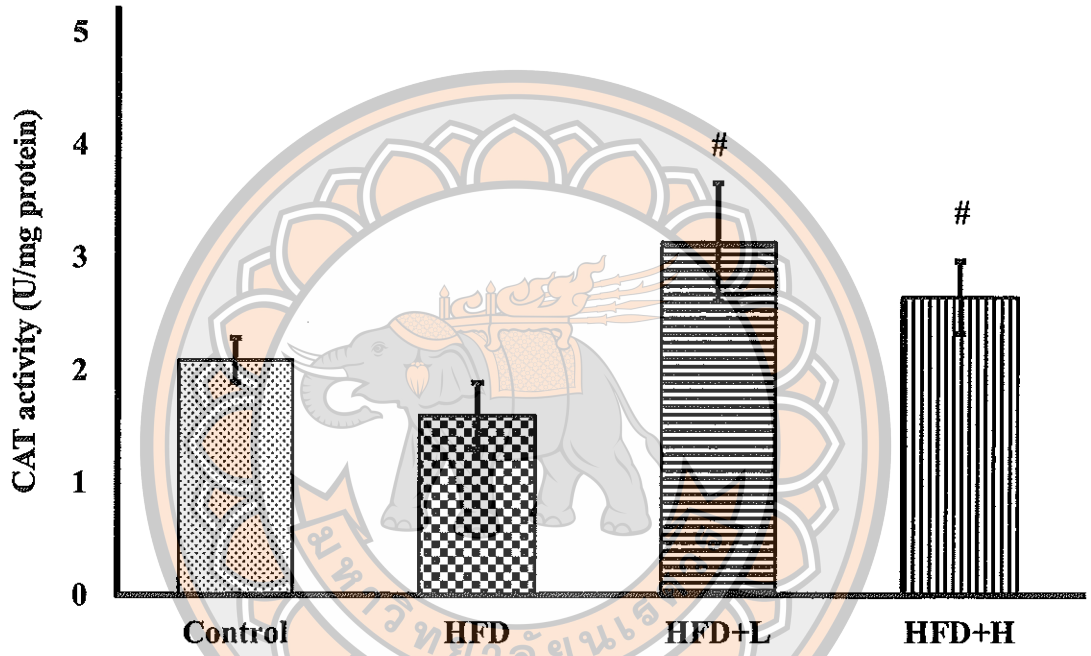


Figure 33 Effects of *Hibiscus sabdariffa* extracts on testicular CAT activity in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 7

Note: $^{\#}p < 0.05$ as compared to HFD group

Effect of Roselle on testicular SOD activity

The SOD activity in the HFD group (3.93 ± 0.3 U/mg protein) was significantly decreased to compare with the control group (6.59 ± 0.2 U/mg protein) ($p < 0.05$). There was no significant change the SOD activity in the HFD, HFD+L and HFD+H groups. However, the SOD activity in HFD+L and HFD+H groups (5.02 ± 0.52 and 4.97 ± 0.32 U/mg protein, respectively) were higher than HFD group.

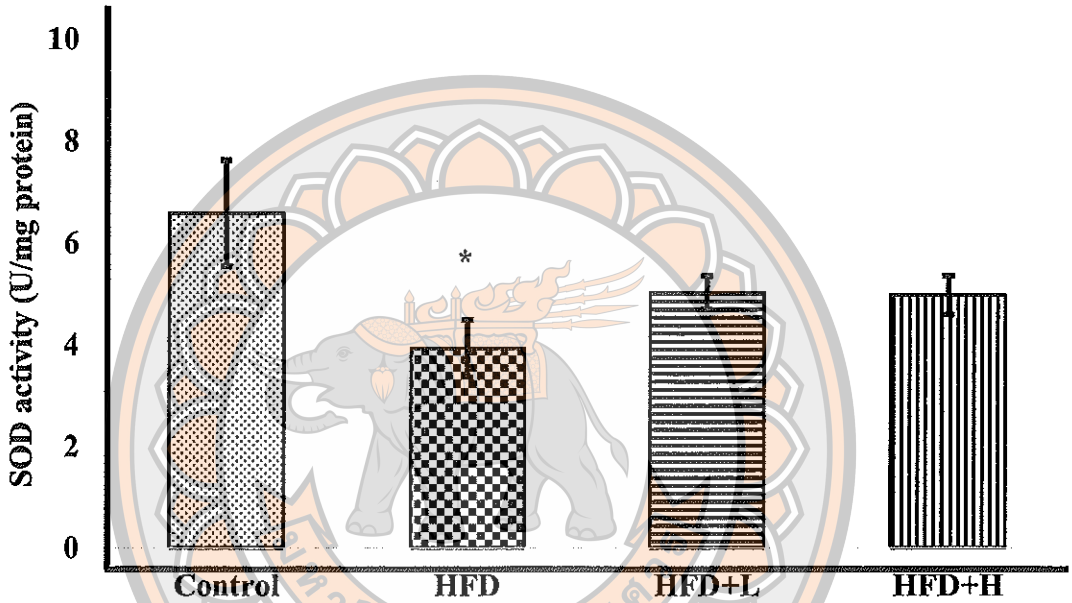


Figure 34 Effects of *Hibiscus sabdariffa* extracts on testicular SOD activity in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 6

Note: * $p < 0.05$ as compared to control group

# $p < 0.05$ as compared to HFD group

Effect of Roselle on histological changes

Histology of the testes was evaluated using H&E staining. The control group showed that the testis composed of compactly arranged, semi-round seminiferous tubules with different sizes. The seminiferous tubules consisted of cells in various stages of spermatogenesis, including spermatogonia, spermatocytes, spermatids and spermatozoa, and Sertoli cells. The lumen of tubule was entirely occupied by spermatids and spermatozoa. Leydig cells located in the interstitial space between the convoluted seminiferous tubules, constitute the endocrine component (Figure 37: 1A and 2A).

The testis of HFD group showed that the seminiferous tubule appeared smaller and lost its normal arrangement. Some of the tubules showed the elongated spermatids/spermatozoa observed in the lumen were very few or absent. Potential space was seen between the basal and adluminal compartments in many tubules. The interstitial space between the tubules was enlarged (Figure 37: 1B and 2B).

The seminiferous tubules of HFD+L group showed that the interstitial space and potential space between the basal and adluminal compartments in some of the tubules were reduced when compared with those in HFD group. Moreover, the spermatozoa were observed in the lumen (Figure 37: 1C and 2C).

The HFD+H group showed that the histological morphology of seminiferous tubule was similar to the control group. However, the seminiferous tubule appeared smaller and its all the components of spermatogenesis were normal (Figure 37: 1D and 2D).

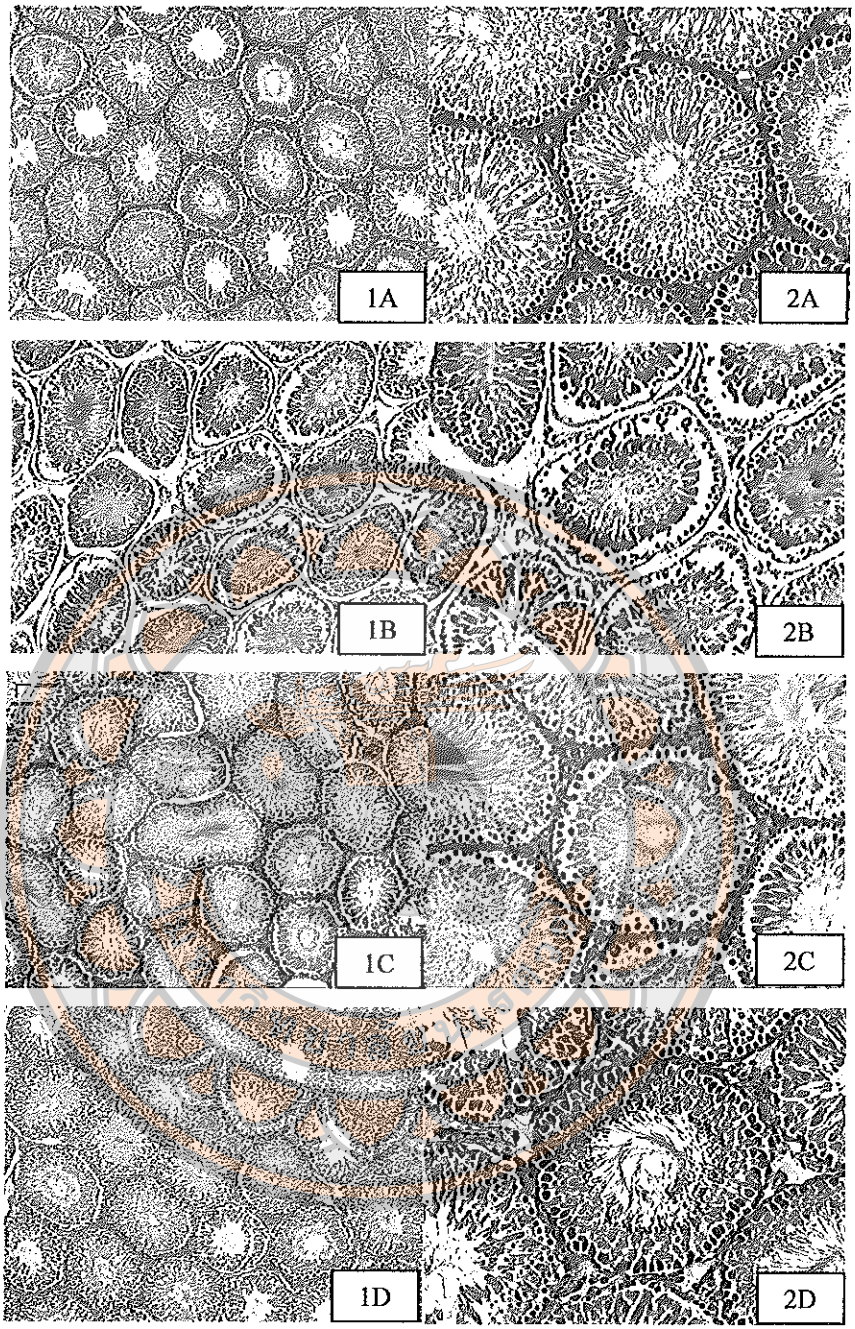


Figure 35 **Photographs of several seminiferous tubules in rats stained with H&E. 1A, 2A: seminiferous tubules in control group which contains more than three and/or four layers of differentiated germinal cells. 1B, 2B: HFD group; seminiferous tubule which contains lower than three and /or four layers of differentiated germinal cells. 1C, 2C: HFD+L group. 1D, 2D: HFD+H group Hematoxylin and Eosin staining. Magnification: 1A, 1B, 1C & 1D 40x; 2A, 2B, 2C & 2D 100x**

Effect of Roselle on seminiferous tubule diameter

The diameter of the seminiferous tubule in the HFD group (121.74 ± 12.31 μm) was significantly decreased compared with the control group (215.40 ± 26.85 μm) ($p < 0.05$). There was no significantly different in the diameter of the seminiferous tubule in the HFD, HFD+L and HFD+H groups. However, the diameter of the seminiferous tubule in HFD+L and HFD+H groups (125.16 ± 12.77, 135.04 ± 9.89 μm, respectively) were higher than HFD group.

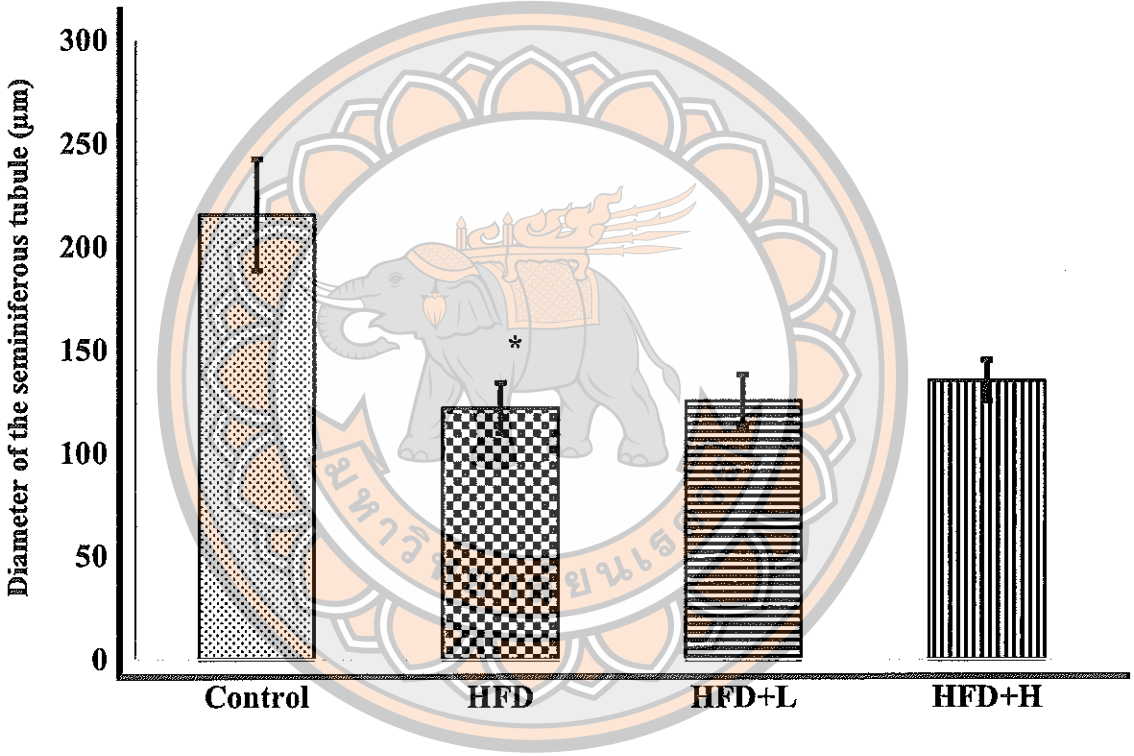


Figure 36 Effects of *Hibiscus sabdariffa* extracts on diameter of the seminiferous tubule in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 7

Note: * $p < 0.05$ as compared to control group

Effect of Roselle on seminiferous epithelium thickness

The thickness of the seminiferous epithelium in the HFD group (18.32 ± 1.16 μm) was significantly decreased compared with the control group (36.97 ± 4.65 μm) ($p < 0.05$). There was no significantly different in the thickness of the seminiferous epithelium in the HFD, HFD+L and HFD+H groups. However, the thickness of the seminiferous epithelium in HFD+L and HFD+H groups (19.07 ± 0.29, 25.35 ± 0.81 μm, respectively) were higher than HFD group.

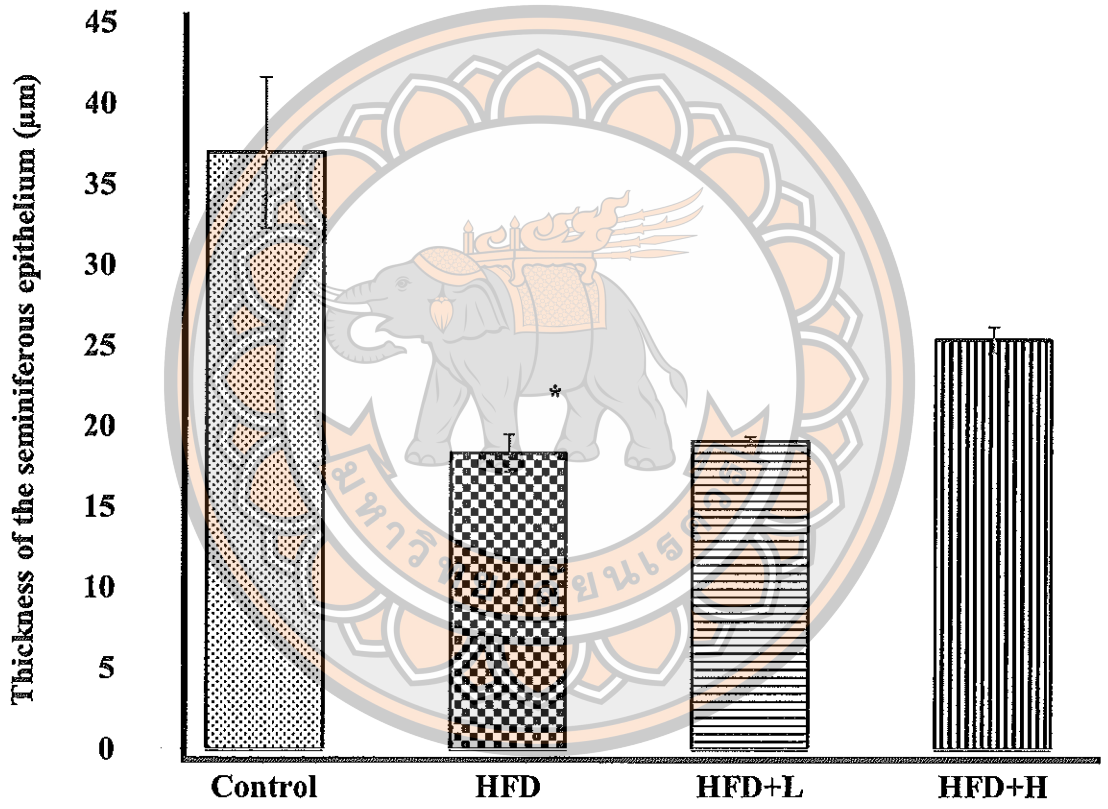


Figure 37 Effects of *Hibiscus sabdariffa* extracts on thickness of the seminiferous epithelium in rats fed with high fat diet, Data were expressed as mean ± SEM, n = 7

Note: * $p < 0.05$ as compared to control group

Effect of Roselle on tubular differentiation index (TDI)

Table 8 Effects of *Hibiscus sabdariffa* extracts on the percentage of TDI in rats fed with high fat diet

Groups	TDI (%)
Control	100
HFD	100
HFD+L	100
HFD+H	100

The result of TDI showed that the TDI in all experiment groups were 100 % indicating that all seminiferous tubules in all groups contained more than three layers of germinal cells.

CHAPTER V

DISCUSSION AND CONCLUSION

Obesity is a person carries excess weight or body fat that has a negative effect on health. The common cause of obesity is overeating and physical inactivity. The occurrence of obesity affects the physiological functions and leads to various diseases, including metabolic syndrome, arthritis, cardiovascular disease. Furthermore, it has an effect on the male reproductive system which may result in erectile dysfunction and male infertility (Cabler et al., 2010).

This study used a high-fat-diet-induced obesity rat model. High fat diet produce oxidative stress insults an imbalance between antioxidants and free radicals. This imbalance can damage the cells and affect the function of various systems (Oyeyipo et al., 2018). The testicular tissue and spermatozoa are very sensitive to free radicals attack and induce lipid peroxidation. Oxidative stress shows the negative effect on the quality of spermatozoa including sperm motility and viability and the structure of the testis which may result in male infertility (Aitken, & Roman, 2008; Oyedrngbe et al., 2016; Wu et al., 2015).

Initially, an obese rat model was successfully established by feeding a high fat diet which leads to development a significant increase in body weight (Janson, & Tunsophon, 2018). The results from the present study have revealed that the high fat diet decrease the relative testes weight, sperm quality, and testicular antioxidant activity of SOD and CAT while it increases the MDA level. In addition, we have found the alterations of histological structure of testis in obese rat. Treatment of roselle could have the protective effects on male reproductive system by reverse an imbalance of oxidative status in testes.

In this study, the relative testes weight of rats fed with a high fat diet is decreased. The association between high fat diet and weight loss has been previously reported in several studies. In 2014, Vendramini et al. published that high-fat diet feeding decreased the relative testicular and epididymis weights in the fatty rats (Vendramini et al., 2014). The decrease of testicular and epididymal weights might be

a consequence of the atrophy of seminiferous tubules and small testes (Zhao et al., 2014; Fernandez et al., 2011). It might have an impact on the spermatogenesis and Sertoli cells (Miao et al., 2018). In 2018, Ghosh and Mukherjee found that the high fat diet produced tissue damages leading to disruption of the seminiferous epithelium and appeared vacuole in seminiferous tubules and interstitial spaces resulting in an impairment of steroidogenesis of Leydig cells (Ghosh, & Mukherjee, 2018). In the testes of obese rats, we have found that the seminiferous tubules are small, the potential space between the basal and adluminal compartment is wide. The interstitial space between the tubule is more spaced and seminiferous tubule diameter and seminiferous epithelium thickness are decreased. The significant decrease in the relative testicular weights in HFD group rats, suggested that testicular weight could also be due to testicular atrophy resulting in the diameter of seminiferous tubule reduced indicated diameter. Seminiferous lining thus increased intratubular spaces lead to reduced relative testicular weights (Seethalakshmi, et al. 1978). Moreover, TDI in all experiment groups were 100 % indicating that all seminiferous tubules in all groups demonstrates that affect the organized germinal cells and all types of cells had normal cellular attachment. Moreover, three or more cell layers were seen in the epithelium of seminiferous tubules. In contrast, the TDI were diminished in diabetic testes rats by confirm this change in cellular activities of seminiferous tubules (Kianifard, Sadrkhanlou, & Hasanzadeh, 2011). Spermatogenesis in seminiferous tubule and sperm morphology could also have been impaired. It has been reported that high fat diet consumption results in the spermatogenesis impairment and affects disrupted sperm quality (Anthony, Stefan, & Agarwal, 2012). The data presented here showed that high fat diet decreased sperm quality involving, sperm concentration, motility, and viability. In addition, high fat diet decreased normal sperm morphology and increased abnormal sperm morphology of tail parts while there was no defect in head and middle piece. These results are in agreement with the previous studies showing that obesity was associated with lower volume of semen, concentration of spermatozoa, sperm count, progressive motility and total motility and significant changes were also found in sperm morphology (Ghangyem et al., 2010; Ferramosca, et al. 2016; Ramaraju et al., 2017). Reduction of normal sperm morphology when fed with high fat diet led to sperm qualities disruption

(Anthony, Stefan, & Agawal, 2002). In addition, obese men had higher rate of sperm head defects, thin heads and pyriform heads (Ramaraju et al., 2017).

As a result, the present study demonstrated that high fat diet induced-obesity may induce oxidative stress resulting in testicular damage. These changes may alter testicular functions and consequently the decrease in relative weights of testes and sperm parameters. These may be speculated that obesity can be important causative factor in the etiology of the male reproductive dysfunction and sperm damage.

Previous studies have been found that low testosterone production in obese rats and human as the result of high fat diet could lead to Leydig cell dystrophy (Fan et al., 2015; Vigueras-Villasenor et al., 2011; Yan et al., 2015) Testosterone is produced by Leydig cells is critical for initiation maintenance and regulation of spermatogenesis. Moreover, high fat diet affected Sertoli cell functions owing to reduction in FSH receptor. Sertoli cells are involved in developing sperm cell number and regulating of seminiferous tubules through blood-testis barrier and reduction of the number spermatogenic output (Niewoener, 2004)

Oxidative stress may be a key mechanism linking obesity with male reproductive dysfunction and inducing sperm damage. Obesity stimulates inflammatory cytokines and processes mitochondria respiration and energy production leads to increased ROS. Several earlier informations have shown that oxidative stress is one of the factors in male infertility, which it affects sperm functions, resulting in decrease sperm quality and damage the structure and function of testes (Aitken and Roman, 2008; Oyedrngbe et al., 2016; Wu et al., 2015). Obesity causes the elevation of free fatty acids and PUFAs and accumulation in male reproductive organs. Cell membranes of sperm are rich in PUFAs, which it very sensitive to ROS attack. Oxidative stress could induce a rapid loss of intracellular ATP, causing axonemal damage, low sperm viability and mobility, and increased defects of sperm. Lipid peroxidation of sperm membrane is a key mediator of ROS-induced abnormal mitochondria respiration and the deficient DNA repair mechanism, leading to sperm damage (Aitken, 1995; Galay et al., 2014). This data is in agree with several studies where significant increase in the levels of MDA as well as decreased SOD and CAT activities were observed in testes rat fed with high fat diet (Zhao et al., 2014; Fernandez et al., 2010). As previous study showed that the decrement of SOD levels and elevation of MDA levels in the testicular tissues of obese

rats, demonstrating that the oxidative stress may impact sperm quality (Jia et al., 2018). The results indicated that high fat diet induce oxidative stress insults an imbalance between antioxidants and free radicals. This imbalance can affect sperm functions, resulting in decrease sperm quality and damage the structure and function of testes.

Several studies have been postulated that the balance between oxidative stress and antioxidant activity has an important role in preventing the negative effect of obesity. Roselle is traditionally used as folk and herbal medicines and it has abundant content of polyphenolic compounds, including anthocyanins, phenolic acid, flavonoids which were reported to have strong antioxidant properties (Mazza, 1995; Lin et al., 2012; Lim et al., 2017). The total anthocyanin content of roselle sample was approximately 21 mg/g extract (data not shown). Anthocyanins are natural colorants that belong to the flavonoid family. They are water soluble and generally found in roselle (Tsai et al., 2002; Castaneda-Ovando et al., 2009). High antioxidant activity of anthocyanins is a significant feature, which is paramount in the prevention diabetes and obesity (Konczak, & Zhang, 2004). Studies have established that anthocyanins in plants have higher antioxidant activity than exogenous antioxidants such as Vitamin C and E (Bagch et al., 1998). The antioxidant abilities of anthocyanins act directly or indirectly upon intracellular ROS generation in oxidative stress. Anthocyanins stopped the ROS-induced damage and blocked intracellular ROS generation. They acted indirectly by up-regulating antioxidant enzymes expression or protein activation such as SOD and CAT (Ray, Huang, & Tsyji, 2012). Many studies have shown that roselle has prevented male infertility in several animals' models. In 2012, Muhd Hanis Md Idris, et al. found that roselle (100 mg/kg B.W.) ameliorated diabetes-induced sperm damage (Muhd Hanis Md Idris, et al. 2012). Also, roselle (0.2 g kg-1/day B.W.) showed the protective effect on oxidative damage markers in rats exposed to cadmium (Omonkhua, et al. 2009). Suleiman and colleague found that co-administration of roselle (200 mg/kg B.W.) and vitamin E could reduce the alterations in semen characteristics of sub-chronic carbamazepine (CBZ)-induction (Suleiman, et al. 2015). In addition, roselle (1 g/kg B.W.) could against cisplatin-induced reproductive toxicity in rats (Amin and Hamza, 2006).

The present findings showed that roselle administration resulted in marked restoration in epididymal sperm count, sperm motility, sperm concentration and sperm abnormalities towards control levels. Also, it had beneficial effect against the relative testes weight loss and could improve histological changes in testes induced by high fat diet. However, it did not against obesity induce small seminiferous tubules nor did it change seminiferous epithelium thickness and TDI. Furthermore, roselle extract were significantly decrease MDA levels and increased CAT and SOD activity. The observed increase in antioxidant activities and decrease in the lipid peroxidation in groups treated with roselle suggest its potential anti-lipid peroxidative and antioxidant effects. The protective effects of this extract may be due to its free radical scavenging properties. Polyphenol is the principal source of exogenous antioxidant property and found in roselle. Previous studies reported that the antioxidant property of roselle could reduce the reactive oxygen species generation and activities and improve the antioxidant activity of SOD and CAT. These actions of roselle is able to prevent oxidant injury of testis in diabetic rats (Murali et al., 2011; Kim et al., 2016; Budin et al., 2018). These results indicated that protective effects of this roselle extract may be due to its free radical scavenging properties, which leads to the reduction in the oxidative damage of testicular damage and consequently improving the sperm quality and morphology.

Conclusion

The high fat diet induced rats produced an imbalance of free radicals and antioxidant activity leading to the elevation of oxidative stress consequently testicular damage. Administration of roselle could enhance antioxidant enzyme activity and alleviate MDA level and improve histological changes in high fat diet induced rats (Figure 38). However, further investigation is required to understand the possible involvement of another mechanism of roselle regarding to male reproductive system.

In conclusion, roselle supplementation is able to protect against oxidant damage to the testes in obesity by possibly inhibiting the oxidative stress through antioxidant activities.

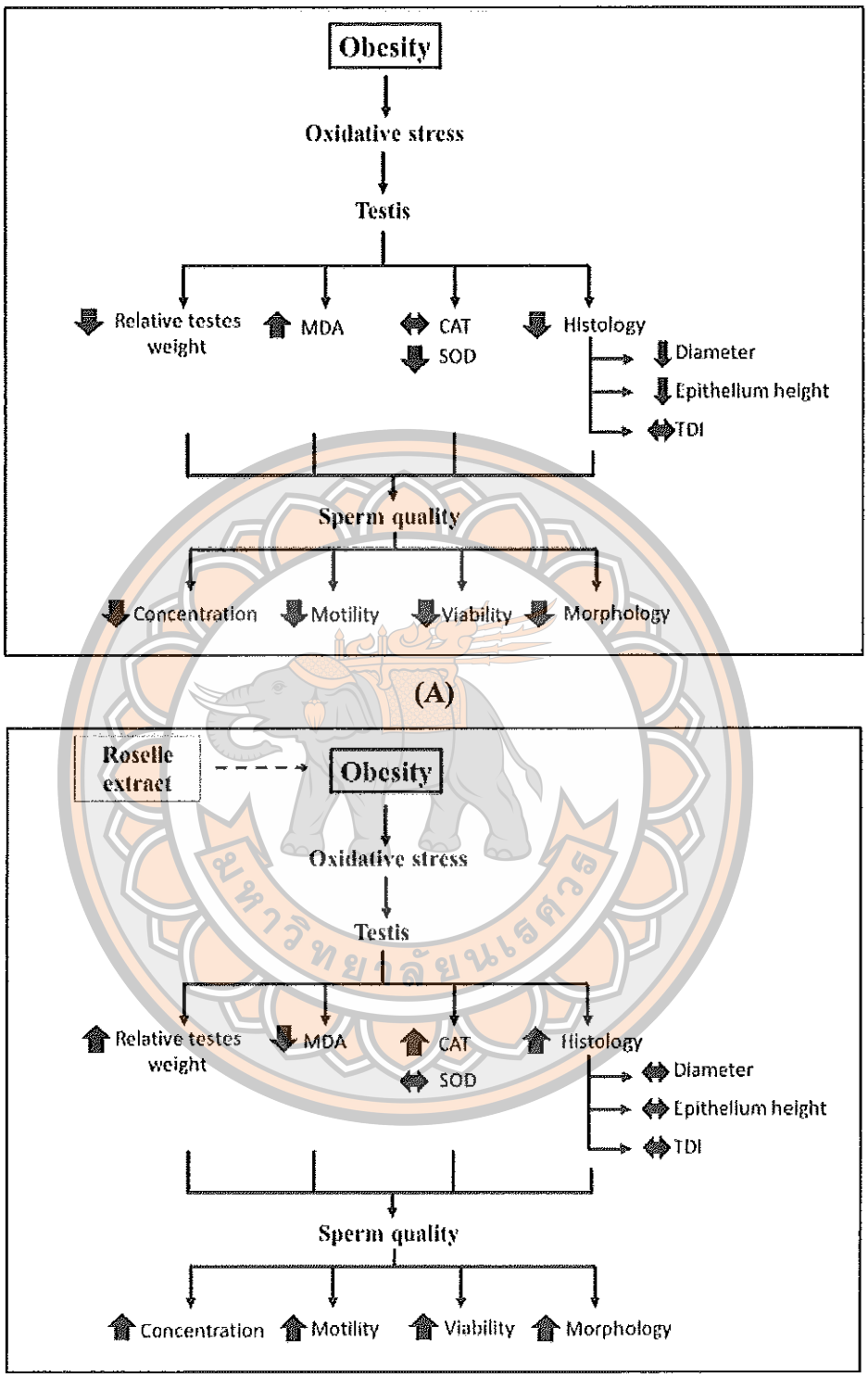


Figure 38 Effects of high fat diet (A) and high fat diet with *Hibiscus sabdariffa* extracts (B) on relative testes weight, SOD activity, MDA level, histological changes in testis, sperm quality and morphology in rats

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APPENDIXS

APPENDIX

Ratio and preparation of solutions and buffers

20% Acetic acid solution

20 ml	Pure acetic acid
80 ml	Distilled water

Catalase assay buffer (0.5 M, pH 7)

500 ml	Na_2HPO_4
250 ml	K_2HPO_4

70% Ethanol

1,105 ml	95% Ethanol
395 ml	Distilled water

80% Ethanol

1,263 ml	95% Ethanol
237 ml	Distilled water

85% Ethanol

1,342 ml	95% Ethanol
158 ml	Distilled water

90% Ethanol

1,421 ml	95% Ethanol
79 ml	Distilled water

1M HCl

83 ml	HCl
1 L	Distilled water

Hydrogen peroxide working solution

340 µl	H_2O_2
100 ml	Catalase assay buffer

10% Nigrosin

100 g	Nigrosin
1 L	Distilled water

1% Lithium carbonate

1 g	Lithium carbonate
100 ml	Distilled water

Pyrogallol stock solution

0.025 g	Pyrogallol
1 ml	50 mM Tris-HCl

Pyrogallol working solution

50 µl	Pyrogallol stock solution
50 ml	50 mM Tris-HCl

8.1% SDS

8.1 g	SDS
100 ml	Distilled water

1% TBA

0.5 g	TBA
50 ml	Distilled water

TMP

16.4 µl	TMP
100 ml	Distilled water

50 mM Tris-EDTA buffer (pH 8.2)

0.6 g	Tris
0.0372 g	EDTA
80 ml	Distilled water

50 mM Tris-HCl (pH 7.4)

0.6057 g	Tris
100 ml	Distilled water

Effect of roselle on reducing body weight gain in high-fat diet induced obese rats (Janson and Tunsophon, 2018).

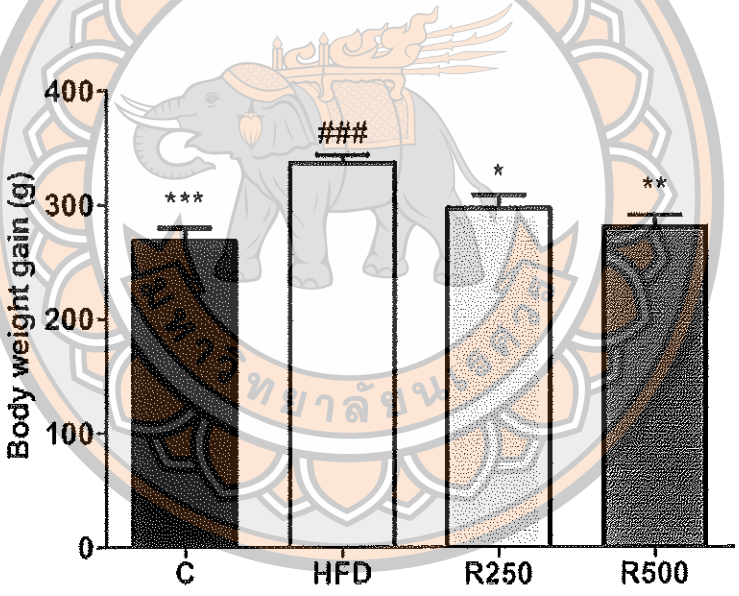

Figure 39 Effects of *Hibiscus sabdariffa* extracts on reducing body weight gain in high-fat diet induced obese rats for eight weeks, Data are expressed as mean ± SEM (n =7)

Note: *,**,*** indicated significant difference at $P < 0.05$, 0.01 and 0.001 respectively, when compared to HFD group.

$^{###}$ indicated significant difference at $P < 0.001$ when compared to control group