Effect of Nigella Sativa Seeds Extract on The Reproductive System In Normal and Streptozocin Induced Diabetic Male Rats

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CERTIFICATE

This is to certify that this thesis entitled *"Effect Of Nigella Sativa Seeds Extract On The Reproductive System In Normal and Streptozocin Induced Diabetic Male Rats"* is a bonafide work of Mrs. Haseena S and was carried out under our supervision and guidance in the Department of Physiology, Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka, India.

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DEDICATED WITH AFFECTION &
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To

Lord Almighty

My Teachers
&
My Family Members

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S.

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SYMBOLS	ABBREVIATIONS & NOMENCLATURE
A	Absorbance
AchE	Acetylcholinesterase
ADP	Adenosine Diphosphate
ANOVA	One-way analysis of variance
ATP	Adenosine tri phosphate
B	Blank
BW	Body weight
CAT	Catalase
CLIA	Chemiluminescence immunoassay
CPCSEA	Committee For the Purpose of Control and Supervision of Experiments on Animals
Cu	Copper
$CuSO_4$	Copper Sulphate
CVD	Cardiovascular diseases
DPA	Dihydroxyacetone phosphate
DC	Diabetic Control rats
D-GAL N	D-Glalactosamine
DHB	Dichloro hydroxyl benzene sulfonic acid
dl	Deci liter
DM	Diabetes Mellitus
DNA	Deoxy ribo nucleic acid
DNS	Diabetic rats treated with Nigella sativa seed powder
DPX	Distyrene plasticizer and xylene
DTQ	Diabetic rats treated with thymoquinone
DW	Distilled Water
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immuno Absorbent Assay
E2	Estradiol
Fe	Iron
FeCl3	Ferric Chloride
FSH	Follicular Stimulating Hormone
GAA	Glacial Acetic Acid
GLUT2	Glucose transporter 2
GPX	Glutathione peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GST	Glutathione – S - Transferase
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HbA1c	Glycosylated Hemoglobin
HCl	Hydrochloric acid
HDL	High Density Lipoproteins
IAEC	Institutional Animal Ethics Committee
Ig G	Immunoglobulin
IU	International Unit
LDH	Lactate dehydrogenase
LDL	Low Density Lipoprotein

SYMBOLS	ABBREVIATIONS & NOMENCLATURE
LH	Luteinizing Hormone
LHP	Lipid Hyper Peroxidase
LPO	Lipid Peroxidation
LPS	Lipo polysaccaride
MDA	Malondialdehyde
MDH	Malate dehydrogenase
mg	Milli Gram
Mg^{+2}	Magnesium ion
Min	Minute
mL	Milli Liter
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogenase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaNO3	Sodium Nitrate
NC	Normal control rats
nm	Nano meter
NO	Nitric Oxide
NNS	Normal rats treated with Nigella sativa seed powder
NS	Nigella Sativa
NTQ	Normal rats treated with thymoquinone
OD	Optical density
OGTT	Oral Glucose Tolerance Test
OSI	Organosomatic Index
PH	Potential of hydrogen
POD	Peroxidase
RNS	Reactive Nitrogen Species
RLU	relative light units
ROS	Reactive Oxygen Species
Sec	Second
SOD	Super oxide dismutase
SOP	Standard Operating Procedure
Std.	Standard
STZ	Streptozocin
T	Test
TBA	Thio Barbituric Acid
TCA	Tri Chloro Acitic Acid
TG	Triglycerides
TQ	Thymoquinone
THQ	Thymohydroquinone
U	Unit
USA	United States of Americax
USD	United State Dollars
Vit-C	Vitamin-C
Vit-E	Vitamin-E
VLDL	Very Low Density Lipoproteins
β-cells	Beta cells of Islet of Langerhans
µL	Micro Liter
4AAP	4-Aminoantipyrine

ABSTRACT

ABSTRACT

Introduction: Diabetes mellitus is a condition in which there are high levels of glucose in the blood. Diabetes develops when pancreas, the gland that makes insulin, is either unable to make insulin or the insulin does not work properly or cells not responding to insulin, without enough insulin blood glucose levels rise and lead to health problems. There are two main types of diabetes. Type 1 diabetes can begin at any age once known as juvenile diabetes or insulin-dependent diabetes mellitus, is a condition in which the pancreas produces little or no insulin. Insulin is a hormone needed to allow glucose to enter into cells to produce energy. About 85 per cent of people with diabetes have type 2 diabetes, which is linked to diet, lack of exercise, obesity and family history. If undetected or poorly controlled, diabetes can lead to a shorter life. Diabetes can cause blindness, kidney failure, nerve damage, reduced blood circulation that may lead to lower limb amputation and can increase the chance of cardiovascular diseases. Men with diabetes also have a higher chance of developing sexual and reproductive health problems including erectile dysfunction, testosterone deficiency, low sexual desire, retrograde ejaculation, poor seminal quality etc. Diabetes can be treated by medicines, diet balance and physical activities. However, plant-derived and herbal remedies continue to be popular alternatives for treatment of diabetes. Nigella sativa is an extensively used herb in Arab medicine and Indian system of medicine, Ayurveda. The present study was conducted to assess the effect of Nigella sativa seed and thymoquinone (major bio-active component of Nigella sativa seed) in the treatment of reproductive dysfunction in streptozocin induced diabetic albino Wister rats.

Material and Methods: Laboratory bred adult albino wister rats weighing between 175 - 250gm were used in the study. The acclimatized animals were divided into six groups of six rats each. Group I rats were normal control rats; group II were normal rats treated with Nigella sativa seed powder (300mg/kg body weight), group III were normal rats treated with thymoquionone(4mg/kg body weight), group IV were streptozocin induced diabetic control rats, group V were streptozocin induced diabetic rats treated with Nigella sativa seed powder(300mg/kg body weight) and group VI were streptozocin induced diabetic rats treated with thymoquinone(4mg/kg body weight). The duration of study was 45 days. At the end of 45 days, blood was collected for biochemical parameters such as glucose, insulin, MDA, SOD, Vitamin C & E, total protein and seminal analysis. For the histopathological observations, testicular and epididymal tissues were collected and processed. IAEC was taken and CPESEA guidelines were followed.

Results: The diabetic untreated rats showed significant increase in levels of serum glucose and MDA compared with normal control rats. After treatment with Nigella sativa seed powder and thymoquinone in induced diabetic rat groups, the above mentioned parameters were lowered significantly. Levels of Insulin, SOD, Vitamin C, Vitamin E, Total proteon and all reproductive parameters (Levels of Testosterone, LH and FSH) were decreased significantly in diabetic untreated group. Sperm count and motility were decreased significantly in diabetic untreated group. After treatment with Nigella sativa seed powder and thymoquinone, the same parameters were increased significantly in induced diabetic rat groups. There was no significant change of any

parameter between normal control rat group and normal rat groups treated with Nigella sativa seed powder and thymoquinone. Histopathological observations of testis and epididymis of induced diabetic treated rat groups revealed that treatment with Nigella sativa seed powder and thymoquinone reversed the histopathological changes which have been seen in induced diabetic control rats. There were no significant histopathological changes observed between normal control rats and normal rat groups treated with Nigella sativa seed powder and thymoquinone. This indicates non-toxic effect of Nigella sativa seed and thymoquinone.

Conclusion: The biochemical parameters and seminal analysis in induced diabetic rats were normalised with treatment of Nigella sativa seed powder and its major bioactive component, thymoquinone. There was no toxic effect observed in normal groups treated with Nigella sativa seed powder and thymoquinone. This observation was supported by non-significant changes in biochemical parameters between normal control rat and normal rat groups treated with Nigella sativa seed and thymoquinone and furthermore supported by histological observations. The biochemical results and histopathological observations clearly showed beneficial effect of Nigella sativa seed powder and thymoquinone in diabetic treated groups. Hence, these phytochemical substances may be considered as antidiabetic agents, favourable to treat reproductive dysfunction in diabetes mellitus as well as beneficial to the overall health of diabetics.

Key words: Nigella sativa seed, Thymoquinone, Diabetes mellitus, Streptozocin, Hyperglycaemia, Antioxidants, Seminal analysis, Testis, Epididymis and Leydig cells.

CHAPTER 1

INTRODUCTION

1.1.Diabetes mellitus

Diabetes Mellitus is a disease that occurs when blood glucose is too high. Blood glucose is main source of energy and comes from the food. Insulin is a hormone secreated by pancreas, helps glucose transport into cells which will be used by them for energy. Sometimes, body does not make enough or no insulin or does not use insulin well. Glucose then loads in blood and may not be transported or utilized by cells. Over time, having too much glucose in blood can cause health problems. The reason for diabetes is either the pancreas is not releasing sufficient insulin or the cells of the body are not responding properly to the insulin[1, 2]. Although diabetes has no cure but one can take steps to manage diabetes to stay healthy. If left untreated, diabetes can cause many complications. Diabetes mellitus could induce long-term damages, dysfunction and failures of various organs including retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers, amputations, Charcot joints, autonomic neuropathy causing gastrointestinal, genitourinary, cardiovascular symptoms and sexual dysfunction[3,4].

In diabetes mellitus, there are three important types:

- In type 1 diabetes, body does not make insulin. Immune system attacks and destroys the beta cells in pancreas that secrete insulin. Type 1 diabetes is usually diagnosed in children and young adults, although it can appear at any age. People with type 1 diabetes need to take insulin every day. Earlier, it was called as ``Insulin-Dependent Diabetes Mellitus``(IDDM).

- In type 2 diabetes, pancreas does not produce enough insulin or cells resistance to insulin. Type 2 diabetes mellitus develops at any age. Usually, this type of diabetes occurs most often in middle-aged and older people. Type 2 is the most common type of diabetes. Type 2 diabetes mellitus in which the cells of the body fail to respond to insulin properly due to insulin resistance. As the disease progresses, lack of insulin may also develop. Earlier, it was called as "Non-Insulin Dependent Diabetes Mellitus"(NIDDM). The primary cause is excessive body weight and not enough exercise[1].
- Gestational diabetes mellitus develops in some women when they are pregnant. Most of the time, this type of diabetes goes away after the baby is born. Gestational diabetes may lead to greater chance of developing type 2 diabetes later in life. Sometimes, diabetes diagnosed during pregnancy is actually type 2 diabetes. In this type, there need not any previous history of diabetes but a high glucose level is developed during pregnancy[5].

Prevention and treatment of diabetes involve a healthy diet, physical exercise, weight management and avoiding smoking. The diabetic persons should control the blood pressure and proper care of their foot is very important. Type 1 diabetes mellitus may be managed with insulin injections[1]. Type 2 diabetes mellitus may be treated with proper medications with or without insulin and proper physical workouts[5]. Gestational diabetes usually resolves after the birth of the baby[6]. In 2014, an estimated 387 million people had diabetes throughout world[6], out of which Type 2 diabetes mellitus were about 90%. This represents 8.3% of the adult population with equal rates

in both women and men[7]. In between 2012 and 2014, diabetes resulted in an estimated 1.5 to 4.9 million deaths each year[5]. The number of people with diabetes is expected to increase to 592 million by 2035[8]. The economic cost of diabetes worldwide in 2014 was estimated around 612 billion United State Dollors[9].

1.2. Risk Factors for Type 2 Diabetes Mellitus [10, 11]

People who develop type 2 diabetes are more likely to have the following characteristics:

- Age 45 years or more
- Some patiens develop diabetes at a young age, usually driven by insulin resistance due to obesity and ethnicity.
- Body Mass Index (BMI) >25 kg/m^2
- Parent or sibling with diabetes
- History of gestational diabetes
- History of giving birth to a baby weighing >4kg
- High blood pressure—140/90 or above—or being treated for high blood pressure
- High-density lipoprotein (HDL)cholesterol below 35 milligrams per dL (mg/dL) or a triglyceride level above 250 mg/dl
- Polycystic ovary syndrome (PCOS)
- Prediabetes— HbA1C level of 5.7 to 6.4 %; fasting plasma glucose level of 100–125 mg/dl (impaired fasting glucose) or 2-hour oral glucose tolerance level of 140–199 (impaired glucose tolerance)

1.3.Effect of diabetes mellitus on male reproductve system

Reproductive dysfunction is one of the major secondary complications in diabetes millitus. Diabetes millitus has recently broken the age barrier and has been heavily diagnosed in children and young people of reproductive age. In the past few years, many studies on diabetes millitus in reproductive functions in both diabetic men and experimental diabetic animals have been conducted. It is recognized that sustained higher glucose level impairs reproductive function in men. Diabetes millitus harmfully affects male reproductive function in multiple areas. They may include spermatogenesis, sperm maturation, fertility capability, penile erection and ejaculation. Glucose metabolism is an important event in spermatogenesis[12]. A large number of studies both in diabetic men and animal models indicate that diabetes mellitus causes male infertility based on impotency, retrograde ejaculation and hypogonadism. Diabetes mellitus may affect male reproductive functions at multiple levels including variations in sperm quality, altered spermatogenesis, morphological changes in testes, altered glucose metabolism in Sertoli-blood testes barrier, reduced levels of testosterone, ejaculatory dysfunction and reduced libido[13,14,15,16,17,18,19]. Several clinical and animal studies have focused on the molecular mechanism responsible for the alterations induced by diabetes mellitus in male reproductive potential including endocrine disorders, neuropathy and increased oxidative stress[20]. Diabetes mellitus induced adverse effects on male reproductive functions might be mediated through hormonal alterations in the hypothalamo-pituitary-gonadal axis or through the direct interaction of insulin with the

testes and sperm cells, as both the testes and sperms themselves produce insulin[21]. Insulin expression in the testes also seems to be affected by diabetes[22].

Both diabetic men and mice had notably impaired spermatogenesis, increased germ cell depletion and Sertoli cell vacuolization suggesting that insulin may have an important role in spermatogenesis[23]. It is still unclear whether the effects of diabetes on male fertility are due to testicular insulin insufficiency or through systemic effects of diabetes[24,25]. Furthermore, oxidative stress may play a pathogenic role in diabetes related male reproductive function abnormalities[20]. Studies have shown that men suffering from diabetes have sperms with greater DNA fragmentation and an increase in advanced glycation end products and their receptors leading to deterioration of sperm quality, sperm functions coupled with changes in testicular metabolite levels and spermatogenic gene expression[26,27]. Several studies have shown that antioxidant treatment improves glycemic index, reduces diabetic complications and protects components from oxidative damage[28,29].

1.4. Effect of diabetes mellitus on various biochemical parameters

Homeostasis of blood glucose

The principal level of control on glycaemia by the Islets of Langerhans depends largely on the coordinated secretion of glucagon and insulin by α- and β-cells respectively. Both cell types respond oppositely to changes in blood glucose concentration. Hypoglycaemic conditions induce secretion from α-cells and β-cells

release insulin when glucose levels increase[30]. Insulin and glucagon have opposite effects on glycaemia as well as on the metabolism of nutrients. Insulin acts mainly on muscle, liver and adipose tissue with an anabolic effect inducing incorporation of glucose into these tissues and its accumulation as glycogen and fat. By contrast, glucagon induces a catabolic effect mainly by activating liver glycogenolysis and gluconeogenesis which results in the release of glucose to the blood stream. An abnormal function of these cells can generate failures in the control of glycaemia which can lead to the development of diabetes. Actually, diabetes is associated with disorders in the normal levels of both insulin and glucagon. An excess of plasma levels of glucagon relative to those of insulin can be determinant in the higher rate of hepatic glucose output which seems to be critical in maintaining hyperglycaemia in diabetic patients[31].

Most digestible carbohydrates in the diet ultimately form glucose. The dietary carbohydrates that are actively digested contain glucose, galactose and fructose residues that are released in the intestine and transported to the liver. Galactose and fructose are readily converted to glucose in the liver. The liver has the primary metabolic function of regulating the blood concentration of most metabolites particularly glucose. In the case of glucose, this is achieved by taking up excess glucose and converting it to glycogen or fat. Skeletal muscle utilizes glucose as a fuel. It stores glycogen as a fuel for its use during muscular contraction. Liver cells appear to be freely permeable to glucose, whereas cells of extra hepatic tissues are relatively impermeable. As a result, the passage through the cell membrane is the rate limiting step in the uptake of glucose in extra

hepatic tissues and glucose is rapidly phosphorylated by hexokinase on entry into the cells. The concentration of glucose in the blood is an important factor controlling the rate of uptake of glucose in both liver and extra hepatic tissues[32].

Role of Insulin in glucose homeostasis

In addition to the direct effects of hyperglycemia on the uptake of glucose into both the liver and peripheral tissues, the hormone Insulin plays a central role in regulating the blood glucose concentration. The islet cell is freely permeable to glucose via GLUT-2 transporter and the glucose is phosphorylated by the high-KM glucokinase. Therefore, the blood glucose concentration determines the flux through glycolysis, the citric acid cycle and the generation of ATP. The concentration of insulin in the blood parallels that of the blood glucose. Insulin has an immediate effect of increasing glucose uptake in tissues such as adipose tissue and muscle. This action is due to an enhancement of glucose transport through the cell membrane by requirement of glucose transporter from the interior of the cell to the plasma membrane. Insulin does indirectly enhance long-term uptake of glucose by the liver as a result of its actions on the synthesis of enzymes controlling glycolysis, glycogenesis and gluconeogenesis. Insulin has an immediate effect in activating glycogen synthase[32].

In general, the glucose level in the blood is expressed in terms of milligrams per decilitre (mg/dl), with the normal range of 70 to 110 mg/dl(Fasting). In general, in healthy individuals, if glucose level is out of this range, the amount of insulin and glucagon released by the pancreas will be used to bring glucose level back within the normal range. When the system is functioning properly, there is always some insulin and

glucagon being produced by the pancreas that is acting to bring a balance between glucose release into the blood and glucose uptake into cells[33,34].

Diabetes Mellitus-Oxidative stress

Oxidative stress defined as an imbalance between oxidants and antioxidants which leads to many biochemical changes. It is an important causative factor in several chronic diseases in human beings. Diabetes mellitus is one such disease[35]. The elevated levels of blood glucose in this disease produce ROS. The oxidative stress associated with diabetes mellitus may play an important role in the initiation and progression of diabetic complications. Excessive generation of free oxygen radicals may damage various tissues in the body[36]. ROS can attack vital cell components like polyunsaturated fatty acids, proteins and nucleic acids. To a lesser extent, carbohydrates are also the targets of ROS. These reactions can alter intrinsic membrane properties like fluidity, ion transport etc. and may result in loss of enzyme activity, protein cross linking, inhibition of protein synthesis, DNA damage and ultimately cell death. Glucose itself and hyperglycemia related increased protein glycosylation are important sources of oxygen free radicals. They will cause membrane damage due to peroxidation of membrane lipids and protein glycation. Increased free oxygen radical activity can initiate peroxidation of lipids. They will inturn stimulate glycation of protein, inactivation of enzymes and alterations in the structure and function of collagen, basement and other membranes. Eventually, they may play a role in the long term complications of diabetes. It has also been suggested that there is a link between the development of microvascular and macrovascular diabetic complications and oxygen free-radical damage[37,38]. The level of

lipid peroxidation in cells is controlled by various cellular defense mechanisms which consist of enzymatic and non-enzymatic scavenger systems[39]. The concentrations of the ROS are modulated by antioxidant enzymes glutathione reductase (GR), glutathione peroxidase (GPX), glutathione-s-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and non- enzymatic scavengers like reduced glutathione (GSH)[40]. Pancreatic beta cell death underlies the pathogenesis of type I diabetes mellitus. Liver is an important organ which offers an adequate site for various metabolic functions. ROS have been implicated in both beta cell destruction as well as in liver injury[41]. Antioxidants and polyphenolic compounds have been shown to scavenge free radicals and reduce oxidative stress[42]. Therefore, phytochemicals appear to manipulate by various indirect mechanisms. The complications of diabetes are mediated through oxidative stress. Most of the studies have shown oxidative stress in diabetes mellitus and complications of diabetes mellitus related to eye, heart, kidneys and liver. Thus, oxidative stress is a more worrying factor in metabolic disorders specially type 2 diabetes mellitus[43,44,45]. In diabetic mellitus, oxidative stress is produced. This is likely to result progression of pancreatic beta cell dysfunction as they are highly vulnerable to oxidative stress. This is associated with low levels of antioxidant enzymes. Pancreatic beta cell dysfunction leads to decrease in circulating levels of insulin and resulting in hyperglycaemia. The cells show intolerance to glucose. Higher levels of glucose lead to increased percentage of HbA1c[46]. Superoxide dismutase(SOD) provides first line of defence against ROS mediated cell injury by catalysing the proportion of superoxide (Primary ROS in oxygen metabolism) to molecular oxygen and peroxide. Hence in

diabetes mellitus, people show low levels of SOD[47]. Vitamins are very important because they play an important role in different biochemical processes. Vitamins A, C and E act as antioxidants by detoxifying the free radicals. The changes in vitamin levels represent as significant biomarkers of oxidative stress. When they are utilized more in the body, their levels in blood will be automatically reduced [48].

Lipids

Diabetes mellitus produces abnormal changes in the lipid profile. It will lead the cells to become more susceptible to lipid peroxidation[49]. Experimental studies show that the presence of polyunsaturated fatty acids in cell membrane leads to attack by free radicals due to the presence of multiple bonds[50]. Lipid hyperperoxides (LHP) through intermediate radical reactions produce such fatty acids that generate highly reactive and toxic lipid radicals. They form new lipid hyperperoxides[48]. A critical biomarker of oxidative stress is lipid peroxidation. It is the most explored area of research when it comes to ROS[51]. Malondialdehyde (MDA) is formed as a result of lipid peroxidation that can be used to measure lipid peroxides after reaction with thiobarbituric acid[52]. These conditions lead to increased levels of markers of oxidative stress and dyslipidemia.

Liver function tests

Theories behind elevation in liver function parameters in diabetes state that the liver helps to maintain normal blood glucose concentration during fasting and postprandial states. Loss of insulin effect on the liver leads to glycogenolysis and increase in production of hepatic glucose. Abnormalities of triglyceride storage and lipolysis in insulin-sensitive tissues such as the liver are an early manifestation of conditions characterized by insulin resistance. It can also be detectable earlier than fasting hyperglycaemia, the precise genetic, environmental and metabolic factors and sequence of events that lead to the underlying insulin resistance[53]. The excess in free fatty acids found in the insulin-resistant state is known to be toxic to hepatocytes. Putative mechanisms include high concentration of cell membrane disruption, toxin formation, mitochondrial dysfunction and inhibition and activation of key steps in the regulatory of metabolisms[54]. Other potential explanations for elevated transaminases in insulin-resistant states include peroxisomal beta-oxidation, recruited inflammatory cells and oxidant stress from reactive lipid peroxidation.

1.5. Therapeutic properties of medicinal plants

Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. Medicinal plants have been the subjects of man's concern since time

immemorial. Every civilization has a history of use of medicinal plant. Most of the people in the world's developing countries put their faith in traditional medicine for their primary health care needs. About 85% of traditional medicine involves the use of plant extracts. Medicinal plants have always been considered a healthy source of life for all people. Therapeutic properties of medical plants are very useful in healing various diseases. The advantage of these medicinal plants is being 100% natural. Plants and their active constituent or phytochemicals are having many therapeutic properties[55].

1.6. Diabetes Mellitus – Herbal care

There are two important principles in the management of type 2 diabetes mellitus: one is to increase the production and effectiveness of insulin as a part of therapeutic management and the other is to decrease the glucose load to the body as part of dietary management. The drugs are of two types: some drugs are managing diabetes by increasing the insulin production from pancreatic β- cells (Sulfonylurea) and some other drugs are working like insulin (Biguanides). These drugs play a very important role in treatment of diabetes. But due to failures in achieving ideal result and increasing side effects, there has always been a need and desire for a natural, more effective and economically feasible alternatives with fewer side effects. Herbs are the main say in the alternative treatment, as they are very satisfying for the patients to get their aliments treated with traditional recipes. They are safe and easy to administer, provided these are properly recognized and their pharmacological properties established.

1.7. NIGELLA SATIVA

Scientific classification [56]

Kingdom : Plantae

Division : Angiosperms

Class : Eudicots

Order : Ranuculales

Family : Ranuculaceae

Genus : Nigella

Species : Nigella Sativa

Common Names

English : Black-caraway, Black-cumin, Fennel-flower, Roman-coriander.

Hindi : Kalonji, Kalajira,

Sanskrit : Mugrela, Upakuncika, Kalajaji

Kannada : Kari jirige

Bengali : Kalo jira

Figure 1. Nigella sativa flower and fruits[57]

Figure 2. Nigella sativa seeds[57]

Medicinal plants have been used for curing diseases for many centuries in different indigenous systems of medicine as well as folk medicines. Moreover, medicinal plants are also used in the preparation of herbal medicines as they are considered to be safe as compared to modern allopathic medicines. Among various medicinal plants, Nigella sativa is emerging as a miracle herb with a rich historical and religious background as many research works revealed its wide spectrum of pharmacological potential. Nigella sativa is commonly known as ``black seed``. Nigella sativa is native to Southern Europe, North Africa and Southwest Asia. It is cultivated in many countries in the world like Middle Eastern region, South Europe, India, Pakistan, Syria, Turkey and Saudi Arabia[58].

The seeds of Nigella sativa and their oil have been widely used for centuries in the treatment of various ailments throughout the world. It is an important drug in the

Indian traditional system of medicine like Unani and Ayurveda. Among Islamic population, it is considered as one of the greatest forms of healing medicine available. It was mentioned that black seed is the remedy for all diseases except death in one of the Prophetic hadith. Nigella sativa has been extensively studied for its biological activities and therapeutic potential and shown to possess wide spectrum of activities such as diuretic, antihypertensive, antidiabetic, anticancer and immunomodulatory, analgesic, antimicrobial, anthelmintic, analgesic and anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, reno-protective and antioxidant properties. The seeds of Nigella sativa are widely used in the treatment of various diseases like bronchitis, asthma, diarrhoea, rheumatism as well as skin disorders. Most of the therapeutic properties of this plant are due to thymoquinone, which is a major active chemical component of its essential oil. [59,60,61,62,63,64].

Administration of Nigella sativa oil in hyperlipidemic rats can increase their reproductive eeficiency[65]. Aqueous extract of Nigella sativa have enhanced spermatogenesis in male rats[66]. Plant mixture containing Nigella sativa can improve semen characteristics and reduce free radicals in the seminal plasma[67]. Nigella sativa can produce favourable effects on fertility potential, plasma Gonadotropins and testosterone in male rats[68].

Nigella sativa seeds contain large no of phytochemicals. Important compounds are thymoquinone (TQ), thymohydroquinone (THQ) and thymol[69]. Thymoquinone is

one of most important components which have been studied during last five decades. Thymoquinone is known to have anti-oxidant, anti-inflammatory and anticancer properties and used in treatment of diabetes mellitus, encephalomyelitis, asthma and cancer[70]. Thymoquinone is acting as an effective superoxide radical scavenger. In addition, it also helps to preserve antioxidant enzymes glutathione peroxidase and glutathione-S-transferase. They are major detoxifiers and play an important role in cellular antioxidant defence systems due to their hepatoprotective nature against toxins[71,72]. Thymohydroquinone is an important natural acetylcholinesterase(AChE) inhibitor. These inhibitors are chemicals that stop enzyme activity and play a very important role in treatment of Alzheimer's disease, autism, glaucoma, schizophrenia, Parkinson's disease and other neurodegenerative diseases[73]. Thymol is one of the active ingredients of Nigella sativa seed which acts as natural monoterpene that has a number of useful qualities. Nigella sativa and thymoquinone can induce hepatoprotective effect in experimental animals[74]. Nigella sativa and thymoquinone can cause partial regeneration of pancreatic beta cells. They can increase the lowered serum insulin levels and decrease the elevated glucose levels. They can act like as metformin which in turn can increase glucose tolerance[75]. Thymoquinone can reverse serological and histopathological changes in the testes of experimental animals with oxidative stress and can also cause increase in number of healthy sperms. Thymoquinone like antioxidants may improve fertility by means of increasing the healthy sperm number and preventing sperm anomalies[76]. Thymoquinone can produce protective effect in experimental animals with testicular torsion[77].

Hence, the present study was attempted to investigate the effects of Nigella sativa seed powder and its major bioactive component, thymoquinone on reproductive activity, various biochemical parameters and histopathological changes in reproductive organs in normal and streptozocin induced diabetic rats.

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CHAPTER 2

REVIEW OF LITERATURE

2.1 Diabetes Mellitus

2.1.1. Historical Background

Knowledge of diabetes dates back to centuries before Christ. The Egyptian Papyrus Ebers (1500 B.C) described an illness associated with passage of more urine. Celsus (30 B.C to 50 A.D) recognized the disease but it was not until two centuries later that another Greek physician, the renowned Areataeus of Cappodocia, gave the name diabetes. He made the first complete clinical description, describing it as "melting down of the flesh and limbs into urine." In the 3rdand 6th centuries AD, scholars in China, Japan and India wrote of a condition with polyuria in which the urine was sweet and sticky, although it had been known for centuries that diabetic urine tasted sweet, it remained for Willis in 1674 to add the observation "as if imbued with honey and sugar". The name diabetes mellitus (mellitus-honey) was thus established. A century after Willis, Dobson demonstrated that the sweetness was, indeed due to sugar. From the time of the earliest recorded history with diabetes, progress in the understanding of the disorder came slowly until the middle of the 19th century. However, over these centuries, gradually the course and complications of the disease were recognized. Within the past century, an association was established with a disturbance in the beta cells clustered as tiny islets of tissue in the exocrine pancreas. These islets were first noted in fish by Brockman early in the 19thcentury, but they bear name of Langerhans who described them in mammals in 1869. Soon after, the German Scientists, Von Mering and Minkowski found that surgical removal of the pancreas produced diabetes in dogs. At the turn of the century, Opie, an American noted the beta cells in the islets to be damaged in humans leading to the disease. Before tracing the biochemical changes in diabetic

patients, physiologic role of insulin needs to be discussed in simple terms. Insulin is the body's signal whose concentration controls both storage and mobilization of fuels. Insulin is a polypeptide hormone produced by the beta cells of islets of Langerhans of pancreas. It has profound influence on the metabolism of carbohydrate, fat and protein. Insulin is considered as an anabolic hormone as it promotes the synthesis of glycogen, triacylglycerols and proteins[1,2].

2.1.2. Types of Diabetes Mellitus

Diabetes Mellitus is a clinical syndrome characterized by an increase in plasma levels of glucose. According to International Diabetes Federation Report of 2011, an estimated 366 million people had diabetes mellitus. By 2030, this number is estimated to almost around 552 million.There are three types of diabetes: Type 1 diabetes, Type 2 diabetes and gestational diabetes. All these three types of diabetes mellitus have some common features. Normally, our body breaks down the sugars and carbohydrates we eat into a special sugar called glucose. Glucose fuels the cells in body. High levels of blood glucose can damage the tiny blood vessels in kidneys, heart, eyes or nervous system. That is why diabetes if left untreated can eventually cause heart disease, stroke, kidney disease, blindness and damage to nerves in the feet.

Type 1 Diabetes: Type 1 diabetes mellitus is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute insulin deficiency. Type 1 is usually characterized by the presence of antiglutamic acid decarboxylase,

insulin antibodies which identify the autoimmune processes that lead to beta cell destruction. Eventually, all Type1 diabetic patients will require insulin therapy to maintain normoglycemia[3,4]. Type 1 diabetes can affect major organs in body including heart, blood vessels, nerves, eyes and kidneys. Keeping blood glucose level in control can dramatically reduce the risk of many complications. When this type of diabetes is untreated, it can gradually develop long term complications. Management of blood glucose within normal range can help to lower the risk of complications.

Clinical features and complications of Type I diabetes: Some of the symptoms include weight loss, polyurea, polydipsia, polyphagia, constipation, fatigue, cramps, blurred vision and candidiasis. Patients with long lasting Type 1 diabetes mellitus may be susceptible to microvascular complications and macrovascular diseases like coronary artery, heart and peripheral vascular diseases. The major long term complications of Type 1diabetes are atherosclerosis, neuropathy, nephropathy, retinopathy and diabetic foot ulcers. Treatment for Type 1 diabetes involves subcutaneous injection of insulin[4,5,6,7].

Type 2 Diabetes: The relative defect in insulin secretion or in the peripheral action of the hormone result in occurrence of Type 2 diabetes mellitus. Type 2 diabetes mellitus comprises 80% to 90% of all cases of diabetes mellitus. Most of the individuals with Type 2 diabetes exhibit obesity, which is closely associated with insulin resistance. In addition, hypertension and dyslipidaemia are often present in these individuals. This type diabetes mellitus is mostly associated with a family history of diabetes, older age, obesity and lack of exercise[4]. Often, it is considered that Type 1 diabetes is more dangerous than Type 2 diabetes. Nevertheless, Type 2

diabetes can still cause major health complications, particularly in the smallest blood vessels in the body that nourish the kidneys, nerves and eyes. Type 2 diabetes also increases the risk of heart disease and stroke. In Type 2 diabetes, the pancreas usually produces small amount of insulin. But either the amount of insulin produced is not enough for the body's needs or the body's cells are resistant to it in type 2 diabetes mellitus. Insulin resistance or lack of sensitivity to insulin happens primarily in fat, liver and muscle cells. People are at high risk of developing type 2 diabetes, particularly who are overweight or obese (having more than 20% over than their ideal body weight). There is no cure for diabetes. Type 2 diabetes can be controlled by increasing physical activity, nutritional modification and weight management. Type 2 diabetes tends to progress and medications are often needed.

Clinical features of Type II diabetes; Most of the cases are diagnosed because of complications or incidentally. It carries a high risk of atherosclerosis of large vessels commonly associated with hypertension, hyperlipidaemia and obesity. The symptoms of diabetes type 2 include increased hunger, increased thirst, dry oral cavity, frequent urination, unusual weight loss, weakness, blurred vision, recurrent infections etc. Most of the patients with type 2 diabetes mellitus die from cardiovascular complications and end stage renal disease. Geographical variation can contribute in the magnitude of the problems and to overall morbidity and mortality. As a result of diabetes mellitus (Type 2), glucose builds up in the blood instead of going into cells. Eventually, cells are not able to function properly. The increased glucose in urine results in frequent urination and causes dehydration. A life threatening complication of diabetes mellitus (Type 2), diabetic coma may occur in

some individuals and may develop severe fluid loss with marked dehydration. Long term exposure to elevated blood glucose levels may damage the nerves and capillaries and may cause stroke, cardiac and renal diseases [4, 5,6,7].

Gestational Diabetes

Gestational diabetes mellitus is identified in women who develop diabetes mellitus during gestation. Women who develop Type 1 diabetes mellitus during pregnancy and women with undiagnosed asymptomatic Type 2 diabetes mellitus that is discovered during pregnancy are termed as Gestational Diabetes Mellitus. In most of the women who develop gestational diabetes mellitus, disorder has its onset during third trimester of pregnancy[4]. As maternal blood with high blood sugar is circulated through the placenta to the baby, gestational diabetes must be controlled to protect the baby`s growth and development. Treating gestational diabetes can help both mother and baby stay healthy. Mother`s blood with elevated glucose circulated through the placenta to the baby can affect the health of baby and growing tissues and risks to the unborn baby are even greater than risks to the mother. The risks in gestational diabetes to baby include abnormal weight before birth, respiratory problems at birth and higher obesity and risk of diabetes in later part of life. In gestational diabetes, mother can face risks like damage to heart, kidney, nerves, eye and overweight baby may need delivery by cesarean section[4].

Other Forms of Diabetes – Other types of diabetes mellitus of various known etiologies are grouped together to form “Other Specific Types”. This group includes people with genetic defects of beta cell function or with defects of insulin action;

people with diseases of the exocrine pancreas such as pancreatitis or cystic fibrosis; people with dysfunction due to other endocrinopathies and people with pancreatic dysfunction caused by drugs, chemicals or infections. They constitue less than 10% of cases of diabetes mellitus[4,8].

2.1.3. Pathophysiology of Type 2 Diabetes Mellitus

Type 2 diabetes has a different pathophysiology and etiology as compared to type 1 diabetes. The existence of many new factors, for example, the increased prevalence of obesity among all age groups and physical inactivity and poor diet (both sexes) means that the number of patients diagnosed with type 2 diabetes is rising[9]. Type 2 diabetes is described as a combination of low insulin production from pancreatic beta cells and peripheral insulin resistance[10]. Insulin resistance leads to elevated fatty acids in the plasma causing decreased glucose transport into the muscle cells, increased fat breakdown and subsequent elevation in production of hepatic glucose. For the development of type 2 diabetes mellitus, insulin resistance and pancreatic beta cell dysfunction must occur simultaneously. Anyone who is overweight and/or obese has some kind of insulin resistance, but diabetes develops only in those individuals who lack sufficient insulin secretion to match the degree of insulin resistance. Insulin in those people may be high, yet it is not enough to normalize the level of glycemia. Dysfunction of beta cells is a main factor across the progression from prediabetes to diabetes. After the progression from normal glucose tolerance to abnormal glucose tolerance, postprandial blood glucose level increases initially. Thereafter, fasting hyperglycaemia may develop as the suppression of

hepatic gluconeogenesis fails. Despite the fact that the pathophysiology of diabetes mellitus differs between type 1 and type 2 diabetes, most of the complications are similar, which may include macrovascular and microvascular complications[11]. Abnormal glycemia appears to contribute to microvascular and metabolic complications. However, macrovascular complications appear to be unrelated to glycemic abnormalities. Insulin resistance with lipid abnormalities, thrombotic abnormalities as well as atherosclerotic risk factors determine the cardiovascular risk in a patient. Cardiovascular risk is associated with the development of insulin resistance even before frank hyperglycaemia occurs. A hypothesis of complications called the "ticking clock" developed by Haffner et al in 1999 showed that the onset of hyperglycaemia puts one at risk for developing macrovascular complications at an earlier point, perhaps at the onset of insulin resistance and before hyperglycemia[12,13].

2.1.4. Mechanism of Diabetes Mellitus and its complications

Carbohydrates, the major component of our food are broken down into simpler sugars like glucose and then absorbed into the body. Glucose is a source of energy, is transported into the blood and has to enter into the cell for metabolism. Insulin facilitates the entry of glucose into the cell, where it burns through Krebs cycle in the mitochondria under the influence of multiple enzymes and energy is released in the form of adenosine tri phosphate (ATP). Body tissues like muscles get energy from ATP to execute mechanical functions. In the absence of or ineffective insulin or a disturbance at the insulin receptor level or its destruction by the antibodies, the body is deprived of energy as it cannot use the ideal source of energy, the glucose. The result is under utilization of glucose which leads to its accumulation

in the blood. It will in turn lead to hyperglycemia, a hallmark of diabetes mellitus. The body constantly requires energy to perform different functions like walking, working, sitting as well as sleeping. Even during sleep, the organs are constantly at work like digesting food and breathing. All these functions need energy. In diabetes mellitus, being unable to use glucose, the body resorts to other sources of energy like lipids / fats and proteins. When lipids and fats are used in more quantity, their breakdown products like cholesterol and triglycerides are produced in abundance. The excess of cholesterol accumulates in blood. A close relationship exists between levels of blood cholesterol or other lipids and the development of atherosclerosis. In this disorder, plaques containing cholesterol are deposited on the walls of arteries. It happens usually in small and medium sized vessels. It will lead to the reduction in the diameter of lumen and flow of blood. Clotting of blood as in the coronary arteries which causes heart attack, is most likely to develop at places where arterial walls are roughened by such plaques. This blockade of the blood vessels is the main cause for complications of diabetes mellitus. This chronic condition requires careful management to avoid multiorgan damage and derangements in lipid metabolism, in order to prevent its complications[7, 8].

2.1.5. Diabetes Mellitus and Reproductive system dysfunction

As incidence of diabetes mellitus is rising worldwide, proportion of men in reproductive age affected is also inevitably increasing. Infertility is a major health problem in both developed and developing countries[14].Animal studies using rodent models of streptozocin induced diabetes mellitus have demonstrated a reduction in quantity and quality of sperms[15]. In addition, a marked reduction in fecundity has

been observed after as little as 15 days following the injection of streptozocin[16]. Ballester et al[17] in their studies have reported similar findings after longer periods of induced diabetes. The associated reduction in fertility is more pronounced when diabetes mellitus is induced in prepubertal animals, furthermore, spontaneously occurring diabetes mellitus in the Wistar rat, is also associated with a significant reduction in fertility[18], thus eliminating any possible confounding effects of diabetogenic agents as a primary cause. These studies support the hypothesis that diabetes mellitus impairs male reproductive function. A reduction in all semen parameters like semen volume, sperm count, motility and morphology may be observed in diabetic men. Some studies showed lower semen volume and total sperm count in diabetic male individuals. Others studies on diabetic men showed an increase in sperm concentration and total sperm output but a concomitant reduction in motility[19,20,21,22].

Diabetes mellitus may affect male reproductive functions at multiple levels including its detrimental effects on endocrine control of spermatogenesis and/or by impairing erection and ejaculation[23,24]. Ricci G et al[25] found that diabetes is accompanied by reduced semen volume and decreased vitality and motility of the spermatozoa, but no change in seminal viscosity. In a study, it was observed that hyperglycaemia may affect sperm quality and decreases male fertility potentials[26]. There are some confirmations indicating higher rates of infertility in diabetic men and poor reproductive outcomes in comparison with healthy men[27]. Petroianu et al[24] study didn't see any differences in seminal concentration and rates of motile spermatozoa between diabetic and healthy men. Agbaje et al[26] examined

spermatozoa in diabetic and non-diabetic men with the same average age. They found that even though semen volume in diabetic men was significantly lesser than controls, there were no significant differences in sperm concentration, total sperm count, morphology and their ability to move between two groups. Arikawe et al[27] showed that in Alloxan induced diabetic rats, all of the sperm parameters were significantly different in comparison with controls. La Vignera et al[28] presented that clinical and experimental verifications suggest that sperm parameters are affected in cases of diabetes mellitus. They also suggested that the involved mechanisms in the beginning of these alterations are hormonal changes, accelerated neuropathy and enhanced oxidative stress. Oxidative stress is harmful to sperms and is considered as a main factor in male infertility. It is generally accepted that the oxidative stress impairs male fertility by changing the cell function like sperm motility and increased DNA damage by induction of gene mutations, DNA denaturation, base pair oxidation and DNA fragmentation[29,30,31].

2.1.6. Streptozocin induced diabetes model – Reproductive dysfunction

Experimentally induced diabetes through the administration of beta cytotoxic drugs such as Streptozocin is well characterized. Streptozocin induced diabetes is caused by the specific necrosis of the pancreatic beta cells. This agent is the first choice for induction of diabetes mellitus in animals. For induction of severe diabetes, streptozocin is administered at a dose of 40–50 mg/kg body weight intravenously or intraperitoneally during adulthood. Approximately after three days, these animals present with blood glucose levels greater than 300 mg /dL[32]. The male tastes and epididymis of streptozocin induced diabetic rats were subjected to significant

oxidative stress. It may contribute to the development of testicular dysfunction and may lead to altered steroidogenesis and impaired spermatogenesis. In diabetes, Leydig cell function and testosterone production may decrease due to the absence of stimulatory effect of insulin on the respective cells. An insulin-dependent decrease in FSH may occur which in turn may reduce LH levels. Sperm output and fertility are reduced due to decrease in FSH which in turn is caused by a reduction in insulin secretion[17,33,34]. Hyperglycaemia has an adverse effect on sperm concentration and motility via the changes in energy production and free radical management. Diabetes mellitus may cause male subfertility by altering steroidogenesis, sperm motility and GLUT expression[33]. In a study by Kim S T, fertilization rate was distinctly lower in streptozocin injected male group compared with the normal group. Furthermore, in fertilized zygotes, embryo developmental rate to the blastocyst stage in diabetic group was lower than that in controls[35]. Mallidis et al. described the changes in the testicular metabolism after administration of streptozocin in an experimental model which developed diabetes and identified the perturbations in several important metabolites. Specifically, diabetic animals showed the decreased carnitine, creatine and choline and the increased lactate, alanine and myo-inositol. Epidermal growth factor deficiency is a potential cause for the pathogenesis of oligozoospermia in diabetic mice[36]. Streptozocin induced diabetes also notably reduced the concentrations of testosterone, androgen binding protein, sialic acid and glyceryl phosphorylcholine epididymal tissue, suggesting its adverse effects on the secretory activity and the concentrating capacity of epididymal epithelium. Impaired cauda epididymal sperm motility and fertility in streptozocin induced diabetic rats implied the defective sperm maturation. Insulin replacement prevented these changes

either partially or completely[17]. From the above findings, it is evident that streptozocin induced diabetes has an adverse effect on sperm maturation, which may be caused by the decrease in the bioavailability of testosterone and epididymal secretory products. Some studies indicated that the germ cell abnormalities in the hyperglycaemic group could be interpreted as the primary effect of streptozocin[36,37]. In another study, it was explained that streptozocin induced diabetes mellitus may influence the male fertility potential via affecting sperm parameters and DNA integrity[38].

2.1.7. Management of Diabetes Mellitus

Effective management of diabetes requires sustained glycemic control to lower the risk of diabetic complications. There are different classes of anti-diabetic drugs and their preference depends on the nature of diabetes, age and condition of the person. Therefore, treatment of Type 2 diabetes must include agent which lowers blood glucose by increasing the amount of pancreatic insulin secretion, increase sensitivity of target organs to insulin or decrease the rate at which glucose is absorbed from the gastrointestinal tract. Amongst antidiabetic drugs, Metformin, a biguanide has become the most commonly used and the first drug of choice for treatment of type 2 diabetes mellitus[39].

Type 1 diabetes mellitus can be treated by insulin therapy only.

In Type 2 diabetes, efforts are made to convert the supply of insulin or reduce the glucose load to the body. Thus, the following principles of management of diabetes can be proposed and practiced.

- Providing insulin to the body through injections like Humulin or NPH insulin etc.
- Increasing the production of effective insulin within the body, which can be achieved through:
    - Drugs that stimulate the production of insulin from the beta cells of islets of Langerhans in pancreas like Sulphonylureas. They act mainly by augmenting insulin secretion and consequently are effective only when some residual pancreatic beta-cell activity is present. During long term administration, they may produce an extra pancreatic action causing hypoglycaemia. But, this is uncommon and usually indicates excessive dosage. The hypoglycaemia may persist for many hours and must always be treated after hospitalization.
    - Use of herbs may increase the production of insulin by maintaining the health and integrity of insulin producing cells and modulating the immune function.
- Use of drugs that increase the glucose utilization like Biguanides (Metformin) which work effectively by decreasing gluconeogenesis and by increasing peripheral utilization of glucose. As it acts only in the presence of endogenous insulin, it is effective only when there are few active functioning beta cells present in pancreas.

- Decreasing the production of glucose from non-carbohydrate sources like amino acids and fat gluconeogenesis especially in the liver.
- Decreasing the glucose load in the body by
  - Restricting the carbohydrates in the diet.
  - Decreasing the absorption of glucose from the gut by Acarbose an inhibitor of intestinal alpha glucosidases which delays the digestion and absorption of starch and sucrose[40].

2.2. Nigella sativa

2.2.1. Historical Background

According to Zohary, archeological evidence about the earliest cultivation of Nigella sativa "is still scanty", but they reported that Nigella sativa seeds have been found in several sites from ancient Egypt, including Tutankhamun's tomb. Although its exact role in Egyptian culture is unknown, it is known that items entombed with a pharaoh were carefully selected to help him in the afterlife [41,42,43].

The earliest reference to Nigella sativa seed is found in the book of Isaiah in the bible. The Nigella sativa cumin is not threshed with a threshing sledge, nor a cart wheel rolled over the cumin. It is beaten out with a rod[44]. The Hebrew word for Nigella sativa seed, "ketsah," refers to Nigella sativa, belongs to the order Ranunculaceae which was cultivated in Egypt and Syria for its seed. This was clarified in Easton's Bible Dictionary[45]. Dioscoredes, the Greek physicist at unity century reported that Nigella sativa seed was used to treat headaches, nasal congestion, toothache and intestinal worms. In addition, it is also used to promote menstruation and increase milk production in nursing mothers[46]. A Biruni who composed a treatise on the early origin of Indian and Chinese drugs, mentions that the Nigella sativa seed is a kind of grain called ``Al wanak`` in the Sigzi dialect[47]. Later, this statement was justified by Suhar Bakht who explained that the Nigella sativa seed as habb-i-Sajzi (viz. Grains Sigzi) allows people to use it as a nutritional ingredient in the 10th and 11th century. Nigella sativa seed is a valuable remedy in treating gastrointestinal dysfunction, hepatitis etc. It is described as a stimulant to different conditions and reliever of high grade fever[48].

Nigella sativa has been used for medicinal purposes for centuries, both as a herb and pressed into oil, in Asia, Middle East and Africa. It has been traditionally used for a variety of conditions and treatment related to respiratory health, stomach and intestinal health, kidney and liver function, circulatory and immune system support and for general well being. In Islam, it is regarded as one of the greatest forms of healing medicine available. It is an important medicine of Tibb-e-nabwi (prophetic medicine)[41].

As per the saying of the Prophet Muhammad (Peace be upon Him) about Nigella sativa seed quoted in Al-Bukhari and Sahih Muslim. Abu Huraira (Radi Allah Anhu) reported that he heard Allah's Messenger as saying: "Nigella seed is a remedy for every disease except death".(Sahih Muslim, 5490)[41].

Ibn Sina (980-1037), most famous for his volumes called "The Canon of Medicine," refers to Nigella sativa seed as the seed "that stimulates the body's energy and helps recovery from fatigue or despiritedness". Nigella sativa seeds and their oil have along history of folklore usage in Arabian and Indian civilization and are used in food as well as medicine. The seeds are used as flavouring, to improve digestion and produce warmth, especially in cold climates. They are sometimes scattered in the fields of woolen fabrics to preserve them from insect damage[49,50].

Many research works conducted recently have provided evidence which indicates that Nigella sativa seed having an ability to improve and boost human immune system significantly if used over a period of time. The prophetic phrase, "hold on to the use of the seed" also emphasizes consistent usage of the seed[51].

In the Middle and Far East countries for centuries, Nigella sativa seeds have been used traditionally and successfully to treat ailments including rheumatism and related inflammatory diseases, bronchial asthma and bronchitis, to treat digestive disturbances, to increase milk production in nursing mothers, to support the body's immune system and to fight against parasitic infestation. Its oil has been used to treat skin diseases such as eczema and boils. Multiple uses of Nigella sativa seed have earned for this medicinal herb the Arabic approbation ``habbatul barakah`` meaning "the seed of blessing"[52,53].

Nigella sativa has been used as a natural remedy for many ailments in various parts of Asia and Africa for centuries. It is now well known in USA and Europe also. Seeds of Nigella sativa had been in use for over 4000 years all over the world[54,55]. According to Birdwood, Nigella sativa is the Nigella sativa cumin of Bible. Analic mentioned its use as carminative, external application for skin eruptions[56]. The ancient Greek and Roman clinicians were aware of the beneficial effects of Nigella sativa. It is used in combination with honey[57,58]. Although native place of Nigella sativa is not exactly known, this herbaceous plant belongs to countries around the Mediterranean sea like Egypt, Turkey and Italy[59]. Roxburgh believes this plant to be a native of India so also called as Nigella indica[60].

2.2.2. Morphological features of Nigella sativa

Nigella sativa belongs to the buttercup family, Ranunculaceae. It is an annual flowering plant, native to Southwest Asia. The dry roasted seeds are used in flavouring curries, vegetables and pulses. The Nigella sativa seeds taste like oregano and have bitterness like mustard seeds. It can be used as a "pepper" in recipes with

pod fruit, vegetables, salads and poultry. Nigella is a genus of about 14 species of annual plants in the family Ranunculaceae, native to Southern Europe, North Africa and Southwest Asia. The plant grows to 20-90 cm tall with finely divided leaves. The leaf segments are narrowly linear to threadlike. The flowers are white, yellow, pink, pale blue or pale purple with 5-10 petals. The fruits are with capsule composed of several united follicles, each containing numerous seeds. Several species are grown as ornamental plants in gardens, popular for their seed capsules, which are used in dried flower arrangements. The flowers are the best to add texture to any dried flower arrangement. The delicate purple striped pods are used in several arrangements for an airy effect[42].Dried fruit and seeds are the main plant components and are mostly used medically. Seeds of Nigella sativa are in color and triangular in shape. The seed is about one eighth inch long having a rough interior and a white oily kernel. On rubbing, the seed diffuses a pleasant odor of lemon with a slight soupcon of carrot[61,62].

2.2.3. Chemical Composition of Nigella sativa

According to Ahmed Aftab et al[63], many active compounds have been isolated, identified and reported in Nigella sativa seeds. The most important active compounds are thymohydroquinone, dithymoquinone, thymoquinone (30%-48%), 4-terpineol (2%-7%), sesquiterpene longifolene (1%-8%), α-pinene, t-anethol (1%-4%), p-cymene (7%-15%), carvacrol (6%-12%), thymol etc. Nigella sativa seeds also contain some other compounds in traces. They contain two different types of alkaloids: isoquinoline alkaloids like nigellicimine- N-oxide and nigellicimine and indazole ring bearing alkaloids or pyrazol alkaloids like nigellicine and nigellidine. The seeds also contain alpha-hederin, a water soluble pentacyclic triterpene and

saponin, a potential anticancer agent[64]. Other compounds like carvone, limonene, and citronellolare also found in traces. Most of the pharmacological properties of Nigella sativa are mainly due to quinine constituents of which thymoquinone is the most important one and responsible for medicinal properties of seed. On storage, thymoquinone yields dithymoquinone and higher oligocondensation products. The seeds of Nigella sativa contain protein (26.7%), fat (28.5%), carbohydrates (24.9%), crude fiber (8.4%) and total ash (4.8%). Various vitamins and minerals like Cu, P, Zn and Fe are also contained in seeds of Nigella sativa in good amount. The seeds contain carotene which is converted by the liver to Vitamin A. Root and shoots are reported to contain vanillic acid[65]. Many previous studies reported that the Nigella sativa seeds contain a fatty oil, rich in unsaturated fatty acids mainly linoleic acid, eicodadienoic acid, oleic acid and dihomolinoleic acid. Saturated fattyacids are present in Nigella sativa seeds to an extent of about 30% or less. α-sitosterol is a major sterol which accounts for around 55% of the total sterols present in Nigella sativa seeds. Stigmasterol is one of the major sterols after α-sitosterol[66,67]. In some studies, it is reported that the other components such as nigellone, avenasterol-5-ene, avenasterol-7-ene , camp esterol, cholesterol , citrostadieno l, cycloeucalenol, gramisterol, lophenol, obtusifoliol, stigmastanol, stigmasterol-7-ene, beta-amyrin, butyro-spermol, cycloartenol, 24-methylene-cycloartanol, taraxerol, tirucallol, volatile oil (0.5-1.6%), fatty oil (35.6-41.6%), oleic acid, esters of unsaturated fatty acids with C15 and higher terpenoids, esters of dehydrosteari, hederagenin glycoside, aliphatic alcohol, linoleic acid, melanthin, melanthigenin, beta unsaturated hydroxy ketone, tannin, resin, reducing sugar, glycosidal saponin and proteins are also present in Nigella sativa seeds [68,69,70].

2.2.4. Nutritional value of Nigella sativa

The earlier studies have shown Nigella sativa to have a high nutritional potential i.e. protein (22%), fat (38-40%) and carbohydrates (32%)[71]. Haq et al in their study mentioned regarding fractionation of whole Nigella sativa seed proteins and their characterisation by Sodium dodecylsulphate polyacrylamide gel electrophoresis. The minerals and vitamins found in seeds are: iron, copper, zinc, phosphorus, calcium, thiamin, niacin, pyridoxine and folic acid[72]. A qualitative study of Nigella sativa and a number of other plant extracts of Saudi origin used in folk medicine has revealed the presence of sterols, triterpenes, tannins, flavonoids, cardiac glycosides, alkaloids, saponins, volatile oils, volatile bases, glucosinolates and anthraquinones[73].

2.2.5. Thymoquinone

Thymoquinone is a phytochemical compound found in the plant Nigella sativa. In laboratory experiments in cells and in animals, it has shown anti-inflammatory and antioxidant effects in models of cardiovascular diseases, diabetes, neurodegenerative diseases and stroke and cancer. It has been considered as a ``pan assay interference compound`` which binds indiscriminately to many proteins[74].

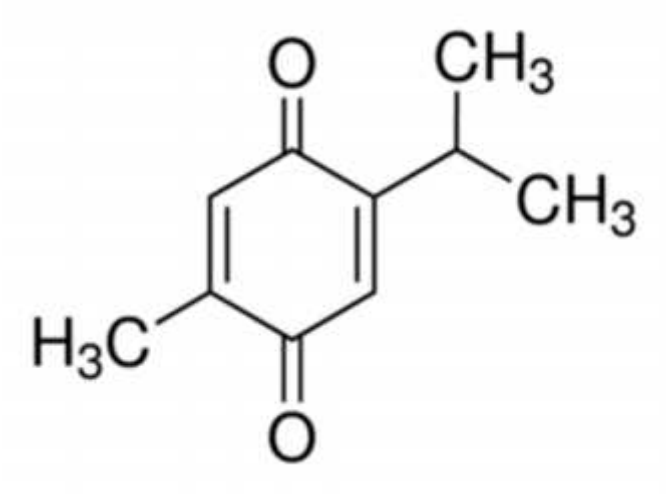

Figure 3. Thymoquinone molecule[75]

Thymoquinone, the major component of Lamiaceae family and found in Nigella sativa seed, exhibits protective effects against diabetes, oxidative stress, coronary artery diseases, failing respiratory and urinary systems, hypertension, neuro degenerative diseases, apoptosis and inflammation. The antioxidant and anti inflammatory activities of thymoquinone may impart its clinical effects against various diseases. The anti-inflammatory effect of thymoquinone is associated with its inhibitory effects on cyclo-oxygenase and 5-lipoxygenase. Its anti-oxidant effect is associated with scavenging activity against reactive oxygen species (ROS)[74,75]. Thymoquinone penetrates through physiological barriers, become accessible to subcellular compartments and exhibits radical scavenging effect[74,76]. Thymoquinone also reacts with glutathione (GSH), NADH and NADPH to form glutathionyl-dihydro-thymoquinone and combats with free radicals[74,77].

2.3. Antidiabetic effects of Nigella sativa

Of the traditional remedies, prescriptions of Nigella sativa for the treatment of various diseases are well known since prehistoric era. Nigella sativa is one of the plants commonly used in Moroccan folk medicine for treatment of various ailments including diabetes mellitus. Previous studies showed that its anti-diabetic effect is multifaceted like regulating blood glucose, insulinotropic, prevention of gluconeogenesis and preserving the integrity of beta cells of islets of Langerhans in pancreas etc[42].

2.3.1. Effect of NigellaSativa and Thymoquinone on Blood Glucose

Majority of studies on the hypoglycemic effect of Nigella sativa and thymoquinone in diabetic animals showed positive results. In study of Eskander EF et al[78], a plant mixture which contained Nigella sativa seeds produced a strong hypoglycemic effect in streptozocin induced diabetic rats. The researchers attributed this hypoglycemic effect of the plant mixture to a decrease in glucogenesis. Another group of researchers using another plant mixture which contained Nigella sativa reported a significant glucose lowering effect. According to Shamshun Nehar et al[79] study, Nigella sativa administration to diabetic animals revealed a significant decrease in the levels of blood glucose and elevation in the levels of insulin. According to Merel L et al[80], oral administration of Nigella sativa to streptozocin induced diabetic rats decreased fasting blood glucose significantly with increase in levels of insulin. In a study of Abdelmeguid NE et al[81], administration of Nigella sativa for 30 days in streptozocin induced diabetic rats produced a significant reduction in fasting blood glucose associated with decreased malondialdehyde

(MDA), elevating glutathione (GSH) and protected beta cells. In a study of Al-Hader Aetal[82], Nigella sativa volatile oil was used in diabetic animal models and found hypoglycaemic action, increased levels of insulin, increased number of insulin immune reactive beta cells and their granules. Kanter M el al[83] in their study reported that hypoglycemic effect of Nigella sativa associated with amelioration of oxidative stress and the preservation of integrity of pancreatic beta cells against streptozocin induced beta cell damage.

According to Fararh K.M et al[84] study, reported regarding the effects of thymoquinone, which is the main active constituent of the volatile oil of the Nigella sativa seeds on hyperglycemia in streptozocin induced diabetic animals. The results of study indicated thymoquinone is beneficial in producing pharmaceutical care against diabetes. The results of this study clearly showed that administration of thymoquinone, a most abundant component of the nigella sativa, is alone sufficient to produce a decrease in blood glucose levels in streptozocin induced diabetic animals. This study also stated that the glucose lowering effects of the Nigella sativa seeds would largely be attributable to thymoquinone. The decrease in total glycosylated hemoglobin levels observed in this study reflects the adequate and effective action of thymoquinone in long term reduction of diabetic hyperglycemia. In Pari L et al[85] study, thymoquinone was intragastrically administered to streptozocin Nicotinamide induced diabetic rats for 45 days showed significant hypoglycemic effect with a decrease in HbA1c, elevation in insulin and increased tolerance to the oral glucose. According to study of El-Dakhakhny et al[86] who found that the hypoglycemic effect of Nigella sativa seed may be mediated by extrapancreatic actions rather than by stimulated insulin release. In Gupta et al[87] study, it has been demonstrated that in

diabetics the increased gluconeogenesis is related, to increased expression of key gluconeogenic enzymes like phosphoenolpyruvate carboxykinase, glucose-6-phosphatase and fructose-1,6-bisphosphatase in the liver. It is thus possible that decreased gluconeogenesis by thymoquinone may be due to suppression of synthesis of the gluconeogenic enzymes. In few studies, it is reported that Nigella sativa seeds can be widely used as anti-diabetic remedies in alternative medicine[88,89]. In a study of Abdel Rahaman et al[90], powdered Nigella sativa did not produce significant effect on normal rat's blood glucose level. In study of Hawsawi ZA et al[91] reported no effect in normal rats by Nigella sativa and thymoquinone. In Le PM et al[92] study reported no significant effect of Nigella sativa on blood glucose in alloxan and in streptozocin induced diabetic rats. These conflicting results in normal animals are most probably due to use of different doses in the studies reported. Hence, majority of researchers reporting negative results in normal animals in which sub-therapeutic doses of Nigella sativa were used[93] indicating its non toxic nature. In a study, Khanam M[94] reported that Nigella sativa showed the lowering of blood glucose in diabetic group but the change was not statistically significant. In studies of Salama RH[95], Qidwai Wet al[96], Sabzghabaee AM et al[97] and Datau EA et al[98], blood glucose lowering effect was observed in diabetic treated groups with Nigella sativa.

2.3.2. Insulinotropic effect and Hypoglycemic mechanism of Nigella sativa and Thymoquinone

Balance concept for any variable in the body is based on equalization of its input to its output. Plasma input of glucose could be through intestinal absorption,

endogenous production like glycogenolysis and gluconeogenesis, while output from plasma is contributed by tissue glucose utilization like glycolysis, pentose phosphate pathway, tricarboxylic acid cycle and glycogen synthesis. Insulin being the most important hormone plays a major role in glucose homeostasis. The normal function of insulin releasing cells as well as other cellular mechanisms are disturbed by oxidative stress which would contribute in induction of diabetes mellitus and its complications[93]. The enhancement of antioxidant power in Nigella sativa treated diabetics might lead to preservation of pancreatic beta cells. Indeed, numerous histopathological studies on different species with different diabetic models have reported preservation of beta cells in animals treated with Nigella sativa and its derivatives. In studies, the preservation of islet cells was associated with significant increase in secretion of insulin[95,99,100,101,102].

In study of Meddah et al[102] reported direct inhibition of electrogenic intestinal absorption of glucose in vitro by Nigella sativa extract. Fararh K M et al[103] reported that Nigella sativa seed extract given orally has insulinotropic properties and it increases the production of insulin from the beta-cells of the Islets of Langehans. This effect has been extensively studied in Streptozocin and Nicotinamide induced diabetic animal models. This was evidenced by the increase in serum insulin levels as measured by enzyme immunoassay and increased staining areas with positive immune reactivity for the presence of insulin in Islets of Langerhans. Studies of Omar et al[104] and Rchid et al[105] have shown significant decrease in blood glucose levels and increased Islet cell regeneration as evidenced by insulin immune-histochemical staining. Oxidative stress is believed to play a role in its pathogenesis.

Studies of Kanter et al[106], Badary et al[107] and Hajhashemi et al[108] showed the evidence of insulinotropic effect by increased intensity of staining for insulin and preservation of beta-cell number in the Nigella sativa treated experimental diabetic animals. Its anti inflammatory effect also plays a role in preserving the integrity of beta cells. Moneim et al[109] reported that the Nigella sativa and sulfonylureas like Gliclazide have synergistic action as for insulinotropic effect is concerned. An extra pancreatic hypoglycemic effect of Nigella sativa has been observed in the study of Le et al[92] with an insulin sensitizing action of Nigella sativa extracts by enhancing the activity of the two major intracellular signal transduction pathways of the hormone's receptor and decrease in hepatic gluconeogenesis. In a study by Ahmad Bilal et al[110]reported that Nigella sativa showed the change in insulin levels but the change was not statistically significant.

2.3.3. Nigella sativa and Histopathology of Pancreatic Beta cells

Kanter et al[83] investigated the effect of Nigella sativa on histopathology of pancreatic beta cells in streptozocin induced diabetic rats. Blood insulin and glucose concentrations were also measured. Researchers observed that Nigella sativa treatment caused decrease in the elevated serum glucose, increase in the lowered serum insulin concentrations and partial regeneration or proliferation of pancreatic beta cells in streptozocin induced diabetic rats. The study concluded that the hypoglycemic action of Nigella sativa could be partly due to amelioration in the beta-cells of pancreatic islets causing increase in insulin secretion. Another study of same researchers evaluated the possible protective effects of Nigella sativa against beta cell damage in streptozocin induced diabetic rats. Pancreatic beta cells were examined by immunohistochemical methods. Streptozocin induced a significant

increase in lipid peroxidation and serum NO concentrations and decreased antioxidant enzyme activity. Nigella sativa treatment provided a protective effect by decreasing lipid peroxidation and serum NO and increasing antioxidant enzyme activity. In the same study, cells of islets of Langerhans showed weak insulin immunohistochemical staining and degeneration in untreated streptozocin induced diabetic rats was observed. Increased intensity of staining for insulin and preservation of beta cell number were apparent in the Nigella sativa treated diabetic rats. This study stated that Nigella sativa treatment decreasing oxidative stress and preserving pancreatic beta-cell integrity. Thus, Nigella sativa is shown to produce therapeutic protective effect in diabetes mellitus. It was also suggested that Nigella sativa may be clinically useful for protecting beta cells against oxidative stress[106]. In study of Nabila E M et al also found similar findings[111].

2.3.4. Effect of Nigella sativa and Thymoquinone on Lipid Peroxidation and Anti-oxidant Defense System

The study of Bahram et al[112]showed significant decrease in the development of hyperlipidemia in Nigella sativa seed treated group and serum lipid profile and malondialdehyde were significantly lower compared to control group. In study of Al-Hader et al[52] showed significant decrease in the concentration of total cholesterol and triglycerides. In an another study, Nigella sativa administration to rats significantly decreased serum total cholesterol, LDL-C and triglycerides and increased HDL-C[86]. Le P M et al[92] reported a significant decrease in plasma triglycerides and an increase in HDL-C levels in Nigella sativa seed extract orally treated rats compared to the control group. Morikawa et al[68] in an in vitro study investigated hypotriglyceridemic effect of nigellamines, that is Nigella sativa seed diterpene alkaloids, equivalent to

the hypolipidemic agent, clofibrate. Blunden and Ali[113] stated in their study that the hypolipidemic effect of Nigella sativa seed does not seem to be due to single component, but rather to the synergistic action of its different constituents including thymoquinone and nigellamine, soluble fiber mucilage, sterols, flavonoids and high content of polyunsaturated fatty acids. The hypolipidemic action of thymoquinone and its mechanism is not fully established. However, it is proposed that antioxidant property of thymoquinone may be responsible for its hypolipidemic action.

Studies were conducted to observe effect of Nigella sativa seed on lipid peroxidation and antioxidant defense system. Kanter et al[83] found that treatment with Nigella sativa decreased blood MDA levels and increased the antioxidant defense system activity in carbon tetrachloride treated rats. In an another study conducted by same authors in rats with the experimental spinal cord injury in rats proved that Nigella sativa treatment can reduce the spinal cord MDA and treatment also prevented from inhibitory effects of CAT, SOD and GPX enzymes[106]. The study of Meral et al[114] indicated that Nigella sativa extract decreased the elevated blood MDA concentrations and increased the lowered glutathione and ceruloplasmin concentrations in diabetic group. The antioxidant effect of Nigella sativa seed seems to be due to thymoquinone, flavonoids and antioxidant Vitamins such as Ascorbic acid. It has been shown that Nigella sativa and TQ inhibit non-enzymatic lipid peroxidation in liposomes and both work as scavengers of various reactive oxygen species including superoxide anion and hydroxyl radicals[73]. In addition, flavonoids are considered as polyphenolic compounds that seem to have antioxidant property by suppressing formation of reactive oxygen and nitrogen species, scavenging reactive oxygen, nitrogen species and protecting the antioxidant defense system[115].

Hosseinzadeh et al[116]reported that thymoquinone and Nigella sativa may have protective effects on lipid peroxidation process during ischemia-reperfusion injury in rat hippocampus. Several studies have shown various therapeutic actions of Nigella sativa. Some studies conducted to find out hepato-protective[117,118,119] and reno-protective[120,121,122,123] effects of Nigella sativa as well as thymoquinone against chemically induced damage and reported positive results.

2.3.5. Effect of Nigella sativa and Thymoquinone on male reproductive system

In Mohammad et al study investigated on effect of Nigella sativa seed extract on reproductive organs of male albino rats. It has been revealed that the thickness of the germinal layer of seminiferous tubules increased significantly, whereas the thickness of epithelial layer that lined the tubules decreased[124]. In study of AL-Zuhairy showed that there was a significant increase in the weight of seminal vesicle in rats administered with crude Nigella sativa in comparison to control group[125]. According to Al Saaidi et al study reported that treatment with Nigella sativa caused significant increase in reproductive parameters such as thickness and diameter of seminiferous tubules, number of spermatogonia, primary and secondary spermatocytes, spermatids, free spermatozoa, Sertoli and Leydig cells, diameter of Leydig cells and levels of hormones: Testosterone, LH and FSH[126]. In Tawfeek study reported that Nigella sativa counteracted the impairment that occured in the epididymal sperm characters caused by Hydrogen Peroxide (H2O2) treatment due to its antioxidant nature[127].

In study of Al-Mayali, Nigella sativa seeds were used for treating infertility in male rats which were subjected for the effects of Cadmium Chloride. It was observed

that Nigella sativa extract caused enhancement in testicular histological architecture with decrease in the abnormalities of sperms [128]. In study of Al-Zubiady, observed that Nigella sativa led to activation of reproductive function in male rats with decrease in sexual desire time, increase in concentration viability of sperms and decrease in sperm abnormalities[129]. In study of Al-Zamily, Nigella sativa seed suspension caused significant increase in ejaculation volume, sperm activities and motility[130]. In another study, it was shown that Nigella sativa increased the number of Leydig cells and their nuclear diameter in rat testes[131]. In study of Rahmatollah Parandin showed that Nigella sativa treatment protected spermatozoa from oxidative injury by encouraging scavengers of reactive oxygen species. In addition, this study stated that the observations may be connected to the presence of rich unsaturated fatty acids in Nigella sativa seed[132]. The unsaturated fatty acids stimulated the activity of 17 ß-hydroxysteroid dehydrogenase, the most important key enzyme in testosterone biosynthesis pathway[133]. The study of Samir Bashandy found that administration of Nigella sativa to hyperlipidemic rats improved their reproductive efficiency and produced additional protection against hyperlipidemia induced reduction in fertility[134]. Some studies showed that Nigella sativa treatment caused increment in hormonal level of testosterone. It may be due to the effect of Nigella sativa seed on main enzymes which affect the metabolism and steroid secretion in the testis. The increase in sperm concentration was partly due to increase in the level of testosterone, FSH and LH in testicular tissues. These hormones were responsible for spermatocytogenesis and spermiogenesis in seminiferous tubules, while testosterone is responsible for epididymal function[135,136,137].

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CHAPTER 3

HYPOTHESIS

AIMS & OBJECTIVES

HYPOTHESIS

Nigella sativa seed powder and Thymoquinone can reduce diabetes mellitus induced alterations in reproductive hormonal profile and various biochemical parameters in male albino rats.

AIMS & OBJECTIVES

a. To evaluate the effect of Nigella sativa seed powder and Thymoquinone in normal and Streptozocin(STZ) induced diabetic rats on reproductive hormonal profile, various biochemical parameters and morphological effect on reproductive organs(Testis and Epididymis) .

i. Reproductive hormonal profile:

Serum levels of Testosterone, LH, FSH, Progesterone and Estrogen.

ii. Semen Analysis:

Sperm count and motility.

iii. Biochemical Parameters:

Serum levels of Glucose, Insulin, MDA, SOD, Total protein,

Vitamins – C & E.

iv. Histopathological changes: Testis and Epididymis.

b. Purpose of the present study was to arrive at some additional information supporting already existing information in regard of effect of Nigella sativa seed powder and Thymoquinone in treating diabetes mellitus induced male infertility.

CHAPTER 4

MATERIALS & METHODS

4.1. Procurement and Rearing of Experimental Animals

Laboratory inbred adult albino Wister rats fed with laboratory stock diet and water *ad libitum* and weighing 175- 250g were used in the study. Rats were acclimatized a week to the laboratory conditions at 22- 24^{o} C and a 12 h light: dark (circadian) cycle. The acclimatized rats were housed in polypropylene cages (32cm x40 cm x18 cm) three animals in each. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), BLDE(Deemed to be University), Shri B M Patil Medical College, Hospital and Research Center, Vijayapura and the experiments were performed as per norms of Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA).

4.2. Experimental Design

Above said acclimatized animals were divided into six groups. Each group comprised of six animals in each group.

Group I: Rats were healthy normal controls.

Group II: Normal healthy rats treated with Nigella sativa seed powder at a dose of 300mg/kg body weight.

Group III: Normal healthy rats treated with thymoquinone at a dose of 4mg/kg body weight.

Group IV: Streptozocin induced diabetic control rats.

Group V: Streptozocin induced diabetic rats treated with nigella sativa seed powder at a dose of 300mg/kg body weight.

Group VI: Streptozocin induced diabetic rats treated with thymoquinone at a dose of 4mg/kg body weight.

Animals were treated for 45days and later were sacrificed on day, 46. Regularly, weight of the rats and levels of glucose were measured.

4.3. Preparation of Nigella sativa seed powder

Seeds of Nigella sativa were procured from Safa Honey Co Ltd, Bengaluru (recognized Nigella sativa seed seller), authenticated by botanist from DRM Science College, Davangere. The Nigella sativa seeds were grained into fine powder as per the procedure followed by Department of Pharmacognancy, Bapuji College of Pharmacy, Davangere. The powder was stored in air tight containers. Nigella sativa seed powder was administered orally[1,2] (at the dose of 300mg / kg body weight).

4.4. Thymoquinone

Thymoquinone is a phytochemical compound found in the Nigella sativa seed. Thymoquinone procured from Sigma – Aldrich, Bangalore. Its stock solution was prepared by dissolving 1g in 10mL ethanol. Diluted solution of thymoquinone at concentration of 4mg mL^{-1} was prepared by dissolving 400 µL of stock solution in 10mL sterile distilled water. This freshly prepared solution was administered to rats through intraperitoneal injection [3] (4mg / kg body weight).

4.5. Streptozocin Induced Diabetes Mellitus

Streptozocin

Streptozocin is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas. In medical practice, streptozocin is used in

treatment of certain cancers of Islets of pancreas. In medical research, it is used to produce an animal model for diabetes mellitus. Streptozocin is a glucosamine-nitrosourea compound. Like other alkylating agents, it is in class of nitrosourea. It is toxic to cells by causing damage to the DNA, though other mechanisms may also contribute. DNA damage of pancreatic beta cells induces activation of poly ADP-ribosylation, which plays an important role in the induction of diabetes mellitus induction than DNA damage itself. Streptozocin is similar enough to glucose to be transported into the cell by GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to beta cells, which have relatively high levels of GLUT2[4, 5, 6].

Citrate Buffer

- Citric acid – Dissolve 2.101gm of citric acid in 100ml of distilled water.
- Sodium Citrate solution 0.1M: Dissolve 2.941gm of Sodium Citrate in 100ml distilled water.
- Preparation of Citrate Buffer with 4.4 pH: In to 49.5ml of citric acid solution, add 50.5 ml of Sodium Citrate solution.

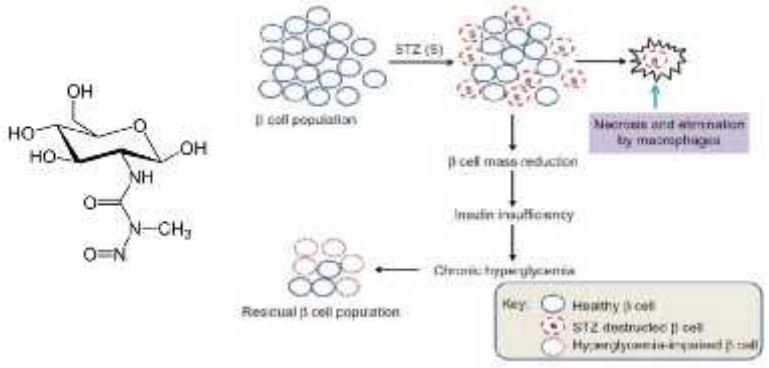

Figure 4: Mechanism of action of Streptozocin[7]

Streptozocin induced diabetes mellitus

- Streptozocin procured from SRL lab, Hyderabad. 50mg/kg body weight streptozocin used for inducing diabetes mellitus in albino rats with single rapid intraperitoneal injection.
- Preparation : 15mg of streptozocin dissolved in 1ml of citrate buffer

  = 50mg/1000 X body weight (gm) = Result / 1.5 = result in ml
- Confirmed Diabetes mellitus after 24 hrs >180mg/dl[1]. Repeated fasting glucose checkup first 7 days. After 1 week of induction, confirmed diabetic(>180mg/dl) rats only were included in the study[8].

4.6. Gravimetry:

Body weight as well as weights of reproductive organs (Testis and Epididymis) of all the groups of animals were measured. Testis of both sides were collected and weighed using digital electronic balance. The average weight was calculated. The same method followed for measurement of weight of epididymis. The organosomatic index was calculated.

$$\text{Organosomatic index} = \frac{\text{Weight of organ (both sides) X 100}}{\text{Body weight of rat}}$$

4.7. Method of euthanasia

Euthanasia is the act of humanely putting an animal to death or allowing it to die. Euthanasia is designed to cause minimal pain and distress. Euthanasia is distinct from animal slaughter and pest control. Animal slaughter is performed mainly for the purpose other than an act of mercy, although in some cases the procedure is the same. Gas anaesthetic such as diethyl ether can be used for euthanasia of rats. The animals were placed in sealed chambers in which high levels of ether gas introduced by soaking cotton in 40ml of diethyl ether. Death may also be caused using Carbon Dioxide following achievement of unconsciousness by inhaled anaesthetic. Once after loss of righting reflex approximately after 60 sec, animal may be removed from the chamber and subjected for further processing.

4.8. Blood & Tissue collection

4.8.1. Blood collection

Blood samples were collected by the retro-orbital route on 46^{th} day and processed for biochemical assay.

Procedure for collection of retro orbital venous blood[9] (periorbital, posterior-orbital and orbital venous plexus bleeding)

- The animal is anaesthetized and at an appropriate depth of anesthesia prior to commencement or even can be euthanized. Signs that indicate a satisfactory

plane of anesthesia include a lack of response to a toe pinch and respirations that are regular and relaxed.

- The anaesthetized rat made to lie on its back. Head of the rat is kept towards the edge of table.
- Head was fixed with thumb and forefinger. The skin over the sides of the face were tightened. This led to retraction of the eyelids and protracted the eye ball.
- A micro hematocrit (capillary) tube was inserted into the medical canthus and twisted. It led to a break through the bulbar conjunctiva. Capillary tube should pass towards the medial aspect of the bony orbit.
- The blood was collected into test tubes and they were sealed. Process was continued until unable to collect. If difficulty arises one has to change to other eye.
- Blood flow can be controlled or stopped by applying finger pressure gently to the soft tissue. A finger should be placed over the closed eyelid for approximately 30 seconds. The rat should be checked for post-operative pre-orbital lesions approximately 30 minutes after blood sampling and on at least one more occasion with two hours of sampling.

Advantages of retro orbital venous blood collection

- Large number of animals can be bled within short period of time.
- Medium to large volume of blood can be obtained.

- Potential contamination with topical anesthetic if used should be taken into account (Quality of sample should be good).
- A minimum of 10 days should be allowed for tissue repair before repeating sampling from the same orbit. Otherwise, it may interfere in the healing process with blood flow.
- Alternating orbit should not be attempted until the phlebotomist is proficient in obtaining samples from the orbit accessed most readily by the dominant hand. That is, a right handed person should withdraw samples from the right orbit before attempting to obtain samples from the left orbit.

4.8.2. Tissue collection

During euthanasia rats were kept in supine position and dissected. Reproductive organs such as testis and epididymis were collected and washed in normal saline. Histopathological study of all collected organs was done. For the evalution of histopathology, specimens were kept in 10% formalin. By following routine histological techniques, the samples were put into paraffin and serial sections of 5µm were taken from tissue blocks.

4.8.3. Analysis of Biochemical assay

<u>Glucose</u> (GOD-POD Method) [10]

Principle:

The reaction sequence employed in this assay is as follows:

Glucose is oxidized by glucose oxidase and produces gluconate and hydrogen peroxide. The hydrogen peroxide is oxidatively coupled with 4-amino anti pyrine and phenol. The intensity of coloured complex is proportional to the glucose concentration in the sample and can be measured photometrically at 505nm (500-540nm).

Reagent composition:

Active ingredients	**Concentration**
Phosphate buffer	100 mmol/L
Glucose oxidase	7000 U/L
Peroxidase	6700 U/L
4-Aminoantipyrine	0.7 mmol/L
Phenol	0.1mmol/L

Procedure:

Pipette into test tubes labelled Blank (B), Standard (S) and Test (T) as follows:

	B	S	T
ERBA Glucose reagent	1.0ml	1.0ml	1.0ml
Glucose Standard	-------	1.0ml	-------
Specimen	-------	--------	1.0ml

Table 1: Procedure for estimation of Glucose

Contents were mixed and incubated for 10 minutes at 37^0 C (or) for 15 minutes at room temperature.

Contents were mixed and absorbance of Standard (S) and Test (T) were read against Blank (B) at 505 nm or with green filter (500-540nm). The final colour was stable for 1 hour at room temperature.

Calculations:

$$\text{Glucose Conc. in mg/dl} = \frac{\text{Absorbance of T} \times 100}{\text{Absorbance of S}}$$

Estimation of Serum Malondialdehyde (MDA) (Nadiger et al method 1986)[11]

Principle - Auto oxidation of unsaturated fatty acids leads to the information of semi stable peroxides which then undergo a series of reactions to form short chain aldehydes like malondehyde(MDA). Two molecules of thiobarbituric acid (TBA) react with one molecule of MDA with the elimination of 2 molecules of water to yield pink crystalline pigment with an absorption maximum at 530nm.

Reagents

1. 10% tricholoacetic acid(TCA)

   10gm of trichloroacetic acid in 100ml distilled water

2. 0.67% thibarbituric acid in 100ml distilled water

Reagents	**Blank(ml)**	**Test(ml)**
Distilled water	0.5	----------
Serum	----------	0.5
0.67% Thiobarbituric acid(TBA)	1.5	1.5
10% Trichloroacetic acid	3.6	3.6
Kept in water bath and boiled for 10-15 min and cooled. Centrifuged for 10-15 min		
Absorbance of supernatant at 530nm		

Table 2: Procedure for estimation of MDA

Calculation:

Concentration of serum MDA

$$= \frac{\text{Absorbance of test}}{\text{Nanomolar non co- efficient}} \times \frac{\text{Total Volume}}{\text{Sample Volume}}$$

$$= \frac{\text{Absorbance of test X 5.6}}{1.5 \times 10^5 \times 0.5} \times \frac{10^9}{1000}$$

= Absorbance of test X 73.33

=……nmol/ml

Estimation of serum Superoxide Dismutase (SOD) (Marklund and Marklund method 1974)[12]

Principle – Superoxide anion is involved in auto oxidation of pyrogallol at alkaline pH(8.5). The superoxide dismutase(SOD) inhibits auto-oxidation of phyrogallol, which can be determined as an increase in absorbance at 420nm.

Reagents –

1. Tris buffer, 0.05M, pH 8.5 containing 1mM EDTA

   50ml of tris buffer(50mM of Tris buffer and 1mM of EDTA) was prepared. To this, 50ml HCL was added to adjust the pH 8.5. Volume was made upto 100ml.

2. Pyrogallol(20mM): 25mg pyrogallol dissolved in 10ml distilled water.

Procedure

	Tris Buffer(ml)	Serum(ml)	Pyrogallol(ml)	OD at 420 nm after 1 min, 30 sec	OD at 420 nm after 3 min, 30 sec
Control	2.9	0	0.1		
Test	2.8	0.1	0.1		

The contents of the above tube were mixed and the absorbance at 420nm was measured

$$\text{A/min} = \frac{\text{Absorbance at 3 min 30sec – Absorbance at 420nm was measured}}{2}$$

Table 3: Procedure for estimation of SOD

Calculation:

Serum SOD activity

$$= \frac{\text{A/min of control - A /min of Test}}{\text{A/min of control x 50}} \times 100 \times \frac{1}{\text{Sample Volume}}$$

$$= \frac{\text{C - T}}{\text{C X 50}} \times 1000$$

=……U/ml

one unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of phyrogallol auto oxidation.

Estimation of Vitamin C (*By Roe & Kuether Method)*[13]

Estimation of Vitamin C included standardization of chemicals & analysis of vitamin C in sample.

Standardization of vitamin C (Ascorbic acid)

Principle

The ascorbic acid is oxidized to Diketogulonic acid in presence of strong acid solution reacts with 2,4, dinitrophenyl hydrazine to form diphenylhydrazone which dissolves in strong H_2 So_4 solution to produce red colour which can be measured at 505nm(range of vitamin C 500-520nm) spectrophotometrically.

Reagents:

1. **TCA (10%):** 10 gm TCA dissolved in distilled water(DW) and make volume up to 100ml.
2. **2,4-DNPH:** 2gm 2,4-DNPH dissolved in $9NH_2SO_4$ & make volume up to 100ML.

   9N H_2SO_4 preparation : MW: 98(2+32+64); Density:1.83gm/cc (i.e.1830gm in 1000cc) Molarity= density/MW; 1830/98=18.67M, but here each mole contributes $2H^+$. So concentration of H_2SO_4 is approx. 37 Normal.

   To make 9N H2SO4 solution in 100ml DW: V1N1=V2N2; 100x9=V2x37; V2=24.324ml; i.e. 24.32ml H_2SO_4 added in DW up to 100ml.
3. **Thiourea :** 10gm thiourea dissolved in 100ml of 50% ethanol (store in 4^oC).
4. **1.5% $CuSO_4$:** 1.5 gm $CuSO_4$ dissolved in DW upto 100ml.
5. **Combined C. R. (freshly):** 5ml 2,4-DNPH+0.1 ml $Cu(SO_4)$+ 1ml thiourea.

6. **85%H_2SO_4 :**85 ml H_2SO_4added in DW upto 100ml.
7. **1% Stock Solution of Vitamin C :** 1gm Vit. C makes it 100 ml with DW.
8. **Working standard:** 1ml stock diluted upto100ml DW to 500DW

Preparation of Standard Curve:

Standardization: 100ml DW = 1gm Vit. C

1ml = 0.01gm, 0.1=0.001gm.

Table 4: Procedure for standardization of Vitamin -C

Stock(ml)	DW(ml)	TCA(ml)	Colour Reagent(ml)	Mixed & kept at 56^0C in warm water bath for 1hr then cooled in ice bath for 5 min.	85% H_2SO_4 (cold)(ml)
Blank	--	0.5	0.4		2
0.1	0.5	0.5	0.4		2
0.2	0.4	0.5	0.4		2
0.3	0.3	0.5	0.4		2
0.4	0.2	0.5	0.4		2
0.5	0.1	0.5	0.4		2

All test tubes were mixed and after 20min OD of Stock Std was read against B at 500nm.

Stock(ml)	Concentration(g/ml)	OD
0.1	0.001	0.008
0.2	0.002	0.028
0.3	0.003	0.036
0.4	0.004	0.049
0.5	0.005	0.055

Estimation

1. Principle

Ascorbic Acid is oxidized to Di ketoglutoxic acid in presence of strong acid solution, which reacts with 2, 4-DNPH to form Diphenylhydrazine which dissolves in strong H_2SO_4 solution to produce red colour which can be measured at 500 nm (range of vit. C 500-520 nm) spectophotometrically.

2. Depolarization:

For plasma / serum sample: 1ml sample+ 1ml 10% TCA (1.1 v/v)

1ml plasma / serum in dry centrifuge + 1ml 10% TCA

Mixed well (10-15 sec.)

Centrifuged (10min at 3500 rpm)

Supernatant taken

Table 5: Procedure for estimation of Vitamin C.

Sl.no	**Reagents**	**T(ml)**	**S(ml)**	**B(ml)**
1	Supernatant	1	--	--
2	Working standard(g/ml)	--	(Stock: DDW)	--
3	10% TCA	--	0.5	0.5
4	DDW	--	----	0.5
5	Colour reagent (Fresh)	0.4	0.4	0.4
Mix & all test tubes kept in warm water bath at 56 degree C for 1hr then cooled in ice bath for 5min.				
6	85% cold H2SO4	2	2	2

All test tubes were mixed and after 20min OD of T& S was read against B at 500nm. T= test, S= standard, B= blank

Estimation of Vitamin E(*By Das et al (2012)*)[14]

Reagents

Absolute ethanol (aldehyde free), N-propanol, Xylene (extra pure), Ferric chloride (%), Distilled water, DL α- tocopherol acetate, & 2, 2` - Bipyridyl.

Preparation of reagents

Stock standard of α- tocopherol (0.27% w/v): 270mg of α- tocopherol acetate, diluted in 100ml absolute ethanol and mixed thoroughly.

2, 2` - Bipyridyl (0.12% w/v): 120 mg 2, 2` - bipyridyl dissolved and made volume up to 100ml of n-propanol & kept in brown bottle.

Ferric chloride (0.12% w/v): 120mg $FeCl_3.6H_2O$ dissolved in 100ml ethanol & kept in brown bottle. All the solutions were stable at room temperature.

Preparation of standard curve

Working standard of α- tocopherol (27µg/mL): Diluted 1ml of stock standard to 100mL absolute ethanol to obtain concentration 27 µg/mL (2.7 µg/100mL). This solution was stable at room temperature. In six centrifuged tubes labelled as (blank) B, S_1, S_2, S_3, S_4 and S_5 placed 00, 150, 300, 450, 600 and 750 µL of working standard α-tocopherol (27µg/mL) respectively and added absolute ethanol to make the volume of each tube equal as 750µL. These solutions (S_1-S_5) were equivalent to 4µg/mL, 8µg/mL, 12µg/mL, 14µg/mL, 16µg/mL and 20µg/mL of α- tocopherol respectively. These solutions were used in routine procedure as shown in table no 6. Absorbance was read by using 200µl of solutions prepared including blank putting on plain ELISA micro

plate (non-antibody coated) and read in ELISA reader at 492nm. Standard curve absorbance *vs* α- tocopherol (µg/mL) was plotted.

Table 6: Procedure for standardization of Vitamin E

S. no	Working standard		DW (µL)	Xylene (µL)	Centrifuge Xylene at 3000 rpm. 500 µL Xylene layer taken out	2,2` - Bipyridyl (µL)	$FeCl_3$ (µL)	After 2 min read OD at 492 nm
	α- tocopherol (µL)	Ethanol (µL)						
B	0	750	750	750		500	100	
S_1	150	600	750	750		500	100	
S_2	300	450	750	750		500	100	
S_3	450	300	750	750		500	100	
S_4	600	150	750	750		500	100	
S_5	750	0	750	750		500	100	

The curve is drawn to determine the extent of adherence to the Beer-Lambert law with various photoelectric instruments.

Analysis of serum α- tocopherol

Preparation of Sample: 3ml of blood was allowed to clot in centrifuge tube at room temperature and centrifuged at 3000rpm for 15 min to get serum. Serum for the analysis of α- tocopherol should be protected from sunlight and undue agitation. α- tocopherol darkens on exposure to light and slowly oxidized by atmospheric oxygen. α- tocopherol was found stable in separated serum at 25^{o} C for 1day, at 4^{o}C for 2 weeks and at -20^{o}C for 2 months.

Step -1of procedure: Two centrifuge tubes were labelled as T and B (i.e. sample and blank). To the sample tube, 750µL of absolute ethanol and 750µL of serum were added. Serum was slowly added with shaking to obtain a finely divided protein precipitate. To the blank tube, 750µL of distilled water and 750µL of absolute ethanol were added. Tubes were stoppered tightly by wrap paper and shaken vigorously for at least 30sec. To all these tubes, 750µL of xylene was added. Again the tubes were stoppered tightly by wrap paper and shaken vigorously for at least 30sec and all tubes were centrifuged for 10 min at 3000rpm (Table 7).

Step-2: 500µL of the xylene layer was transferred (supernatant) into properly labelled clean small sized test tubes. To each tube, 500µL of 2, 2- bipyridyl solution and 100 µL of ferric chloride solution were added and waited for 2 min (Table 8).

Step-3: 200 µL of solution was transferred from these tubes to uncoated micro wells respectively. Readings were made in ELISA Reader (Erba, Lisa Scan II) with the rapid

measure mode. Primary wavelength was set as 492nm and the absorbance within 4 min was measured(Table 9).

Table 7: Analysis of α- tocopherol

	T(µL)	**B(µL)**	
Serum	750	---	Mix for 30 sec & centrifuge for 10 min at 3000 rpm
DW	---	750	
Ethanol	750	750	
Mix for 30 sec			
Xylene	750	750	

Table 8: Analysis of α- tocopherol

	T(µL)	**B(µL)**	
Supernatant	500	500	Wait for 2 min
2,2-bipyridyl	500	500	
$FeCl_3$	100	100	

Table 9: Analysis of α- tocopherol

	T(µL)	**B(µL)**
ELISA	200	200
Read the absorbance at 492nm within 2min		

Note: we can take the serum 350µL (in case less availability) by reducing all other reagents by half.

Calculation:

$$\text{Concentration of Vitamin E } \mu g/ml = \frac{\text{OD of test} - \text{OD of Blank}}{\text{Slope}} \times \text{Dilution factor}$$

$$\text{Where Slope} = \frac{Y_2 - Y_1}{X_2 - X_1}$$

X & Y are concentration and absorbance of standards respectively.

Insulin(*By Chemiluminescence immunoassay (CLIA))* [15, 16, 17]

Principle of Assay

The insulin CLIA Kit is based on a solid phase enzyme-linked immunosorbent assay. One anti-Insulin antibody utilizes by the assay system for solid phase (microliter wells) immobilization and another anti-Insulin antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test specimen (serum) and standards were added to the insulin antibody coated microliter wells. Then, anti-Insulin antibody labeled with horseradish peroxidase (conjugate) was added. If Insulin is present in the specimen, it will combine with the antibody on the well and the enzyme conjugate resulting in the insulin molecules being sandwiched between the enzyme-linked antibodies and solid phase. After incubation for one hour at room temperature, the wells were washed with water to remove unbound labeled antibodies. A solution of chemiluminescent substrate was then added and read relative light units (RLU) in illuminometers. The intensity of emitting light was proportional to the amount of enzyme present and was directly related to the amount of Insulin in the serum. By reference to a series of insulin standards assayed in the same way, the concentration of Insulin in the unknown sample was quantified.

Procedure

Preparation of reagent

- All reagents should be at room temperature (18-25°C) and mixed by gently inverting or swirling prior to use.
- To prepare substrate solution, a 1:1 mixing of reagent A with reagent B before use was made. Mixed gently till uniform mixture was obtained. Excess was discarded after use.
- 1 volume of wash buffer concentrate (50x) was diluted with 49 volumes of distilled water. 750 ml of washing buffer (1x) can be prepared by diluting of 15 ml of wash buffer concentrate (50x) with 735 ml of distilled water, mixed well before use.
- Each lyophilized standard with 0.5 ml distilled water was reconstituted. The material of reconstituted was allowed to stand for at least 20 minutes. Reconstituted standards should be stored and sealed at 2-8°C.

Procedure

- Secure Desired number of coated wells were secured in the holder. 50 µl of insulin standards, specimens and controls into the appropriate wells were dispensed. Gently but thoroughly mixed for 10 seconds.
- Dispense 100 µl of enzyme conjugate reagent into each well was dispensed. Mixed well for 30 seconds and incubated at room temperature for 60 minutes.
- The mixture of incubation was removed by emptying the plate content into a waste container. Rinsed and emptied the microliter plate 5 times with 1 x wash

buffer (300 µl each well). The microliter plate was striken sharply onto absorbent paper or paper towels to remove all residual water droplets.

- 100 µl of Chemiluminescence substrate reagent was dispensed into each well. Gently mixed for 10 seconds. Incubated at room temperature in dark for 20 minutes.
- The reaction was stopped by adding 100 µl of stock solution to each well. Gently mixed for 10 seconds until blue color completely changes to yellow.
- The optical density was read at 450 nm with a microliter plate reader within 15 minutes.

Total Protein (Biuret)[18,19]

Principle

Cupric ions in an alkaline solution react with the peptide bonds of proteins and polypeptides containing at least two peptide bonds to produce a violet coloured complex. The absorbance of the complex at 546 nm is directly proportional to the concentration of protein in the sample.

$$Cu^{++} + \text{Protein} \xrightarrow{OH^{--}} \text{Cu-Protein complex}$$

Reagents

1. Reagent(R1)

Potassuim Iodide	30mmol/l
Potassium Sodium Tartrate	100 mmol/l
Copper Sulphate	30 mmol/l
Sodium Hydroxide	3.8 mol/l

2. Total protein standard
Ready for use

Sample: Serum

Procedure - Preparation and stability of working reagent

The reagents are ready for use.

If the absorbance of working reagent is higher than 0.1 at 546 nm the reagent cannot be used.

Assay Conditions

Wave length:	546nm (530-508nm)
Temperature:	37^0 C
Cuvette:	1cm light path
Read agent:	Reagent blank
Method:	Endpoint (increasing)

Pipette into cuvette

	Blank	Standard	Sample
Reagent	1ml	1ml	1ml
Distilled water	10µl		
Standard		10µl	
Sample			10µl

Table 10: Preparation and stability of working reagent

Mixed and read the absorbance (A) was read after a 10-minute incubation.

Calibration (37°C, Biuret method)

S1: Distilled water

S2: Total protein standard

Randox Calibration Serum Level I or

Randox Calibration Serum Level II

Calibration frequency

Two-point calibration is recommended:

- after reagent lot change,

- as required following quality control procedures.

Calculation

$$\frac{A_{sample}}{A_{standard}} \times C_{standard} = C_{sample}$$

A = Absorbance, C = Concentration

Luteinizing Hormone (CLIA)[20, 21, 22]

Principle

The LH CLIA Kit is based on a solid phase enzyme-linked immunosorbent assay. The assay system utilizes one anti-LH antibody for solid phase immobilization and another mouse monoclonal anti-LH antibody in the antibody-enzyme conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in LH molecules being sandwiched between solid phase and enzyme-linked

antibodies. After 60 min incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of chemiluminescent substrate is then added and read relative light units in a Luminometer. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of LH in the sample.

Reagents

Anti-LH Antibody Coated Microtiter Wells, Enzyme Conjugate Reagent, Reference Standard, Wash Buffer, Chemiluminescence Reagent A and Chemiluminescence Reagent B

Reagent Preparation

Substrate solution- 1:1 mixing of Reagent A with Reagent B. Mixed gently. Reconstituted each lyophilized standard and controls with distilled water. Allowed to stand for 20 minutes. Diluted 1 volume of Wash Buffer with 49 volumes of distilled water. Mixed well before use.

Procedure

Secured the desired number of coated wells in the holder. Data sheet with was made sample identification. 50 µl of LH standard, specimens and controls were dispensed into appropriate wells. 100 µl of Enzyme Conjugate Reagent was dispensed into each well. Thoroughly mixed for 30 seconds. Incubated at room temperature (18-25°C) for 60 minutes. The incubation mixture was removed by flicking plate contents into a waste container. Rinsed and flicked the microtiter wells 5 times with washing buffer. The wells were striken sharply onto absorbent paper to remove residual water

droplets. 100 µl of chemiluminescence substrate solution was dispensed into each well. Gently mixed for 5 seconds. Wells were read with a chemiluminescence microwell reader 5 minutes later.

Follicle Stimulating Hormone (FSH) (CLIA)[23,24]

Principle

The assay system utilizes a polyclonal anti-FSH antibody for solid phase immobilization and a mouse monoclonal anti-FSH antibody in the antibody- enzyme conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between solid phase and enzyme-linked antibodies. After 60 minute incubation at room temperature, the wells are washed with water to remove unbound labelled antibodies. A solution of chemiluminescent substrate is then added and read Relative Light Units (RLU) in a illuminometer. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of FSH in the sample.

Reagents

Anti-FSH Antibody Coated Microliter Wells, Enzyme conjugate reagent, Reference Standard, Wash Buffer, Chemiluminescence reagent A & Chemiluminescence reagent B.

Reagent Preparation

Substrate solution- 1:1 mixing of Reagent A with Reagent B. Mixed gently. Diluted 1 volume of Wash Buffer with 49 volumes of distilled water. Mixed well before use.

Procedure

Secured the desired number of coated wells in the holder. Data sheet was made with sample identification. 50 µl of FSH standard, samples, and controls were dispensed into appropriate wells. 100 µl of Enzyme Conjugate Reagent was dispensed into each well. Thoroughly mixed for 30 seconds. Incubated at room temperature (18-25°C) for 60 minutes. The incubation mixture was removed by flicking plate contents into a waste container. Rinsed and flicked the microtiter wells 5 times with washing buffer. The wells were striken sharply onto absorbent paper to remove residual water droplets. 100 µl of Chemiluminescence substrate solution was dispensed into each well. Gently mixed for 5 seconds. The wells were read with a chemiluminescence microwell reader 5 minutes later (Between 5 and 20 min. after dispensing the substrates).

Testosterone (CLIA)[25]

Principle

The microliter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the Testosterone molecule. Endogenous Testosterone of a donor sample competes with a Testosterone horse radish peroxidase conjugate for binding to the coated antibody. After incubation, the unbound conjugate is washed off. The amount of bound peroxidase conjugate is reverse proportional to the concentration of Testosterone in the sample. After addition of the substrate solution, the intensity of emitted light is inversely proportional to the concentration of testosterone in the donor sample.

Reagents & preparation

Microliter wells coated with a monoclonal anti-testosterone antibody, Standard, Enzyme Conjugate and Substrate Solution Reagent A, Reagent B, Reagent C and Wash Solution.

Working Substrate Solution: Mixed 1 part of the chemiluminescence Reagent A with 1 parts of Reagent B. Diluted this mixture 1:1.5 with Reagent C.

Wash Solution: Diluted 30 mL of concentrated Wash Solution with 1170 mL deionized water to a final volume of 1200 mL.

Procedure

Secured the desired number of Microtiter wells in the holder. 20 µL of each Standard, Control and samples were dispensed into appropriate wells. 200 µL of Enzyme Conjugate was dispensed into each well. Thoroughly mixed for 10 seconds. Incubated for 60 minutes at room temperature. Rinsed the wells 5 times with diluted Wash Solution. The wells were striken sharply on absorbent paper to remove residual droplets. Added 100 µL of the freshly prepared Substrate Solution to each well. Incubated for 10 minutes at room temperature. The RLU was read with a microtiter plate luminometer within 20 minutes after incubation time of substrate.

Estrogen (Estradial (E2)) (CLIA)[26]

Principle - The microtiter plate provided in kit has been pre-coated with a monoclonal antibody specific to estradiol. A competitive inhibition reaction is launched between biotin labeled estradiol and unlabeled estradiol (Standards or samples) with the pre-coated antibody specific to estradiol. After incubation, the unbound conjugate is washed off. Next, avidin conjugated to Horse Radish Peroxidase (HRP) is added to each microplate well and incubated. The amount of bound HRP conjugate is reverse proportional to the concentration of estradiol in the sample. Then, the mixture of substrate A and B is added to generate glow light emission kinetics. Upon plate development of the intensity of emitted light is reverse proportional to estradiol level in the sample or standard.

Reagents

- Antibody Coated Microtiter Plate: Microplate coated with goat anti-rabbit IgG (1 plate, 48 wells/96wells)
- Enzyme Conjugate Reagent: Horseradish peroxidase (HRP) labeled estradiol (E2) in Stabilizing Buffer (1vial, 5.5ml/11.0 ml)
- Anti-E2 Reagent: Rabbit polyclonal antibodies to E2 (anti-E2 PAb) in Stabilizing Buffer (1 vial, 2.8ml/5.5 ml)
- Reference Standards: 0, 30, 100, 300, and 1000 pg/ml E2 in Stabilizing Buffer (5 vials, 0.3ml/ea)
- Substrate A: (1 vial, 1.8ml/3.5ml) Substrate B: (1 vial, 1.8ml/3.5ml)
- PBS-T Powder: PBS-Tween (1bag, 5g)

Reagent Preparation

- All kit components and samples were brought to room temperature (18-25^0 C) before use.
- **Standard -** Reconstituted the Standard with 0.5mL of Standard Diluent. Kept for 10 minutes at room temperature. Shaken gently(not to foam). The concentration of standard in the stock solution was 1,000pg/mL. Prepared 5 tubes containing 0.6mL of standard diluent and produced a quadruple dilution series.

  Mixed each tube thoroughly before the next transfer. Set up 5 points of diluted standard such as 1,000pg/mL, 250pg/mL, 62.5pg/mL, 15.6pg/mL, 3.9pg/mL and the last EP tubes with Standard Diluent was the blank as 0pg/mL.
- **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Diluted to the working concentration with Assay Diluent A and B respectively (1:100).
- **Wash Solution** - Diluted 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
- **Substrate working Solution -** Mixed the substrate A and B by the ratio of 99:1 to make the substrate working solution. Mixed thoroughly. For example, prepare 1,000µL Substrate working Solution with 990µL Substrate A + 10µL Substrate B.

Assay Procedure

- Determine Wells for diluted standard, blank and sample were determined. Prepared 5 wells for standard points and 1 well for blank.

- Added 50µL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells respectively. And then added 50µL of Detection Reagent A to each well immediately. Shake The plate was gently shaken (using a microplate shaker is recommended). Covered with a Plate sealer. Incubated for 1 hour at 37^0.
- Detection Reagent A may appear cloudy. Warmed to room temperature and mixed gently until solution appeared uniform.
- The solution was aspirated and washed with 350µL of 1X Washed Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher and let it sit for 1-2 minutes. Removed the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeated 3 times. After the last wash, removed any remaining Wash Buffer by aspirating or decanting. Inverted the plate and bloted it against absorbent paper.
- Added 100µL of Detection Reagent B working solution to each well. Incubated for 30 minutes at 37^0 C after covering it with the plate sealer.
- Added 100µL of substrate working solution to each well. Covered with a new plate sealer. Incubated for 10 minutes at 37^oC. Protected from light.
- Measured the chemiluminescence signal in a microplate luminometer or as appropriate for the instrument used.

Calculation

Calculated average of readings for each of standard, control and samples and subtracted the average zero standard relative light unit (RLU). Created a standard curve

on graph paper, with the log of estradiol concentration on the y-axis and the RLU value on the x-axis. The best fit straight line was drawn through the standard points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Progesterone (CLIA)[25,26]

Principle

Immuno specific progesterone Electro Immuno Assay is based on the principle of competitive binding between progesterone in the test specimen and progesterone HRP(Horse Radish Peroxidage) conjugate for a constant amount of antiprogesterone. Anti IgG-coated wells were incubated with 25 µl progesterone standards, controls, patient sample and 100 µl progesterone-HRP. Reagent and 50 µl antiprogesterone reagent were conjugated at room temperature (18-25°C) for 90 minutes. During incubation, a fixed amount of HRP-labelled progesterone competes with the endogenous progesterone in the standard, sample or quality control serum for a fixed number of binding sites of the specific progesterone antibody. Thus, the amount of progesterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of progesterone in the specimen increases. Unbound progesterone peroxidase conjugate was then removed and the wells were washed. A solution of chemiluminescent substrate was then added and read relative light units (RLU) with a Luminometer. The intensity of the emitting light is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled progesterone in the

sample. By reference to a series of progesterone standards assayed in the same way, the concentration of progesterone in the unknown sample is quantified.

Reagents

- Pre-coated, ready to use 96-well strip plate standard (lyophilized)
- Detection Reagent A (green)
- Detection Reagent B (red)
- Substrate A - Wash Buffer (30 × concentrate)

  Standard Diluent

  Assay Diluent A (2 × concentrate)

  Assay Diluent B (2 × concentrate)
- Substrate B

Reagent Preparation

- To prepare substrate solution, An 1:1 mixing of Reagent A with Reagent B right before use was made. Mixed gently to ensure complete mixing. Discarded excess after use.
- Diluted 1 volume of Wash Buffer (50x) with 49 volumes of distilled water. For example, Dilute 15 ml of Wash Buffer (50x) into distilled water to prepare 750 ml of washing buffer (1x). Mix well before use.
- To prepare Working Progesterone-HRP Conjugate Reagent, added 0.1 ml of Progesterone-HRP Conjugate Concentrate (11x) to 1.0 ml of Progesterone-HRP

Conjugate Diluent (1:10 dilution) and mixed well. The amount of conjugate diluted depends on the assay size. Discarded the excess after use.

Assay Procedure

- The desired number of coated wells were secured in the holder.
- 25 µl of standards, specimens and controls were dispended into appropriate wells.
- 50 µl of anti-progesterone reagent was dispended to each well.
- Gently mixed for 5 seconds. 100 µl of Working Progesterone-HRP conjugate reagent was dispended into each well.
- Thoroughly mixed for 30 seconds. It is very important to mix them completely.
- Incubated at room temperature (18-25°C) for 90 minutes.
- Rinsed and flicked the microwells 5 times with washing buffer(1X).
- Dispensed 100 µl chemiluminescent substrate solution into each well. Gently mixed for 5 seconds.
- Wells with a chemiluminescent microwell reader was read 5 minuters later. (between 5 and 20 min. after dispensing the substrates)

Calculation

Calculated the average. Relative light units (RLU) were read for each set of reference standards, control and samples.

Semen Analysis[26]

Collection of specimen

Sperm count was performed on semen samples collected from the cauda epididymis. The cauda portion was separated and placed in 0.25 M sucrose solution. The epididymal tubes were punctured with a fine hypodermic needle and spermatozoa were extruded by squeezing. Semen also can be collected from the vas deferens by squeezing. 10µl of semen was added to 990µl of sucrose solution and mixed well. 100µl was taken from mixture and neubauer counting chamber was charged.

Preparation of .25M sucrose solution

0.342(molecular weight of sucrose) gm of sucrose was added into 1 ml of distilled water. This formed 1M sucrose solution. 1M sucrose solution was diluted with 4 ml of distilled water. It became 0.25M sucrose solution.

Sperm Count

Sperm Concentration ("Sperm Count") is the number in millions of spermatozoa per milliliter of semen. Sperms are immobilized, diluted and counted in a quality-verified chamber such as a hemocytometer, Makler chamber or fixed-cover slip slide. Sperm heads within a 4 corner square area were counted in the same manner as blood cell counteing done using hemocytometer. Their concentration represented by their number in millions/mL.

Procedure (Neubauer Counting Chamber Method)

A clean and dry counting chamber was taken. Mixed properly & placed a drop (8 to 10µ) on to the centre of the lower glass disk. Placed the cover glass covering four corner squares. Semen should spread over the entire area of the lower glass disk avoid adding excess semen. Viewed Under X20 objective and 10X eyepiece. Counted the number of sperms within a strip of 10 squares. Repeated this count from 2 to 3 other strips and averaged the counts. If sperms were highly concentrated, it may be difficult to count the motile sperms. Sperms can be immobilized by heating an aliquote at 50-60 ^{0}C for 5min or placing the chamber at -20^{0}C for10min and then proceeding with the count.

Calculations:

Length of each square = 1mm

Width of each square = 1mm

Height of the Neubauer chamber = 1/10mm

$$\text{Dilution Factor} = \frac{\text{Final Volume}}{\text{Initial Volume}} = \frac{1000\ \mu l}{10\ \mu l} = 100\mu l$$

$$\textbf{No of Sperms/ml of semen} = \frac{\textbf{N X Dilution factor X 1000}}{\textbf{Areas counted X depth}}$$

Where

N= Number of sperms counted in 4 squares

Dilution factor= 100µl (10µl semen in 990µl sucrose sdution)

Areas counted= 4 corners of Thoma zeiss slide (WBC counting areas in Neubauer Counting chamber)

Depth = Depth of chamber, 0.1mm

1000= Conversion from µl to ml

I.e. No of Sperms/ml of semen = $\frac{N \times 100 \times 1000}{4 \times 0.1}$

Now $= \frac{N \times 25 \times 1000}{1/10}$

$= N \times 25 \times 1000 \times 10$

$= N \times 2{,}50{,}000$

= sperms/ml

Sperm motility

Percentage of motile sperms with time and speed of motility of individual sperm were assessed semi quantitatively and the spermatozoa were evaluated for the forward movement & graded accordingly.

Grading of sperm motility

0= No movement

1= sluggish or tail movement alone

2= intermittent sluggish movement

3-4= fair-good movement

5= maximum movement in forward direction

4.8.4. Histopathology

Manual tissue processing

Procedure was followed as per the Bancroft procedure[27]

Fixation: Fixation was the first step towards the preparation of a histological section from a dead biological specimen. The substances used for fixation were called as fixatives. Tissues were kept in fixative for 24hrs.

Common fixatives - Formalin, Zenker`s fluid.

Washing: After fixation, tissue was washed under running tap water 1-2 hours. It removed fixative from tissue.

Dehydration, clearing and Impregnation

Our aim was to imbibe tissue with paraffin. This was not be done directly as paraffin was not mixable from tissue with water of tissue. Dehydration was done in stages. Firstly, the tissue was immersed in alcohol which removed water from tissue (dehydration). Paraffin was not mixable with alcohol also. Xylene, benzene, chloroform and toluene were the substances which got mixed both with the alcohol and paraffin. Therefore, after alcohol the tissue was treated with one of these liquids (called clearing agents). Later tissue was impregnated in melted paraffin. Following was the list of duration for various manual tissue processings.

Step	Treatment	Hours
Fixation	Formalin (10%)	24
Dehydration	30%	1
	50%	1
	80%	1
	90%	1
	100%	1
	100%	1
	100%	1
Clearing	Xylene -1	1
	Xylene -2	1
Infiltration	Paraffin at 50-56^{o}C	1
	Paraffin at 50-56^{o}C	1
	Paraffin at 50-56^{o}C	1

Table 11: Procedure for manual tissue processing

Embedding: The process of embedding enabled specimens too small and / or too delicate to be surrounded with some suitable materials for example paraffin. Paraffin supported them on all sides with firmness but without producing any injurious effect on the them. The embedded tissue was then sectioned into sufficiently thin slices without distortion.

Preparation of blocks: It was done with the help of L-blocks. L-blocks are L-shaped metallic pieces. Two L-blocks were placed on glass plate, so that enclosed a rectangular

space. The tissue was put in it. The melted paraffin was poured on it which solidified slowly. The L-blocks were removed.

Section cutting: For histological preparation, tissue was cut (sectioned) into thin slices. Trim the upper or cutting surface of the block parallel to the knife edge until the surface of the tissue got exposed. Attached the block holder to the microtome. Clamped the knife firmly in position in microtome. Adjusted the desired thickness scale in the microtome. Moved the microtome fairly constant and fast. Picked up the end of short ribbon with a soft brush and gently pulled it away from the knife as one continues sectioning.

Hematoxylin and Eosin Staining

1. Xylene 2 Minutes
2. Xylene 2 Minutes
3. Absolute alcohol 1 Minute
4. Absolute alcohol 1 Minute
5. 90% Alcohol 2 Minutes
6. 70% Alcohol 2 Minutes
7. 50% Alcohol 2 Minutes
8. Distilled water 5 Minutes
9. Hematoxylin 2 to 5minutes {depending on which of the above types of Hematoxylin Solution was used (Stain for 20 minutes with Erlich`s and for 2-5 minutes with Harris` Hematoxylin)}.

10. Washed well in running tap water for 2-3 minutes. Section was examined under microscope to confirm the sufficiency of staining.
11. Removed excess stain by differentiation in acid alcohol (1% HCL in 70% alcohol) for a few seconds. Blue colour of hematoxylin stained section was changed to red by action of acid.
12. Immediately washed in alkaline tap water for at least 5 minutes to regain the blue colour (`bluing section`) (a few drops of saturated lithium carbonate may be added to the tap water in a large beaker and bluing of section will be done by this. After bluing, the slides should be thoroughly washed in running tap water to remove excess lithium carbonate). If necessary, examine under microscope.
13. 1 % aqueous Eosin 1 to 3 minutes
14. Washed with water to remove excess Eosin
15. Examined the sections microscopically. Cytoplasm and muscle cell were deep pink, collagen fibers a lighter pink, erythrocytes and granules of eosinophils were bright orange red.
16. 90% alcohol for 10 to 15 min
17. Absolute alcohol I for 10 to 15 seconds
18. Absolute alcohol II for 10 to 15 seconds
19. Xylene I – 1 to 2 min
20. Xylene I – 1 to 2 min or until completely clear
21. Mounted in D.P.X. or any other synthetic resin medium.

Results

Muscle cell – deep pink, Collagen fibres – lighter pink, erythrocytes and granules of eosinophils – bright orange red, Cytoplasm – pink, Nuclei – blue.

4.9. Statistical Analysis

Data pertaining to all control and experimental samples had been subjected to statistical analysis for evaluation of the range of significance. Data was entered in Microsoft excel. Mean $\pm$ SD (standard deviation) values were calculated for each group and were represented in the form of appropriate tables and diagrams.

To determine the significance of inter-group differences, each parameter was analysed separately. All the parameters were compared by using proper statistical tests using software SPSS V 20 32bit. Values were analysed by one-way analysis of variance (ANOVA). Post hoc Turkey HSD test was used to study the differences between the groups. The level of statistical significance was set at $P<0.05$.

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CHAPTER 5

RESULTS

5.1. Gravimetry

5.1.1. Weight gain

Weights of the rats were regularly recorded during dosage period. Initial and final weights were considered to calculate the weight gain. All the rats in group I, II and III remained healthy and active with normal behaviour. However, rats of group IV acted as diabetic control, where animals with induced diabetes mellitus without any treatment were found to be lethargic and their body weight gain was found to be negative among all groups. This was statistically significant when compared with normal control rats, normal rats treated with Nigella sativa seed powder, normal rats treated with thymoquinone, induced diabetic rats treated with Nigella sativa seed powder and induced diabetic rats treated with thymoquinone. However, in case of group V(induced diabetic rats + Nigella sativa seed powder) and group VI (induced diabetic rats + thymoquinone), an improvement in body weight was observed as compared with the group IV (diabetic induced control) rats (Table 12).

5.1.2. Weight of organs and Organosomatic Index (Testis and Epidydimis)

Weight of organs was measured with simple electronic balance. The organs were collected, washed in saline and weighed. Testis of both sides were collected and weighed. The average weight was calculated. The same method followed for measurement of weight of epididymis. Organosomatic index was calculated by weight of organ multiplied by 100 and divided by total body weight.

$$\text{Organosomatic index} = \frac{\text{Weight of organ} \times 100}{\text{Total body weight}}$$

Weight of Testis and Epididymis – Weights of testis and epididymis were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The Weights of testis and epididymis were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III)(Table 13).

Somatic index of Testis and Epididymis - Somatic indices of testis and epididymis were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The Somatic indices of testis and epididymis were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III)(Table 13).

Groups ⇨	I	II	III	IV	V	VI		
Parameter ⇩	Normal Rats– Control	Normal Rats – Nigella sativa seed powder	Normal Rats – Thymo quinone	Diabetic Rats– Control	Diabetic Rats – Nigella sativa seed powder	Diabetic Rats – Thymo quinone	F – Value	P-Value
Initial Body weight (g)	225.83± 17.07[a]	219.17± 10.59[a]	232.50± 21.57[a]	164.50± 15.27[b]	171.67± 21.02[b]	178.67± 17.40[b]	18.1	0.006
Final Body weight (g)	340.33± 16.12[a]	322.50± 13.69[a]	332.50± 24.44[a]	135.50± 20.98[b]	236.17± 22.17[c]	252.50± 14.40[c]	97.1	0.001
Change in Body Weight (%)	51.71± 16.53[a]	47.47± 10.12[a]	43.44± 9.12[a]	-17.59± 10.45[b]	38.94± 17.84[a,c]	42.81± 19.43[a,c]	21.4	0.003
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 12: Initial, final body weight & weight gain in each group

Groups ⇨	I	II	III	IV	V	VI		
Parameter ⇩	Normal Rats– Control	Normal Rats – Nigella sativa seed powder	Normal Rats – Thymoquinone	Diabetic Rats– Control	Diabetic Rats – Nigella sativa seed powder	Diabetic Rats – Thymoquinone	F - Value	P-Value
Weight of testis(g)	2.27± 0.13[a]	2.06± 0.18 [a]	2.19± 0.09 [a]	0.68± 0.07 [b]	1.67± 0.16 [c]	1.70± 0.22 [c]	71.5	0.002
Testicular somatic index (%)	0.0067± 0.0004[a]	0.0064± 0.0007 [a]	0.0066± 0.0003 [a]	0.0051± 0.0012 [b]	0.0071± 0.0005 [a,c]	0.0068± 0.0011 [a,c]	6.93	0.003
Weight of Epididymis(g)	0.72± 0.09[a]	0.80± 0.11 [a]	0.75± 0.09 [a]	0.20± 0.04 [b]	0.55± 0.08 [c]	0.53± 0.02 [c]	45.9	0.001
Epididymis somatic index (%)	0.0021± 0.0003[a]	0.0025± 0.0004 [a]	0.0023± 0.0003 [a]	0.0015± 0.0004 [b]	0.0023± 0.0004 [a,c]	0.0021± 0.0002 [a,c]	6.21	0.002
Values with superscripts in each row among various groups were statistically significant with each other (P<0.05).								

Table 13: Organosomatic index of Testis and Epididymis.

5.2. Biochemistry

5.2.1. Glucose - Levels of glucose were increased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of glucose were reduced significantly in diabetic rat groups treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 14).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats– Thymo quinone**	**F – Value**	**P-Value**
Levels of Initial Glucose (mg/dl)	84.50± 11.67[a]	88.17± 14.34[a]	80.33± 9.11[a]	202.67± 23.11[b]	211.17± 28.30[b]	207.83± 17.21[b]	79.60	0.05
Levels of Final Glucose (mg/dl)	88.67± 15.36[a]	80.83± 12.24[a]	84.67± 8.34 [a]	254.67± 34.12[b]	171.50± 23.05[c]	160.33± 18.04[c]	68.08	0.002
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 14: Levels of Glucose

5.2.2. Insulin

Levels of insulin were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of insulin were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 15).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats – Thymo quinone**	**F - Value**	**P- Value**
Levels of Insulin (mu/L)	0.48 ± 0.10^{a}	0.45 ± 0.06^{a}	0.42 ± 0.12^{a}	0.14 ± 0.04^{b}	0.32 ± 0.09^{c}	0.34 ± 0.14^{c}	8.91	0.004
Values with superscripts in each row among various groups were statistically significant with each other($P<0.05$)								

Table 15: Levels of Insulin

5.2.3. Serum levels of Malondialdehyde (MDA)

The MDA levels were increased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of MDA were reduced significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Figure 5).

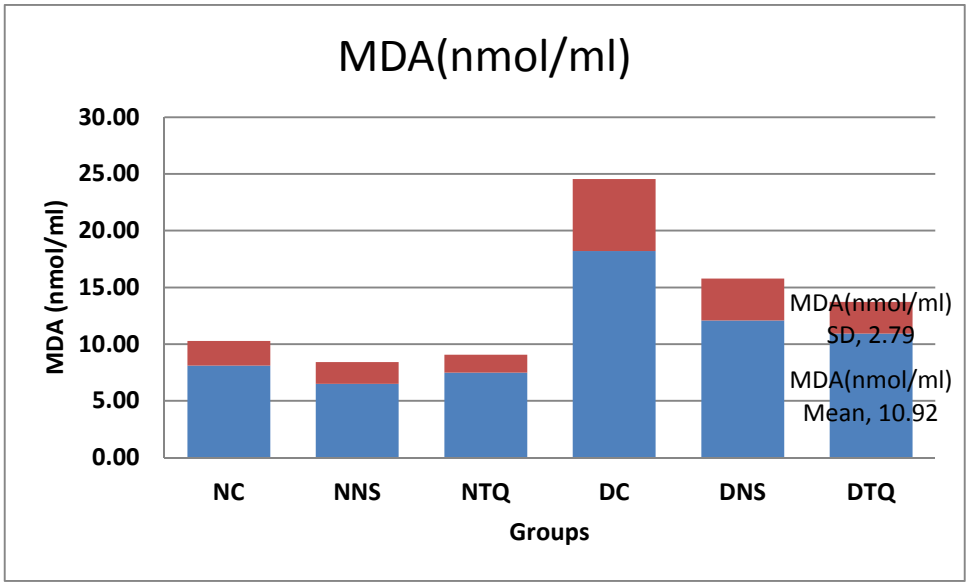

Figure 5: Serum Levels of MDA (nmol/ml)

NC- Normal Control Rats, NNS- Normal rats treated with Nigella sativa, NTQ- Normal rats treated with Thymoquinone, DC- Diabetic Control Rats, DNS- Diabetic rats treated with Nigella sativa, DTQ- Diabetic rats treated with Thymoquinone.

5.2.4. Endogenous antioxidant - Serum Superoxide Dismutase (SOD), Exogenous antioxidants – Serum Vitamins C and E

The levels of Superoxide dismutase (SOD), Vitamins C and E were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of SOD, Vitamins C and E were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Figure 6,7,8).

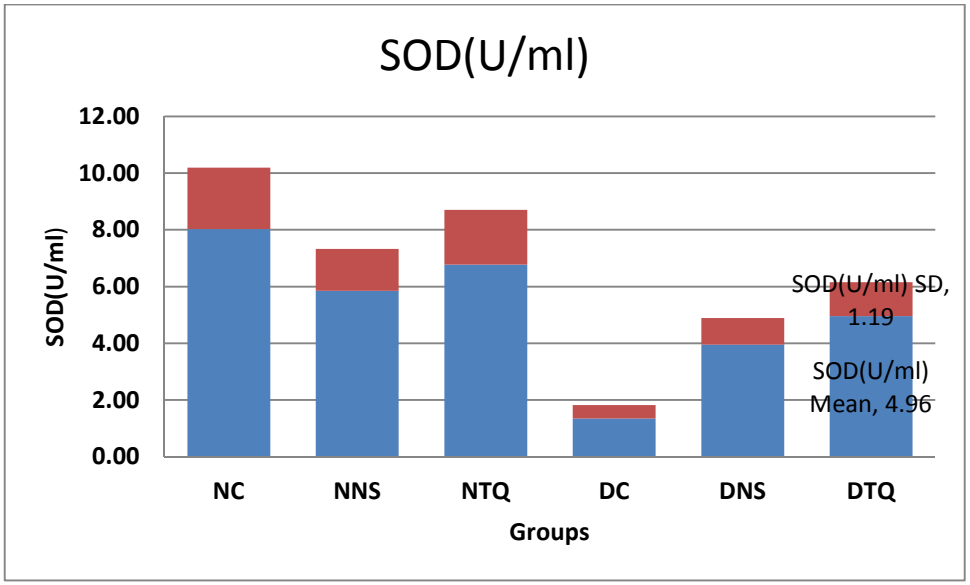

Figure 6: Levels of SOD(U/ml)

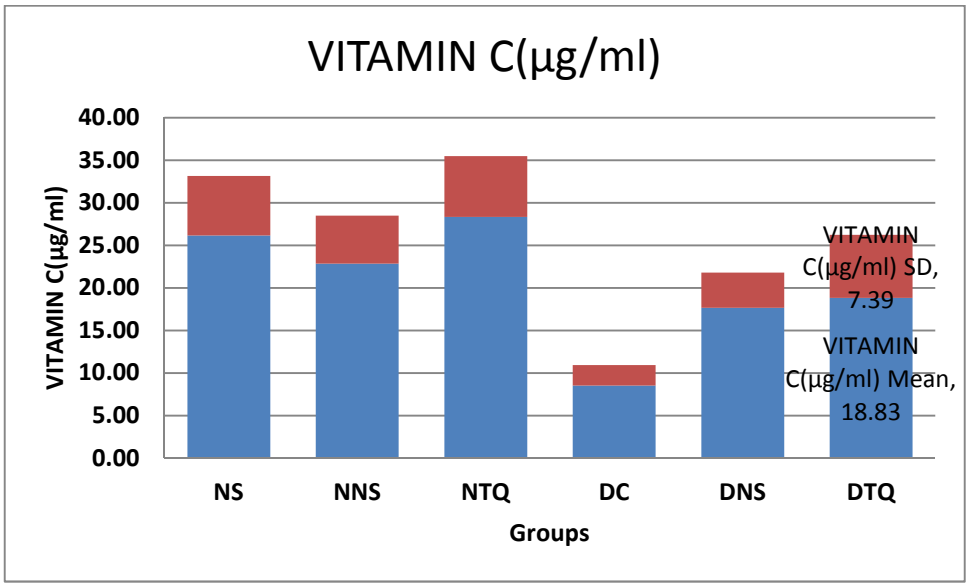

Figure 7: Levels of Vitamin C (µg/ml)

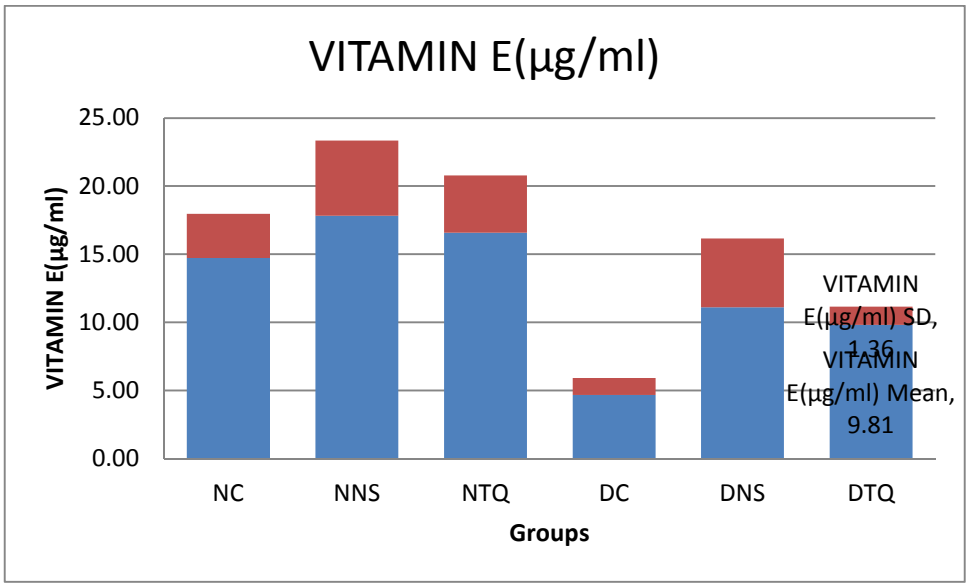

Figure 8: Levels of Vitamin E (µg/ml)

5.2.5. Total protein

The levels of total protein were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of total protein were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 16).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats – Thymo quinone**	**F - Value**	**P-Value**
Levels of Total Protein(g/dl)	9.02 ± 2.55^{a}	12.17 ± 3.93^{a}	10.18 ± 2.61^{a}	3.30 ± 1.32^{b}	6.27 ± 0.82^{c}	7.02 ± 2.13^{c}	10.02	0.02
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 16: Levels of Total Protein

5.3. Reproductive Hormones

5.3.1. Testosterone

The levels of testosterone were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of testosterone were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 17).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats – Thymo quinone**	**F - Value**	**P- Value**
Levels of Testosterone (ng/dl)	84.69± 15.91 [a]	79.57± 11.09[a]	91.38± 20.65[a]	30.48± 6.82[b]	60.82± 18.45[c]	56.32± 9.48[c]	14.35	0.01
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 17: Levels of Testosterone.

5.3.2. LH and FSH

The levels of LH and FSH were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of LH and FSH were increased in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI) but it was not statistically significant. There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 18).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats – Thymo quinone**	**F - Value**	**P- Value**
Levels of LH (mIU/L)	0.50± 0.07 [a]	0.42± 0.11[a]	0.46± 0.08[a]	0.17± 0.06[b]	0.32± 0.09[b]	0.28± 0.07[b]	21.63	0.05
Levels of FSH (mIU/L)	1.31± 0.39 [a]	1.44± 0.26[a]	1.50± 0.43[a]	0.46± 0.22[b]	0.63± 0.31[b]	0.52± 0.26[b]	14.07	0.04
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 18: Levels of LH and FSH.

5.3.3. Progesterone and Estrogen

The levels of progesterone and estrogen were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The levels of progesterone and estrogen were not shown significant change in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 19).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats – Thymo quinone**	**F - Value**	**P- Value**
Levels of Progesterone (ng/ml)	12.76± 2.60 [a]	11.60± 1.52[a]	14.10± 3.01[a]	5.02± 2.21[b]	6.17± 3.18[b]	4.49± 0.87[b]	19.41	0.03
Levels of Estrogen (pg/ml)	27.68± 8.24 [a]	23.15± 4.09[a]	25.77± 6.02[a]	12.91± 3.27[b]	16.35± 5.32[b]	11.62± 1.99[b]	10.33	0.04
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 19: Levels of Progesterone and Estrogen.

5.4. Semen Analysis (Sperm count and Sperm motility)

The sperm count and sperm motility were decreased significantly in diabetic control (group IV) rats compared with normal control (group I) rats, normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III). The sperm count and sperm motility were increased significantly in diabetic rats treated with Nigella sativa seed powder (group V) and thymoquinone (group VI). There was no significant change between normal control (group I) rats and normal rats treated with Nigella sativa seed powder (group II) and thymoquinone (group III) (Table 20).

Groups ⇨	**I**	**II**	**III**	**IV**	**V**	**VI**		
Parameter ⇩	**Normal Rats– Control**	**Normal Rats – Nigella sativa seed powder**	**Normal Rats – Thymo quinone**	**Diabetic Rats– Control**	**Diabetic Rats – Nigella sativa seed powder**	**Diabetic Rats – Thymo quinone**	**F - Value**	**P- Value**
Sperm count (Millions/ml)	136.75± 21.08 [a]	143.17± 25.16[a]	130.04± 14.74[a]	51.13± 15.73[b]	83.83± 10.19[c]	91.54± 20.30[c]	23.14	0.002
Sperm Motility(%)	72.38± 9.32 [a]	66.04± 11.37[a]	75.25± 5.39[a]	27.25± 7.88[b]	45.04± 10.30[c]	49.21± 6.61[c]	27.28	0.01
Values with superscripts in each row among various groups were statistically significant with each other(P<0.05)								

Table 20: Sperm Count and Motility.

5.5. Histopathology

5.5.1. Histopathology of Testis

Normal control rats (group I): Sections studied under H&E stain showed well preserved normal cellular architecture of testis comprised of spermatogonia, sertoli cells, primary spermatocytes, spermatids, spermatozoa with an albugineous layer, complete seminiferous tubule cell series and good number of Leydig cells. (Figure 9,15).

Normal rats treated with Nigella sativa seed powder (group II): Sections studied under H&E stain showed the same architecture as normal control rats. There were no pathological changes (Figure 10).

Normal rats treated with thymoquinone (group III): Sections studied under H&E stain showed the same architecture as normal control rats. There were no pathological changes (Figure 11).

Diabetic control rats (Group IV): Sections studied under H&E stain showed reduction in size of seminiferous tubules and impairment of germinal epithelium. The structure of seminiferous tubules in the diabetic rats was found disrupted and there was a considerable decrease in the number of spermatogenic cell series like spermatogonia, Sertoli cells, primary spermatocytes and spermatids. The atrophy of the tubules with varying degrees of spermatogenetic arrest or reduced formation of spermatozoa also found. The less number of Leydig cells have been observed. Thickness of basement membrane was increased (Figure 12,16).

Diabetic rats treated with Nigella sativa seed powder (Group V) and thymoquinone (Group VI): Section studied under H&E stain showed the normal architecture. There was an improvement in the structure of seminiferous tubules compared with the diabetic group. These sections showed regain of the seminiferous tubules lined by several layers of spermatogenic cell series similar to that of normal rats. (Figure 13,14,17,18).

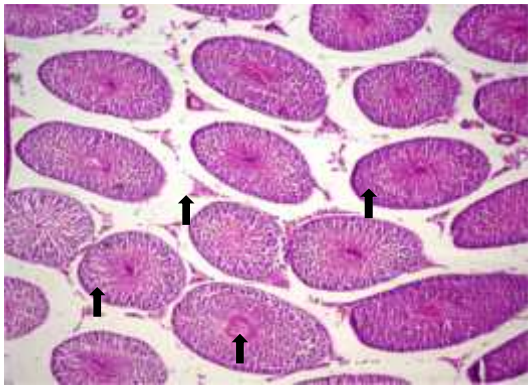

Figure 9: Testis of normal control rats. Section showing classical histological features with spermatogonia, sertoli cells,primary spermatocytes, spermatids, spermatozoa and Leydig cells (H&E10X).

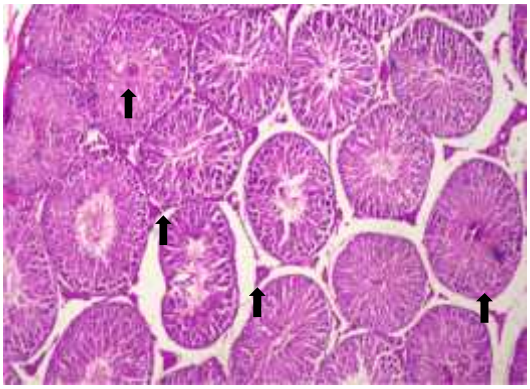

Figure 11: Testis of normal rats treated with thymoquinone. Section showing classical histological features with spermatogonia, sertoli cells, primary spermatocytes, spermatids, spermatozoa and Leydig cells (H&E10X).

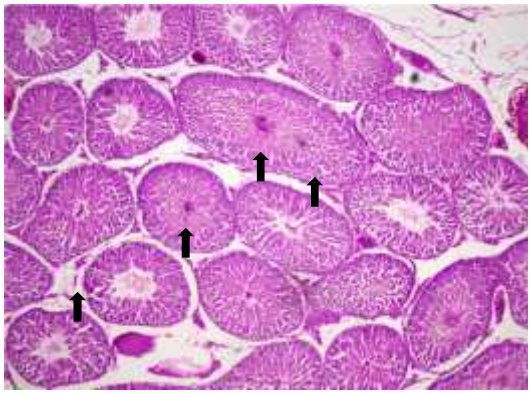

Figure 10 : Testis of normal rats treated with Nigella sativa. Section showing classical histological features with spermatogonia, sertoli cells, primary spermatocytes, spermatids, spermatozoa and Leydig cells (H&E10X).

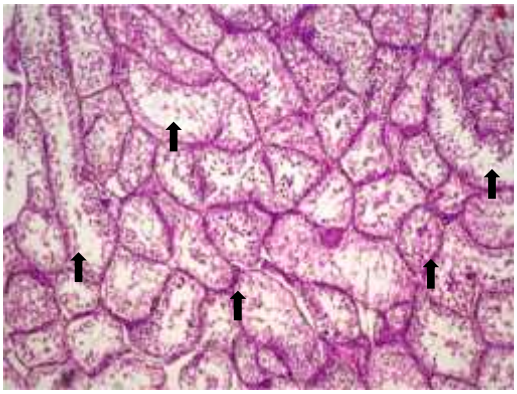

Figure 12: Testis of diabetic control rats. Section showing disrupted seminiferous tubules and decreased spermatogenic cells, spermatogonia, sertoli cells, spermatids and Leydig cells. Thickening of basement membrane(H&E 10X).

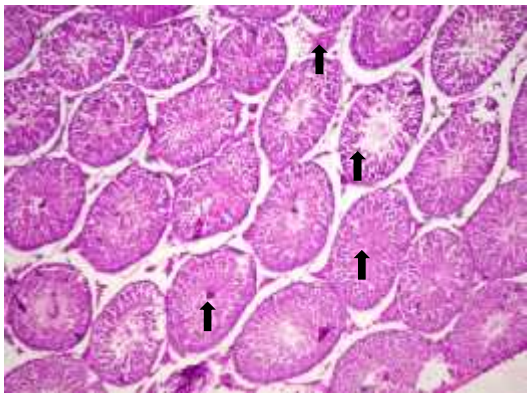

Figure 13: Testis of diabetic rats treated with Nigella sativa. Section showing regain of the seminiferous tubules lined by several layers of spermatogenic cell series similar to that of normal rats (H&E-10X).

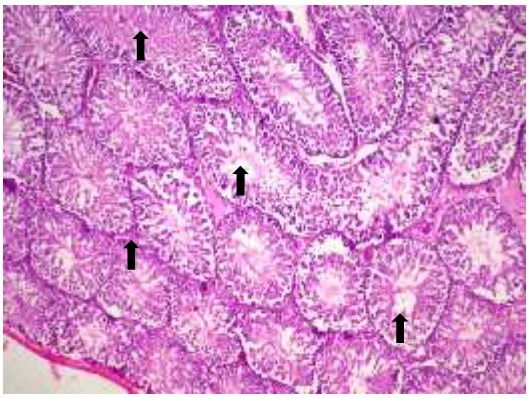

Figure 14: Testis of diabetic rats treated with thymoquinone. Section showing regain of the seminiferous tubules lined by several layers of spermatogenic cell series similar to that of normal rats (H&E-10X).

Figure 15: Testis of normal control rats. Section showing classical histological features with spermatogonia, sertoli cells, primary spermatocytes, spermatids, spermatozoa and Leydig cells (H&E- 20X).

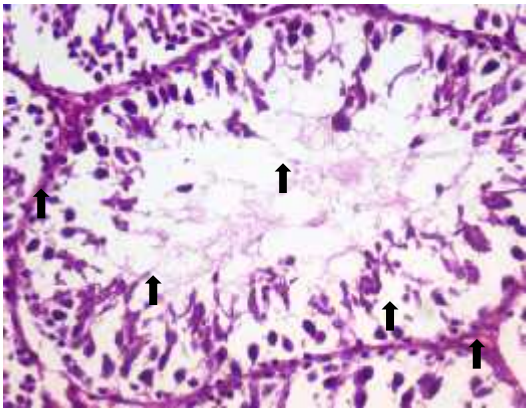

Figure 16: Testis of diabetic control rats. Section showing disrupted seminiferous tubules and decreased spermatogenic cells, spermatogonia, sertoli cells, spermatids and Leydig cells. Thickening of basement membrane (H&E- 20X).

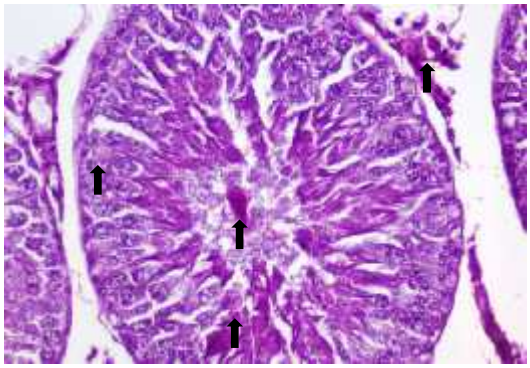

Figure 17: Testis of diabetic rats treated with Nigella sativa. Section showing regain of the seminiferous tubules lined by several layers of spermatogenic cell series similar to that of normal rats (H&E-20X).

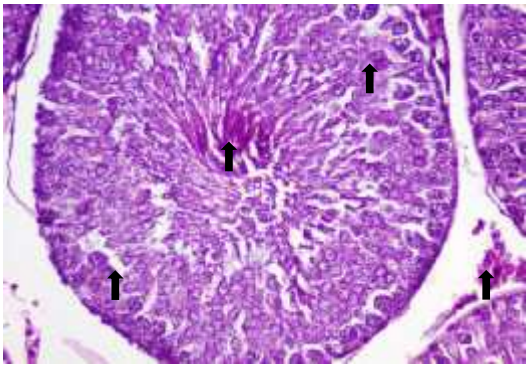

Figure 18: Testis of diabetic rats treated with thymoquinone. Section showing regain of the seminiferous tubules lined by several layers of spermatogenic cell series similar to that of normal rats(H&E-20X).

5.5.2. Histopathology of Epididymis

Normal control rats (group I): Sections studied under H&E stain showed the well preserved normal cellular architecture of the epididymis. The lumen of epididymis ducts were filled with spermatozoa. The basement membrane containing the smooth muscle layer, adjacent to the smooth muscle layer, cells and fibers of the connective tissue were found (Figure 19).

Normal control rats treated with Nigella sativa seed powder (group II): Sections studied under H&E stain showed the same architecture as normal control rats. There were no pathological changes (Figure 20).

Normal control rats treated with thymoquinone (group III): Sections studied under H&E stain showed the same architecture as normal control rats. There were no pathological observations (Figure 21).

Diabetic control rats (Group IV): Sections studied under H&E stain showed the thickening of basement of tubules, degenerative epithelium and very less or no sperm were found in the lumen of ducts (Figure 22).

Diabetic rats treated with Nigella sativa seed powder (Group V): Sections studied under H&E stain showed the thickness of basement membrane was found to come back to normal and epithelium was found to be with few degenerative changes and with good volume of spermatozoa (Figure 23).

Diabetic rats treated with thymoquinone (group VI): Sections studied under H&E stain showed the same features as diabetic rats treated with Nigella sativa seed powder (Group V) (Figure 24).

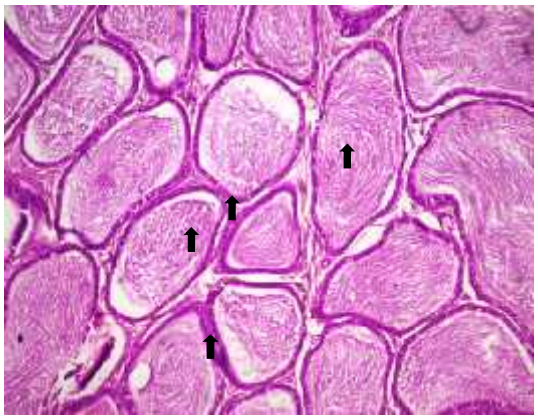

Figure 19: Epididymis section of normal control rats showing the lumen of ducts was filled with spermatozoa. The basement membrane with smooth muscle layer (H&E-10X).

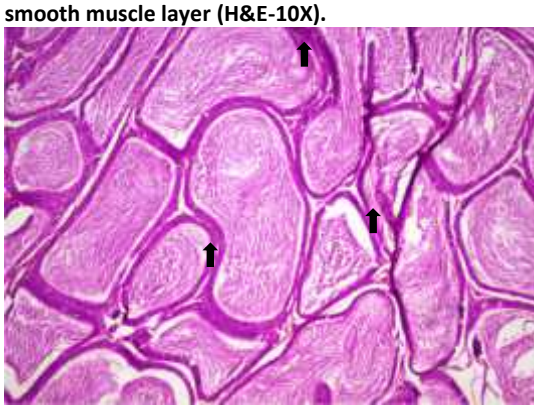

Figure 20: Epididymis section of normal rats treated with Nigella sativa showing the lumen of ducts was filled with spermatozoa. The basement membrane with smooth muscle layer (H&E-10X).

Figure 21: Epididymis section of normal rats treated with thymoquinone showing the lumen of ducts was filled with spermatozoa. The basement membrane with smooth muscle layer (H&E-10X).

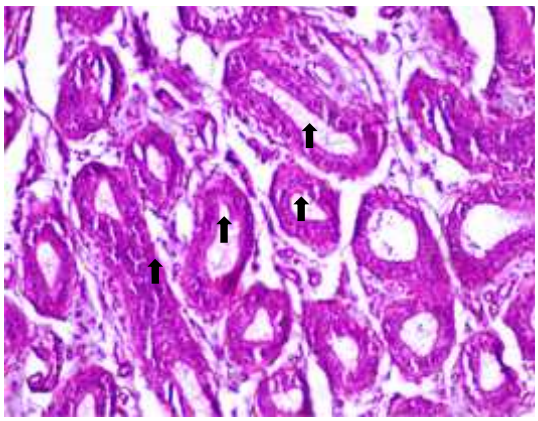

Figure 22: Epididymis section of diabetic control rats showing thickening of basement of tubules, degenerative epithelium and very less or no sperm were found in the lumen of ducts (H&E -10X).

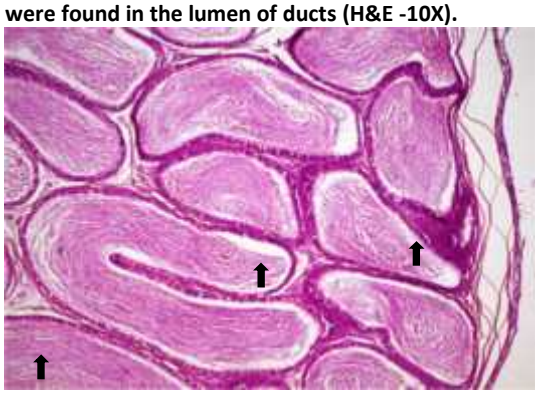

Figure 23: Epididymis section of diabetic rats treated with Nigella sativa showing reduced thickness of basement membrane appearing as normal and epithelium was with few degenerative changes and with good volume of spermatozoa (H&E-10).

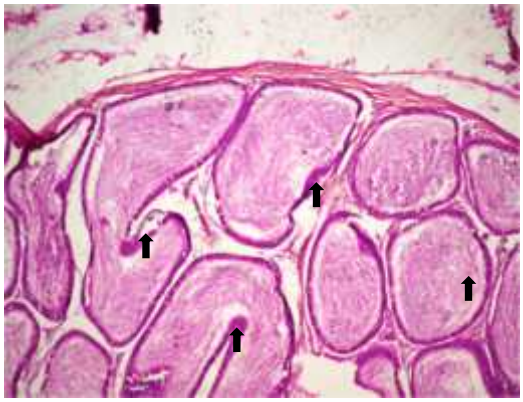

Figure 24: Epididymis section of diabetic rats treated with thymoquinone showing reduced thickness of basement membrane appearing as normal and epithelium was with few degenerative changes and with good volume of spermatozoa (H&E-10).

CHAPTER 6

DISCUSSION

6.1. Gravimetry

6.1.1. Weight gain

Results of the present study showed negative weight gain in case of streptozocin induced diabetic rats. It clearly indicates diabetes mellitus interfering with metabolic pattern of experimental animals. This may be due to increased hyperglycaemia which in turn leads to protein overutilization associated wasting due to inaccessibility of carbohydrate[1]**.** A significant improvement of body weight gain in case of Nigella sativa seed and thymoquinone treated induced diabetic rat groups indicates a beneficial role of both the compounds to overcome the effect of diabetes mellitus[2]**.** A possible explanation for this might be that Nigella sativa seed and thymoquinone reduce hyperglycaemia as well as protein overutilization associated wasting due to inaccessibility of carbohydrate[3]**.** There was no significant change in normal rats treated with Nigella sativa seed powder and thymoquinone compared with normal control rats. Our results are in agreement with the findings of study of Samad Alimohammadi et al[2]. A significant weight loss was observed in the diabetic untreated group, while Nigella sativa seed powder and thymoquinone treated rat groups exhibited significant increase in the body weight in comparison with diabetic untreated group but lower than in the normal controls. In the study of Kanter et al[3], Nigella sativa markedly improved body weight gain in STZ-induced diabetic rats. The study of Eman G. E. et al[4] revealed severe loss in body weight gain in diabetic rats when compared with the normal rats. In this study, they mentioned that this loss may be due to inhibition of synthesis of DNA and RNA in the diabetic

animals and/or it is attributed to different side effects on the ability to use carbohydrates including lipolysis, glycogenolysis and acidosis. This result is mainly due to destruction of β -cells which may lead to sudden decrease in secretion of insulin.

In a study conducted by Thanaa A.A. [5], they observed diabetic rats gained less body weight and showed low feed efficiency ratio as compared to normal control rats. Feeding diabetic rats on diets supplemented with Nigella sativa improved body weight gain and increased feed efficiency ratio when compared with the diabetic control group. Alduwish Manal et al[6] in their study showed significant difference in body weight loss between normal control and diabetic control. In this study, researchers observed significant increase in body weight between diabetic control and diabetic groups treated with thymoquinone. Jabbar A.A.[7] study results indicated reduction in body weight of diabetic untreated rats. This may be due to growth retardation which may be in turn due to the obstruction to glucose uptake caused by the lack of insulin following sterptozotocin injection. After treatment with thymoquinone, concerned group showed significant increase in the body weight. Hui liu et al[8] in their study demonstrated that thymoquinone treatment significantly increased body weight in diabetic rats. The study of El-Mahmoudy et al[9] also presented same results and stated that thymoquinone protects against the streptozocin induced diabetes via inhibition of Nitric Oxide. Pari and Sankaranarayanan[10] study also showed beneficial effect of thymoquionone on body weight in diabetic rats.

Our results are also in agreement with findings of Kaleem M et al[11] that streptozocin induced diabetic rats showed significant decrease in body weight and after treatment with Nigella sativa significant improvement in the body weight and weights of kidney and liver. Ozdemir et al[12] attributed the reduction of body weight may be due to the breakdown of tissue protein in the diabetic group. Diabetes mellitus causes a significant increment in skeletal muscle catabolism along with a decline in protein synthesis[13].

6.1.2. Organ weight and Organosomatic index (Testis and Epidydimis)

Significant decrease in the weights of testis and epidydimis in induced diabetic group of rats indicates an alteration of metabolism in those organs. Decrease in organosomatic index of testis and epidydimis of streptozocin induced diabetic groups may be due to decreased bodyweight. The improvement of organ weight in testis and epidydimis in groups of Nigella sativa seed powder and thymoquinone treated induced diabetic rats indicates a beneficial effect in this regard possibly by resorted altered metabolism of concerned organs[11]. There was no significant change in normal rat groups treated with Nigella sativa seed powder and thymoquinone compared with normal control rat group, which indicates non toxicity of Nigella sativa seed and its major bioactive component, thymoquinone .

Mustafa S. Atta et al[14] study revealed that the rats body weight and relative weight of reproductive glands decreased during the course of diabetes. Relative weights of testicles and epididymis were decreased in diabetic rats and may be attributed to the

testicular deterioration and decline in the weight of epididymis. Growth and secretory action of reproductive organs depend upon testosterone. The study of Soudamani et al[15] has shown that the atrophic alteration in the epididymis is due to reduced tubular diameter, volume and surface density in diabetic rats. Histological findings of present study also observed the same. In this study, diabetic rats showed hyperglycemia and decreased serum insulin level. This finding is in agreement with results of study conducted by Ballester et al[16]. In their study, they found that diabetic rats displayed a reduction in the levels of serum insulin, testosterone, FSH and LH. Decreased insulin levels reflected a sharp weakening of spermatogenesis and consistent with results reported by Bruening[17]. Navarro-Casado L et al[18] study observed the significant reduction in weight of testis and epidydimis. Shrilatha B Muralidhara[19] study also observed the reduction in the weights of reproductive organs. The researchers demonstated the experimental induction of diabetes mellitus by chemical diabetogens to impair testicular function progressively leading to decreased fertility. Based on the occurrence of oxidative impairments in sterptozotocin induced diabetes group of animals both during early and progressive phase, it is hypothesized that oxidative stress mechanisms may be wholly or in part contribute towards the development of testicular dysfunction and degeneration under situations of experimentally induced diabetes in animal models.

6.2. Biochemistry

6.2.1.Glucose hemostasis

Glucose and Insulin

The level of glucose was increased significantly in induced diabetic rats. The level of glucose was reduced significantly in diabetic rat groups treated with Nigella sativa seed powder and thymoquinone. The level of insulin was decreased significantly in induced diabetic rats. This may be due to toxic effects of streptozocin on beta cells of Islets of Langerhans which may in turn lead to insufficient secretion of insulin[1]. The treated groups of induced diabetic rats with Nigella sativa seed and thymoquinone showed significant increase in the levels of insulin. There was no significant change in normal rats treated with Nigella sativa seed powder and thymoquinone compared with normal control rats. The hypoglycemic effect of Nigella sativa seed and it`s bioactive component, thymoquinone may be due to reduction in oxidative stress, thus preserving pancreatic beta cell integrity leading to insulin level to increase[2,5]. In the study of Kaleem M et al[11], treatment with Nigella sativa seed resulted in elevation in the levels of glutathione(GSH). GSH which is present in the beta cells of islets, protects the membranes against oxidative damage by regulating the redox status of membrane protein[20]. In the study of Kanter et al[3], Nigella sativa caused a sharp decrease in serum concentration of glucose and increase in serum concentration of insulin in streptozocin induced diabetic rats. In addition, Nigella sativa treatment protected the majority of beta cells of Islets of Langerhans. In another study conducted by same researchers, treatment

with Nigella sativa in streptozocin induced diabetic albino rats caused a decrease in the elevated serum glucose and an increase in the lowered serum insulin concentrations as well as an improvement in histological appearance of Islets of Langerhans of diabetic rats. They stated that hypoglycemic effect of Nigella sativa could be partly due to amelioration in beta cells of pancreatic islets causing an increase in insulin secretion[21]**.** According to Alsaif MA[22] , the other possible reason for blood glucose lowering effect of Nigella sativa may be due to improved insulin sensitivity in diabetic rats. Furthermore, the Nigella sativa contains many bioactive constituents such as thymoquinone, p-cymene, pinene, dithymoquinone and thymohydroquinone, which act as antioxidants[23].

According to Fararh K.M. et al[24], the elevated gluconeogenesis under the diabetic condition is decreased by treatment with the thymoquinone. The results of this study indicate that thymoquinone is beneficial pharmaceutical care diabetes mellitus. It has been demonstrated that volatile oil of Nigella sativa seeds possesses glucose lowering action. The results of study of Fararh K.M. et al[24] clearly showed that administration of thymoquinone, a most abundant component of the oil, is alone sufficient to produce a decrease in blood glucose levels in STZ-induced diabetic animals. This indicates that the glucose lowering effects of the volatile oil of Nigella sativa seeds would largely be attributable to pharmaceutical usefulness of thymoquinone against diabetes mellitus. The decrease in total glycated hemoglobin levels in study of Fararh K.M. et al[24] reflects the adequate and effective action of thymoquinone in long-term reduction of diabetic hyperglycemia.

According to Fararh K.M. et al[24] , thymoquinone may reduce blood glucose level by affecting several sites, such as enhancement of peripheral glucose uptake or reduction in hepatic gluconeogenesis. The same study clarifies the glucose lowering mechanism and effect of thymoquinone on hepatic glucose production. It may be due to regulation of hepatic glucose production through gluconeogenesis. Their results clearly show that thymoquinone restores elevated glucose output in diabetic condition. This significant decrease in liver glucose output suggests that the observed antidiabetic action of thymoquinone is mediated through a decrease in hepatic gluconeogenesis. El-Dakhakhny et al[25] study also demonstrated that Nigella sativa may be mediated by extrapancreatic actions rather than by stimulated insulin release for its hypoglycemic effect.

According to Gupta et al[26] study, glucose lowering effect of thymoquinone is not directly related to the acute action of insulin. Alternatively, it has been demonstrated that in diabetics, the increased gluconeogenesis is related to increased expression of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase, glucose-6phosphatase and fructose-1,6-bisphosphatase in the liver. According to El-Mahmoudy et al[27], decreased gluconeogenesis by thymoquinone may be due to suppression of synthesis of the gluconeogenic enzymes.

In the study of Labhal et al[28], Nigella sativa alone was very effective restoring glucose homeostasis**.** They showed in another study that Nigella sativa seed given by intragastric gavage was able to correct diabetes. Le et al[29] confirmed the reduction of intestinal transport in vivo and increased insulin sensitivity. They are in agreement with

Al-Awadi et al[30], in their study obtained an improved OGTT response in streptozocin rats treated with a mixture of plants containing Nigella sativa. Bouchra M et al[31] showed that Nigella sativa aqueous extract directly affected intestinal absorption of glucose. Moreover, electrogenic Sodium dependent glucose transport was inhibited. Thus, inhibition of intestinal glucose absorption by Nigella sativa seed is significant and may produce recognized hypoglycemic effect of the plant. Most of the previous studies showed that hypoglycemic activity of Nigella sativa seed in streptozocin induced diabetic rats may be due to its components mainly thymoquinone and nigellone which are antioxidant in nature helping in preserving integrity of beta cells of islets[1,2].

6.2.2. Oxidative stress (MDA) and Anti-oxidative (SOD, Vitamins C & E) markers

The level of malondialdehyde(MDA) was increased significantly in induced diabetic rats. The level of MDA was reduced significantly in diabetic rat groups treated with Nigella sativa seed powder and thymoquinone. The levels of superoxide dismutase(SOD), Vitamins C and E were decreased significantly in induced diabetic rats. The treated groups of induced diabetic rats with Nigella sativa seed and thymoquinone showed significant increase in SOD, Vitamin C and E levels. There was no significant change in normal rats treated with Nigella sativa seed powder and thymoquinone compared with normal control rats. Lipid peroxidation may result in protein damage and inactivation of membrane-bound enzymes either through chemical modification by its end products or through direct attack by free radicals, MDA and 4-

hydroxynonenal[32]**.** Our results are in agreement with the findings of Wolf[33] , El-Missiry etal[34] and Mahmood et al[35]. These studies reported an increase in lipid peroxides and a decrease in antioxidant enzymes in diabetes. Similar to our study, these studies also showed improvement in reduced antioxidant status after treatment with Nigella sativa. In the study of Bahram Pourghassem-Gargari et al[36], Nigella sativa seed powder showed a significant decrease in serum MDA level. The antioxidant effect of black seed seems to be due to its oil, thymoquinone, flavonoids and also antioxidant vitamins like ascorbic acid and tocopherol. It has been shown that Nigella sativa and thymoquinone inhibit non-enzymatic lipid peroxidation in liposomes and both work as a scavenger of various reactive oxygen species including superoxide anion and hydroxyl radicals[37]**.** In addition, flavonoids present in Nigella sativa are a class of polyphenolic compounds that seem to have antioxidant properties by suppressing formation of reactive oxygen and nitrogen species, scavenging reactive oxygen and nitrogen species and protecting the antioxidant defense system[38,39].

Oxidative stress refers to the tissue damage resulting from the disequilibrium of the overproduction of reactive oxygen species (ROS) and the inhibition of the antioxidant defense Mechanisms[8]. Li X et al[40] study suggested that the circulating levels of oxidative stree markers may increase due to ROS damage and the inability to prevent this effect. According to Huang A et al[41] study, the activation of glucose-induced NOS may lead to oxidative stress related endothelial dysfunction. In the Hui Liu et al[8] study, pretreatment with thymoquinone significantly inhibited the MDA levels and increased SOD activity in diabetic rats. Mabrouk and Ben Cheikh[42] study demonstrated that

thymoquinone has protected diabetic rats against the harmful effects of diabetes millitus through a marked enhancement in testicular antioxidant enzymes. According to Amaral et al[43], hyperglycaemia of diabetes mellitus raises the level of ROS that produces DNA destruction in testis. Diabetes provoked a state of oxidative injury that was proved by an increase in levels of testicular MDA and a reduction in SOD activity[8]. Bauche et al[44] reported regarding a noticeable increase in lipid peroxidation product, MDA levels in diabetic rats. According to Nawal M et al [45] study, thymoquinone suppresses the cyclooxygenase-2 enzyme expression and effectively treated liver injury induced by acetaminophen and it also caused increase in levals of SOD.

In the study of Sayed A.A[46]**,** it was observed that thymoquinone has antioxidative properties as evidenced by the significant increase in glutathione and SOD activity and reduction in lipid peroxidation and levels of NO in the treated diabetic groups. So, it might improve the function of beta cells in the diabetic rats and influence insulin effects directly by acting on specific components of the insulin-signaling transduction pathway. Kanter et al[47] studied the effect of black seed on lipid peroxidation and antioxidant defense system. They found that treatment with Nigella sativa decreased MDA level and increased the activity of antioxidant defense system in carbon tetrachloride treated rats. The study of Kanter et al[48] in rats with experimental spinal cord injury showed that Nigella sativa treatment reduced the levels of MDA in spinal cord tissue and prevented inhibition of SOD, GPX and catalase enzyme activities. Schettler et al[49] suggested that the reduced antioxidant production was due to increased oxygen metabolites causing suppression of antioxidant defense system**.**

In diabetic rats, decreased concentrations of Vitamin C and E were observed. Due to enhanced oxidative stress, utilization of Vitamins C and E is more. As a result of their antioxidant activity and when treated with Nigella sativa and thymoquinone, the utilization of Vitamins C and E is being reduced.This leads to an increase in their levels in diabetic rat groups treated with Nigella sativa seed powder and thymoquinone. In addition, there are many fatty acids in Nigella sativa seed. The most important ingradients in Nigella sativa are linoleic acid (55.6%), oleic acid (23.4%) and palmitic acid (12.5%)[50], Tocopherols (α, β, and γ) and Vitamin C[51] (will lead to increase in Vitamin C and E levels).

In study of Punithavathi VR et al[52], the diabetic rats showed decline in Vitamins C and E. According to studies of Baydas et al[53], Bonnefont-Rousselot et al[54] and Danielle et al[55], data indicated that Vitamin E reduced the glycaemic index in diabetes. This could be due to the correction of glycaemia, which was associated with an improved metabolic state in these animals. The body weight was restored in the presence of Vitamin E in streptozocin induced diabetic rats, demonstrating its antidiabetogenic effect. The capacity of Vitamin E to protect against the loss of body weight could be attributed to its ability to reduce hyperglycaemia. This may be achieved via the suppression of hepatic gluconeogenesis and glucose output from the liver, which is associated with the inhibition of lipolysis in adipose tissue[56]. According to Shamsi et al[57] study, Vitamin E caused a reduction in the level of circulating glucagon in diabetic rats. It is essential to preserve glucose homeostasis as it is a key part in regulation of hepatic metabolism[56]. Hence, Nigella sativa seed and it`s major bioactive component,

thymoquinone may have antioxidant properties that may be useful for therapeutic purposes. The results of the present study indicate that the preventive effects of Nigella sativa and thymoquinone may be due to inhibition of lipid peroxidation through its antioxidant property. These findings suggest that treatment with Nigella sativa and thymoquinone has a therapeutic protective effect against diabetes by preserving integrity of pancreatic beta cell population through reduction in oxidative stress. Consequently, Nigella sativa and thymoquinone may be clinically useful for protecting β-cells against oxidative stress and lead to enhanced secretion of insulin.

6.2.3. Total Protein

In present study, the level of total protein was reduced significantly in diabetic control rats. The level of total proteins was increased in groups of diabetic rats treated with Nigella sativa and thymoquinone. A decrease in total protein levels may contribute to the inhibition of oxidative phosphorylation process, leading to a reduction in protein absorption, a decline in protein synthesis and an increase in the catabolic process. A significant decrease in total protein levels reflected the magnitude of damage to hepatocytes in streptozocin induced diabetic rats. A decrease in body weight of diabetic rats is possibly due to catabolism of fats and proteins[58,59,60,61]. The decrease in serum total protein observed in diabetic rats is coinciding with the findings of Ayed Al-Logmani[61], Wanke et al 1991[62], Tragl et al[63] and Ayed Al-Logmani et al[64]**.** They found that treatment with Nigella sativa resulted in improvement in the lowered levels of total proteins in diabetic rats. This effect of Nigella sativa is presumably due to its ability to increase insulin secretion.

6.3. Profile of Reproductive Hormones and Sperm Analysis

6.3.1. Testosterone, LH, FSH, Progesterone & Estrogen and Sperm count & motility

The level of testosterone was decreased significantly in induced diabetic rats. The level of testosterone was increased significantly in diabetic rat groups treated with Nigella sativa seed powder and thymoquinone. The levels of LH and FSH were decreased significantly in induced diabetic rats. The levels of LH and FSH were increased in the treated groups of induced diabetic rats with Nigella sativa seed and thymoquinone. The levels of progesterone and estrogen were decreased significantly in induced diabetic rats. The levels of progesterone and estrogen were not shown any significant change in the treated groups of induced diabetic rats with Nigella sativa seed and thymoquinone. There was no significant change in normal rats treated with Nigella sativa seed powder and thymoquinone compared with normal control rats in any parameter. The results of our study showed promising favourable reproductive effects of Nigella sativa and thymoquinone supplementation in induced diabetic rats. The sperm count and motility were decreased significantly in induced diabetic rats. The sperm count and motility were increased significantly in diabetic rat groups treated with Nigella sativa seed powder and thymoquinone. There was no significant change in normal rats treated with Nigella sativa seed powder and thymoquinone compared with normal control rats. This indicates non toxic effect of Nigella sativa seed and its bioactive component, thymoquinone.

In the present study diabetic rats showed hyperglycemia, decreased serum insulin level, testosterone, LH and FSH. Our results are in agreement with findings of Mustafa S. Atta et al[14] and Ballester et al[16] studies. In their studies, they found that diabetic rats displayed a reduction in the concentration of serum insulin, testosterone, FSH and LH. Brueing et al[17] in their study reported that decreased insulin levels reflected a sharp decline in of spermatogenesis. In our study also, we found similar results. The studies of Abdelmeguid et al[65] and Meral et al[66], researchers observed that Nigella sativa and thymoquinone treatment decreased the serum glucose, HbA1c and increased insulin and testosterone levels in diabetic rats. Hypoglycemia which was observed may be due to improvement in beta cell ultrastructure. This resulted in improvement in insulin levels. This in turn aids glucose uptake with normoglycemia. In study of Mustafa S. Atta et al[14] showed that diabetic rats had a significant increase in HbA1c percentage and decreased insulin and testosterone levels and thymoquinone treated groups showed improvement in insulin and testosterone levels and decresed HbA1c. They also pointed out the importance of HbA1c level in the diagnosis and prognosis of diabetes. According to study of Amaral S et al[43], thymoquinone is a glucose reducing compound that induces hypoglycemia in animals with hyperglycemic state. Hyperglycemia associated with diabetes millitus raises the ROS level that results in DNA destruction in testis and reduction in sperm count and motility. Thymoquinone improves the sperm count and motility by scavenging ROS and activation of testicular enzymatic antioxidant status.

Sperm count plays an important role in the assessment of spermatogenesis. It is associated with fertility status of an individual. In the present study, diabetic rats exhibit a marked decrease in sperm count and motility. In study of Mustafa S. Atta et al[14], diabetic rats showed a marked decrease in sperm concentration, live sperm percentage and increased sperm abnormalities. In study of Scarano et al[67] confirmed that diabetic rats had reduction in sperm quantity and quality due to associated oxidative injury. According to study conducted by Sharpe R.M et al[68], testosterone is crucial for spermatogenesis and high level of testosterone level is essential for the normal physiology of seminiferous tubules. Estradiol is biosynthesized by the action of aromatase enzyme mainly in the Leydig cells. Estradiol functions to control apoptosis of male sperm cells . In the present study, testosterone, LH, FSH and Estradiol were markedly decreased in diabetic rats. Similar results were found in studies conducted by Mustafa S. Atta et al[14] and Farrell et al[69]. In study of Mustafa S. Atta et al[14] also found the mean Johnsen score value was 4.97 in the diabetic rats and it significantly improved in the diabetic rats treated with thymoquinone. This finding explores the protective effect of Nigella sativa sativa and its bioactive component, thymoquinone on spermatogenesis.

Ballester et al[16] study demonstated that the low level of testosterone in diabetic rats may be related to the decrease in number of Leydig cells or decrease in biosynthesis of androgen. In this study explained mechanisms that affect the two important testicular functions in insulin dependent diabetes mellitus. One is: Leydig cell function and testosterone production are diminished in insulin-dependent diabetes due to the absence of the stimulatory effect of insulin on Leydig cells and to an insulin-dependent decrease

in FSH, which, in turn, decreases LH levels. Second one is: sperm output and fertility are reduced due to a decrease in FSH caused by a decrease in insulin. Diabetes provoked a state of oxidative injury that was proved by an increase in levels of MDA and reduction in SOD activity. Similar findings were observed in present study also.

In studies of Bauche et al[70] and Ghosh, J et al[**71**] reported a noticeable increase in lipid peroxidation product, MDA levels due to the oxidative stress caused by diabetes and led to drastic decrease in spermatogenesis. In study of Mabrouk A et al[72], thymoquinone has protected diabetic rats against the harmful effects of diabetes mellitus through a marked enhancement in testicular antioxidant enzymes. In study of Al Wafai R.J[73], thymoquinone suppresses the cyclooxygenase-2 enzyme expression and lipid peroxidation and raises SOD activities in diabetic rats. According to study of Geng X et al[74]**,** the oxidative damage in diabetic rats led to the liberation of acid phosphatase (ACP) and alkaline phosphatase(ALP) into the blood. Their levels were increased and represented as biomarker of testicular injuries. This leakage of testicular ACP and ALP was reduced by treatment with thymoquinone. Therefore, the testicular levels of both ACP and ALP were significantly decreased in response to oxidative injuries in induced diabetic rats. Conversely, thymoquinone treatment had been protecting the testicular injuries and kept the levels of ACP and ALP within normal range in treated diabetic rats similar to normal control rats.

In study of Elham Ghanbari et al[75], diabetes mellitus resulted in male reproductive dysfunctions, decreased weight of testis and seminal vesicles, decreased serum testosterone levels and lowered semen quality and quantity. According to Baccetti B et al[76] study, male sexual dysfunction in streptozocin induced diabetic rats results from the alterations of the pituitary–testicular axis. Our results have shown a decrease in testicular weight and serum testosterone levels in sterptozotocin induced diabetic rats. Treatment with Nigella sativa seed and thymoquinone in diabetic rats increased levels of Vitamins E and C. In study of Nagai T[77] reported that Vitamins E and C increase glutathione followed by decreased MDA levels and increased SOD in diabetic male rats. The increased levels of gluthathione indicating repair of beta cell membrane which helps in stimulation and enhancement of insulin secretion. Ebisch IM et al[78] study reports regarding antioxidant role of Vitamins E & C and shown to inhibit free radical induced damage in sensitive cell membranes of testis and reduced tissue lipid peroxidation.

According to study by South SA et al[79] showed prolonged insulin deficiency reduces LH secretion. The study of David C et al[80] provided an evidence that the LH secretion is modulated by insulin in sterptozotocin induced diabetic animals, that acute and chornic withdrawal of insulin decreased LH pulse frequency and that resupplementation of insulin increased LH pulse frequency. Dong Q et al[81] study showed decreased both body weight and LH secretion in chemically induced diabetic rats. Bucholtz DC et al[82] study determined that reduction in the availability of glucose either peripherally or centrally suppresses LH release transiently / profoundly.

Sudha S et al[83] study indicated that one of the most important regulatory roles of insulin on spermatogenesis is the modulation of serum FSH levels. This strong correlation indicates a direct effect of insulin and/or glucose on the pituitary biosynthesis and/or secretion of FSH. In the study of Mossa M M et al[84], there was significant increase in sperm count, sperm motility, sperm viability, normal sperm morphology and ejaculate volume in infertile men treated with Nigella sativa and the percentage of increment in sperm count after three months of treatment increased significantly. In the studies of Bashandy et al[85] and Buriro MA et al[86] reported that hyperlipidemia may be associated with impaired semen quality. Treatment with Nigella sativa resulted a reduction in serum lipid and improved semen parameters in Albino rats. Manesh M et al[87] study demonstrated that Nigella sativa supplementation to induced diabetic animals caused an increase in sperm motility. In Al-Saaidi JA[88] study reported that oral administration of Nigella sativa led to a clear improvement in fertility in male rats and improvement in the levels of testosterone. In study of Al Dejyli A N[89] reported that the cause of testosterone increment may be due to the effect of Nigella sativa seed on the main enzymes which affect the metabolism and steroid secretion in the testis. The increase in sperm concentration was due to the increase in testosterone and FSH levels in testicular tissue as these hormones were responsible for spermatogenesis and spermiogenesis in seminiferous tubules. Testosterone is responsible for epididymal function associated with maturation of sperms. It was also reported in the study that Nigella sativa seeds contain alkaloids and phenols which stimulate the secretion of FSH and testosterone.

Parandin R et al[90] study results indicated that the Nigella sativa seeds increased fertility in male rats. There was a significant increase in the weight of testes and epididymis, sperm count, serum levels of Testosterone, FSH and LH. In El Khasmi M et al[91] study, it was found that Nigella sativa induced a significant increase in the weight of reproductive organs as well as circulating levels of FSH, LH and Testosterone. In study of Hayfaa MA[92], it was found that Nigella sativa treated rats showed significant increase in total proteins, testosterone, LH, FSH, sperm count and significant decrease in total cholesterol.

According to Mukhallad A M et al[93], spermatogenesis was increased at primary and secondary spermatocyte stages. Epididymis showed elevated number of spermatozoa. Testicular cell population in treated rats showed an increase in number of spermatocytes and spermatids when compared to control animals. The process of spermatogenesis and accessory reproductive organ functions are androgen dependent. In this study, the number of matured Leydig cells were significantly increased. This reflects an increase in androgen level in such animals. It is further confirmed by increase in the number of spermatocytes and spermatids as these stages are completely androgen dependent. In the present study, that there is a significant increase in testosterone level after treatment with Nigella sativa and thymoquinone in strepotozotocin induced diabetic rat groups. It may be attributed to an enhanced release of LH and FSH, which in turn may lead to improvement in sperm count and motility. Increased levels of testosterone help in maintaining normal reproductive activity.

6.4. Histopathology

6.4.1. Testis and Epidydimis

H&E stained sections of testis of normal control rats showed normal semiferous tubules with classical histological features. Cells were present in their normal proportions. Normal group rats showed intact seminiferous tubules with regular spermatogenesis, presence of primary and secondary spermatocytes, spermatids and spermatozoa. Free spermatozoa were present in the lumen of the seminiferous tubules with normal proportion of Leydig cells. There were no pathological observations. H&E stained sections of epidydymis of normal control rats showed normal cellular architecture of the epididymis. The lumen of epididymal ducts were filled with spermatozoa. The basement membrane was containing the smooth muscle layer. Adjacent to the smooth muscle layer, cells and fibers of the connective tissue were found. H&E sections of testis and epidydimis of normal rat groups treated with Nigella sativa seed powder and thymoquinone showed the same architecture as normal control rats. These findings indicate non-toxic effect of Nigella sativa seed and thymoquinone.

H&E stained section of testis of diabetic control rats showed disruption in the structure of seminiferous tubules. There was a considerable decrease in the spermatogenic cell series. Deformed seminiferous tubules represented as histopathological damage of the testicular tissue. Loss and degeneration of all types of the spermatogenic cells within the seminiferous tubules without sperms at their lumens was observed. There was also decrease in numbers of all types of spermatogenic cells with pyknotic Leydig cells. Diabetic rats showed sloughing of the germinal epithelium,

lysis of spermatocytes and necrosis of the spermatids. H&E sections of testis of diabetic rats treated with Nigella sativa showed the normal architecture. There was an improvement in the structure of seminiferous tubules compared with the diabetic untreated group. Nigella sativa treated diabetic rats showed enhancement in the number of seminiferous tubules in the tubular basal compartment with the presence of spermatogonia. Nigella sativa treated rats showed normal seminiferous tubules with spermatogonia. The H&E sections of testis of thymoquinone treated diabetic rats also showed similar features as Nigella sativa treated group. H&E stained sections of epididymis of diabetic control rats sections showed the thickening of basement membranes of tubules, degenerative epithelium and very less or no sperm were found in the lumens of ducts. The H&E sections of epididymis of Nigella sativa treated rats showed thin basement membranes of tubules and tubules with good number of sperms as normal rat epididymis. The H&E sections of epididymis of thymoquinone treated diabetic rats also showed similar features as Nigella sativa treated group.

Mustafa S. Atta et al[14] study revealed histological observations indicated that the testicles of diabetic rats had marked degeneration of seminiferous tubules, sperm giant cells and pyknotic spermatocytes in addition to sloughing of the germinal epithelium, lysis of the spermatocytes and necrosis of spermatids. According to study of Ali Riza et al[94], diabetes mellitus plays a role in the etiology of testicular dysfunction leads to apoptotic cell death and atrophy of the seminiferous tubules, decreased diameters of tubules and reduction of spermatogenetic cell series. Major morphological indicators of spermatogenic failure are of seminiferous tubules atrophy and spermatogenic cell loss.

In Ghafani S et al[95] study of streptozocin induced diabetic rats, testis were found to have disintegration of tubular cells, decreased cellularity, vacuolization of sertoli cells and spermatogonia were seen in most of seminiferous tubules. Vasodilations and congestion of capillaries were also seen in interstitial tissue. In addition, spermatozoa were seen in tubules in comparison with normal rats. In Mehmet F S et al[96] study, in diabetic rats, researchers found that congestion of vessels and hemorrhage, formation of vacuoles in epithelial cells, desquamation of epithelial cells in the lumen, disorder of seminiferous tubules germinal epithelium and multinucleated giant cells and necrosis of some seminiferous tubules. According to Soudamani S et al[97] study suggested based on their gravimetric, histological and histomorphometric observations, it is streptozocin induced diabetes manifested adverse effects on the epididymal histoarchitecture and growth, which are secondary to subnormal testosterone and its action. Amelioration of these changes, either partially or completely by insulin replacement, suggests that insulin is one of the hormones required along with testosterone for the maintenance of epididymal histoarchitecture.

In study of Mustafa S. Atta et al[14] found that Nigella sativa seed`s bioactive component, thymoquinone trated diabetic rats showed normal seminiferous tubules with good number of spermatids, spermatozoa with improvement in the number of Leydig cells. In Study of Yasmina K et al[98] found that Nigella sativa treatment resulted in favourable alteration in testicular morphology and functions in streptozocin induced diabetic rats. In Study of Al-Nailey K. G. Ch[99], Nigella sativa treatment reversed the degeneration of seminiferous tubules, necrosis and impaired functions of testis in

cimetidine induced reproductive toxicity in male mice and also epidydimis found with normal basement membrane and good number of sperms compared to cimetidine induced rats where epidydimis with thick basement membrane with out or less sperms.

According to Danladi et al[100], Nigella sativa has the ability to protect testis against oxidative stress possibly through antioxidant effects of its bioactive compounds. Antioxidant property of thymoquinone is attributed to the quinine structure of thymoquinone molecule[101] and the easy access to sub cellular compartments facilitating ROS scavenging effect[102]**.** Thymoquinone was also shown to inhibit non-enzymatic lipid peroxidation[103]. This leads to decreased oxidative stress and protection of the antioxidant enzymes of testis[100]**.** Moreover, the hypoglycemic effect of Nigella sativa adds to its antioxidant effect by decreasing ROS production due to lowering circulating blood glucose level. In addition, Nigella sativa increases number of Leydig cells in rat testes[93] beside the presence of unsaturated fatty acids in Nigella sativa oil that stimulate 17 ß-hydroxysteroid dehydrogenase activity, increasing testosterone level[104].

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CHAPTER 7

Summary and Conclusion

Summary and Conclusion

Based on the results of the present study, it may be concluded that Nigella sativa exerts therapeutic effect on diabetes mellitus and diminishes the adverse effects of diabetes on the reproductive system and semen quality by preserving pancreatic beta-cell integrity and repairing testicular tissue and decreasing oxidative stress. Consequently, administration of Nigella sativa resulted in better regulation of diabetes and its related subfertility. So, Nigella sativa can be used as an add on therapy to conventional antidiabetic drugs for better glycaemic control and enhancement of fertility state of diabetic patients.

- Glucose levels of induced diabetic rats were increased significantly and were lowered after treatment with Nigella sativa seed and thymoquinone. Insulin levels of induced diabetic control rats were decreased significantly and increased significantly in diabetic rat groups treated with Nigella sativa seed and thymoquinone. This may be due to preservation of integrity of β-cells by their antioxidant properties, which leads to increased secretion of insulin. Increased levels of insulin in diabetic treated rat groups leading to lowering of elevated glucose levels.
- Streptozocin caused degeneration and disruption in structure of seminiferous tubules, vacuolization and reduced intestitial tissue. It may be due to increased oxidative stress due to toxic effects of streptozocin on testicular tissue. This is supported by increased levels of MDA in streptozocin induced diabetic rats and histopathological observations of testicular tissue.
- Increased SOD levels of Nigella sativa seed and thymoquinone treated diabetic rat groups revealed that these components have antioxidant property. This is also supported by

histopathological observations of testicular tissue which showed reduced severity of degenerative and necrotic changes in testicular tissue of diabetic treated rat groups.

- In diabetic rats, decreased levels of Vitamins C and E were observed. It may be because of enhanced oxidative stress and utilization of Vitamins C and E. Increased levels of Vitamins C and E in diabetic treated rat groups with Nigella sativa seed and thymoquinone indicating the antioxidant nature of both components. In addition, there are many fatty acids in Nigella sativa, the most important are linoleic acid, oleic acid, palmitic acid, tocopherols and Vitamin C which lead to increase in Vitamins C and E levels. Increased levels of Vitamin E will be helpful in the enhancement of reproductive activity.
- The level of total protein was reduced significantly in diabetic control rats. The levels of total proteins were improved in diabetic rat groups treated with Nigella sativa seed and thymoquinone. This effect is presumably due to their ability to increase insulin secretion.
- The levels of testosterone, LH and FSH were decreased significantly in induced diabetic untreated rats. The level of testosterone was improved significantly in diabetic rat groups treated with Nigella sativa seed and thymoquinone. The levels of LH and FSH were increased in diabetic treated rat groups. LH and FSH are essential for the release of testosterone and improvement in the functions of germinal epithelium. Testosterone is an important hormone to keep regular and healthy reproductive activity. The improved Leydig cell and testicular histological architecture indicating reproductive activity protecting property of Nigella sativa seed and thymoquinone
- There was no difference in any parameters between normal control rat group and normal rat groups treated with Nigella sativa and thymoquinone. This indicates non-toxic effect

of Nigella sativa and thymoquinone. Our histopathological findings further support this observation as there were no pathological observations in normal rat groups treated with Nigella sativa seed and thymoquinone.

- Nigella sativa seed and thymoquinone showed the same results. This suggests that the active ingredient, thymoquinone is most effective against streptozocin induced diabetes as its administration ameliorated most of the pathological changes.
- All the results clearly indicate that there are beneficial effects in altered parameters which are affected by streptozocin induced diabetes. As the beneficial effects of Nigella sativa seed and thymoquinone are seen in the present study, these phytochemical components may be considered as antidiabetic and favourable in reproductive therapy in sufferers of diabetes mellitus.
- The mechanism of beneficial effect of Nigella sativa seed and thymoquinone, probably through their antioxidant nature, which helps in rectifying oxidant and antioxidant balance in tissues like liver, kidneys, pancreas and testis. Also, it may be due to a cellular protective mechanism through cell signal pathways in pancreas with resultant amelioration of glucose homeostasis. Decreased glucose circulation and increased insulin activity in blood helps in reducing redox status of tissues such as testis, which helps in repair of testicular tissue which suffered with streptozocin induced oxidative stress associated damage. Perhaps, both Nigella sativa seed and thymoquinone improve cellular integrity especially in hepatocytes, β cells of Islets of Langerhans of pancreas and Leydig cells of testis.

Limitations of the present study

- We could not evaluate the exact active compound of Nigella sativa seed which is acting as antidiabetic compound.
- Crude form of seed was used directly.

Scope for future study

- More refined screening of Nigella sativa seed may be done for further conclusion.
- Different extractions of Nigella sativa seed may be used to evaluate their medicinal properties.
- Further evaluation of molecular factors, especially transcriptional factors may be considered.

ANNEXURE - I

PUBLICATIONS

- Effect of Nigella Sativa seed powder on Testosterone and LH levels in Sterptozotocin induced diabetic male albino rats. J. Pharm. Sci. & Res. Vol. 7(4), 2015, 234-237.
- Effect of Thymoquinone on Testosterone and LH levels in Sterptozotocin induced diabetic male albino rats. J. Pharm. Sci. & Res. Vol. 7(8), 2015, 554-556.
- Effect of Nigella Sativa seed and Thymoquinone on reproductive parameters in Sterptozotocin Induced diabetic and normal male albino rats. Int J Intg Med Sci. 2016; 3(3):248-52.

BLDEU'S DEPT. OF PHARMACOLOGY BIJAPUR-586103. OUTWARD No. 603/13 Date 11/09/13 SHRI B.M. PATIL MEDICAL COLLEGE

Chairman,
Institutional Animal Ethics Committee (IAEC),
Prof. & HOD, Dept. of Pharmacology,
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Mr. Shaik Haseena
Ph.D (Physiology) Candidate,
BLDEU's Shri. B.M.Patil Medical College,
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ETHICAL CLEARANCE CERTIFICATE

The Institutional Animal Ethics Committee (IAEC) of this College met on 26.07.2013 at 10.30am to scrutinize the Research Project submitted by Ph.D (Physiology) candidate of this College.

The queries raised by the Ethics Committee have been satisfactorily answered by you, hence the Ethical Clearance is accorded for your Research project.

Title: "Effect of Nigella Sativa seeds extract on the reproductive system in normal and streptozocin induced diabetic male rats".

Principal investigator: Mrs. Haseena S, Ph.D (Physiology) Candidate.

11/9/2013

11.09.2013

Dr. R. S. Wali
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Annexure -I

PLAGARISM VERIFICATION CERTIFICATE

1. Name of the Student: HASEENA S Reg No. 11PhD004
2. Title of the Thesis: Effect of Nigella Sativa Seeds Extract on the Reproductive System in Normal and Streptozocin Induced Diabetic Male Rats
3. Department: PHYSIOLOGY .
4. Name of the Guide & Designation: Dr. MANJUNATHA AITHALA
   Professor & HOD, Department of Physiology, SBMPMCH&RC,Vijayapura.
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Effect of Nigella Sativa Seed Powder on Testosterone and LH levels in Sterptozotocine Induced Diabetes male Albino Rats.

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Abstract

Introduction –

Nigella Sativa belong to the botanical family of ranunculaceae. It has been known as black seed and its seeds are frequently used in folk medicine in Middle East and some Asian countries for the promotion of good health and treatment of many ailments. Nigella Sativa seeds has been used in traditional Iranian medicine as a natural remedy for promotes females menstruation, galactagogue, carminative, laxative and anti-parasitic properties. The present study is conducted to find out effect of Nigella Sativa seed powder on testosterone and LH levels in streptozotocine induced diabetic male albino rats.

Materials and Methods –

This work is conducted as part of Ph.D work under Department of Physiology, Shri BM patil Medical College, BLDE University, Bijapur. University ethical committee and Inistitution Animal Ethical committee are approved the work according to CPCSEA Rules. 18 rats were selected for this study and divided in to 3 groups each contains 6 rats, one group served as normal control, one group served as Diabetic control and one groups served as Treatment group with Nigella Sativa seed powder(300mg/kg BW).

Results –

Testosterone(ng/dl) level of Normal Control rats was 82.78±8.26, Diabetic rats was 41.62±7.28 and treated with Nigella Sativa rats was 71.34±6.58. LH(mIU/L) level of Normal Control rats was 0.46±0.12, Diabetic rats was 0.20±0.06 and treated with nigella sativa rats was 0.30±0.09.

Conclusion –

Compared with normal rats the level of testosterone was decreased in diabetic rats, when it is treated with Nigella Sativa Seed powder the levels of testosterone increased significantly. Compared with normal rats the level of LH was decreased significantly in diabetic rats, when it is treated with Nigella Sativa Seed powder the levels of LH not significant with other groups.

Key Words – Testosterone, LH, STZ, Diabetes, Nigella Sativa.

INTRODUCTION

The insufficient vitamins intake can cause deleterious effects on spermatogenesis and production of normal sperm[1], the sufficient consumption of vitamins and natural antioxidants can protect sperm DNA from oxidative stress and improve male fertility[2]. Vegetable oils contain several natural antioxidant constituents. Some of them such as pumpkin oil[3], celery oil[4], nigella sativa oil [5] and sunflower oil [6] were reported to possess marked antioxidant activity. Natural materials such as medical plants are wieldy accepted as feed additives. Generally, the use of chemical products may cause unfavourable side effects. Many of synthesized chemicals could cause hazards to animal or human. Different studies showed a beneficial positive effect of using Nigella Sativa seeds as feed additive in diet of ruminants. Nigella sativa is a plant of Ranunculaceae family that grows spontaneously and widely in several Southern Mediterranean and Middle Eastern countries.

Its seed has over 100 different chemical constituents, including abundant sources of all the essential fatty acids. Although it is the oil that most often used medicinally, the seeds are a bit spicy and often used whole in cooking curries, pastries and Mediterranean cheeses[7]. Nigella sativa seeds are used extensively in traditional medicine of many countries. It has been used for treatment of many diseases owing to the reported antiviral, antiinflammatory, anti-schistosomiasis and immunomodulatory activities[8]. In recent years, the pharmacological investigations confirmed that most of the therapeutic properties of this plant are due to thymoquinone which is major active component of Nigella sativa oil[9]. Previous data suggest

that the seeds oil and thymoquinone exhibited spermioprotective effect against testes damage.

Reproductive dysfunction is recognized as a consequence of diabetes mellitus[10] represented in decrease in libido, impotence and infertility[11]. In a study poor semen quality has also been reported in diabetic men including decreased sperm motility, sperm concentration, increased abnormal sperm morphology[12] and reduced levels of testicular hormone[13]. Nigella Sativa seed contains a complex mixture of more than 100 compounds[14]. Most of the therapeutic properties of Nigella sativa are due to the presence of the polyphenol Thymoquinone (TQ) [15] which is the major component (28-57%) of Nigella sativa oil[16]. In addition, there are many fatty acids. The most important of which are linoleic acid (55.6%), oleic acid (23.4%) and palmitic acid (12.5%)[17]. Nigella sativa and TQ have been known by their hypoglycemic, hypolipidemic[18]and radical scavenging activity[19].

Nigella sativa oil has the ability to protect testis against oxidative stress possibly through antioxidant effects of its bioactive compounds[20]. Antioxidant property of thymoquinone is attributed to the quinine structure of thymoquinone molecule[21] and the easy access to sub cellular compartments thus facilitating the ROS scavenging effect[22]. Thymoquinone was shown to inhibit non-enzymatic lipid peroxidation. This leads to decreased oxidative stress and protection of the antioxidant enzymes of testis[23]. The hypoglycemic effect of Nigella sativa oil adds more to its antioxidant effect by decreasing ROS production due to lowering glucose circulating in the blood stream. In addition, Nigella sativa oil increase the number of Leydig cells in rat testes[24] beside the presence of unsaturated fatty acids in Nigella sativa oil that stimulate 17 L-hydroxysteroid dehydrogenase activity[25] thus increasing testosterone level. The present study was conducted to study effect of Nigella Sativa seed powder on Testosterone levels in streptozotocine induced diabetic male albino rats.

MATERIALS AND METHODS

Study design - This work is conducted as part of Ph.D work under Department of Physiology, Shri BM patil Medical College, BLDE University, Bijapur. University ethical committee and Inistitution Animal Ethical committee are approved the work according to CPCSEA Rules. 18 rats were selected for this study and divided in to 3 groups each contains 6 rats, one group served as negative control, one group served as Diabetic control and one groups served as Treatment group with Nigella Sativa seed powder(300mg/kg BW), at the end of 45th day blood was collated and measured serum Testosterone and LH by kit method.

Plant material – Nigella sativa seeds were grinded in to fine powder[9] with piston and mortal with help of Bapuji pharmacy college, Davangere. Nigella sativa powder administrated orally according to study of M. Murugesan[26].

Streptozotocine – Induced diabetes -The rats were given Streptozotocine intraperitoneal injection 50mg/BW, Streptozotocine dissolved in ice-cold citrate buffer(PH 4.5)[27]. The diabetes was confirmed by measuring glucose by Code free Glucometer, the glucose level above 250mg/dl considered as diabetes, glucose levels were checked at regular periodical periods.

RESULTS

Testosterone(ng/dl) level of Normal Control rats was 82.78±8.26, Diabetic rats was 41.62±7.28 and treated with Nigella Sativa rats was 71.34±6.58. LH(mIU/L) level of Normal Control rats was 0.46±0.12, Diabetic rats was 0.20±0.06 and treated with nigella sativa rats was 0.30±0.09(Table 1).

DISCUSSION

Infertility is a complex disorder with significant medical, psychosocial and economic aspects. About 25% of couples do not achieve pregnancy within 1 year, 15% of whom seek medical treatment for infertility and less than 5% remain unwillingly childless. Infertility affects both men and women. Male causes for infertility are found in 50% of involuntarily childless couples[28]. A wide majority of medicine plants possess pharmacological principles, which has rendered them useful as curatives for numerous ailments. According to the World Health Organization (WHO) reports, 70-80% of the world population confide in traditional medicine for primary health care[29]. Plants and derivatives of plant played a key role in world health and have long been known to possess biological activity. Thirty percent of all modern drugs are derived from plants [30]. In addition, Plants have a long folklore of use in aiding fertility, including fertility-enhancing properties and aphrodisiacal qualities[31, 32].

The testes, epididymis and other reproductive organs are structurally and physiologically dependent upon the testosterone and other androgens. Testosterone stimulates growth and secretary activity of the reproductive organs[33,34]. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and indirectly stimulates spermatogenesis via testosterone. In our study the testosterone was decreased in diabetes induced rats and at the same time LH was decreased, in nigella sativa treated rats the testosterone levels are increased significantly but LH levels were not significant change. Our results are in agreement with studies of Mukhalad AM et, al.[35], Gokçe A et.al.[36], Rahmatollah Parandin[37].

Table 1. One way results of Testosterone(ng/dl)and LH (mIU/L)

Parameter	Group 1 Normal Rats– Control	Group 2 Diabetic Rats– Control	Group 3 Diabetic Rats – Nigella sativa seed powder	F	P
Testosterone(ng/dl)	82.78 ±8.26[a]	41.62±7.28 [b]	71.34±6.58 [a,c]	33.625	.000
LH(mIU/L)	0.46±0.12 [a]	0.20±0.06 [b]	0.30±0.09 [a,b]	7.088	.000

The difference between groups P<0.05 considered as significant.

According to Phytochemical analytical study of Nickavar B, indicated the rich presence of unsaturated fatty acids (Linoleic acid 55.6%, Oleic acid 23.4%, Palmitic acid 12.5%, Stearic acid 3.4% and else.) in nigella sativa seeds[38]. In study of Fellner et al, the supplementation of rats diets with oils rich in polyunsaturated fatty acids, such as Linoleic acid has positively influenced reproductive functions[39]. The study of Gromadzka-Ostrowska et al shown that the unsaturated fatty acids stimulate the activity of 17 β-hydroxysteroid dehydrogenase, the most important key enzyme in the testosterone biosynthesis pathway[40]. The study of Samir Bashandy found that administration of Nigella Sativa oil to hyperlipidemic rats improved their reproductive efficiency and produced additional protection against hyperlipidemia induced reduction in fertility[41]. According to study of Al-Ali A et al. Thymoquinone is the major active component derived from Nigella sativa and reported that many of the pharmacodynamic effects Nigella sativa are due to Thymoquinone[42]. The study of Gokce et al.[36] confirmed that Thymoquinone treatment has protective effects on testicular parameters. The present study results are correlating with previous studies and confirmed that the testicular favouring results due to thymoquinone content in nigella sativa seed. So the nigella sativa seed may be used for increasing testicular activity.

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Abstract

Introduction **-** Thymoquinone (TQ) and unsaturated fatty acids are the main antioxidant components of Nigella Sativa. Nigella Sativa seeds has been used in traditional Iranian medicine as a natural remedy for promotes females menstruation, galactagogue, carminative, laxative and anti-parasitic properties. The present study is conducted to find out effect of Thymoquinone on testosterone and LH levels in streptozotocine induced diabetic male albino rats.

Materials and Methods **-**This work is conducted as part of Ph.D work under Department of Physiology, Shri BM patil Medical College, BLDE University, Bijapur. University ethical committee and Inistitution Animal Ethical committee are approved the work according to CPCSEA Rules. 18 rats were selected for this study and divided in to 3 groups each contains 6 rats, one group served as normal control, one group served as Diabetic control and one groups served as Treatment group with Thymoquinone(4mg/kg BW).

Results **-**Testosterone(ng/dl) level of Normal Control rats was 82.78±8.26, Diabetic rats was 41.62±7.28 and treated with Nigella Sativa rats was 76.30±8.23. LH(mIU/L) level of Normal Control rats was 0.46±0.12, Diabetic rats was 0.20±0.06 and treated with nigella sativa rats was 0.28±0.10.

Conclusion **-**Compared with normal rats the level of testosterone was decreased in diabetic rats, when it is treated with Thymoquinone the levels of testosterone increased significantly. Compared with normal rats the level of LH was decreased significantly in diabetic rats, when it is treated with Thymoquinone the levels of LH not significant with other groups.

Key Words – Testosterone, LH, STZ, Diabetes, Thymoquinone.

INTRODUCTION

Following an increase in free radicals, DNA damage and lipid peroxidation in human sperm may Occur in STZ induced diabetic rats. The insufficient vitamins intake can cause eleterious effects on spermatogenesis and production of normal sperm[1], the sufficient consumption of vitamins and natural antioxidants can protect sperm DNA from oxidative stress and improve male fertility[2]. Thymoquinone and unsaturated fatty acids are the main antioxidant components of Nigella sativa. Thymoquinone derived from Nigella Sativa can improve male fertility parameters through promoting antioxidant defence.

The sperm cell membrane includes a large amount of polyunsaturated fatty acids and phospholipids which are vulnerable to oxidative stress[3]. Oxidative stress has deleterious effects on the structure, function, motility and survival of sperm. Smoking, alcohol ingestion, infection, exposure to environmental toxins or radiation can trigger rising mitochondrial production of reactive oxygen species (ROS) and oxidative stress. Following a rise in free radicals, DNA damage, lipid peroxidation, protein and biomembrane damage in sperm may occur[4]. Ingredients with antioxidant properties can transfer electrons to oxidizing agents and inhibit free radical production and sperm damage[5]. Antioxidant components have been indicated to improve spermatogenesis and steroidogenesis[6].

Reactive oxygen species that belong to the class of free radicals are highly reactive oxidizing agents. Production of ROS in various tissues like testis is a common event, the abnormal increase in its synthesis could stimulate the DNA damage and oxidation of many cells. The sperm plasma membrane contains a high level of unsaturated fatty acids. Lipid peroxidation could lead to the damage of lipid matrix structure in spermatozoal membranes, and could be associated with impaired motility. Antioxidants are compounds which scavenge and decrease the synthesis of ROS and lipid peroxidation. Biological antioxidants include glutathione (GSH), catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide-dismutase (SOD) that have a very crucial role in scavenging of free radicals.

Therefore, ROS scavenger's application is likely to improve sperm quality[7,8].

Nigella sativa is a plant of the Ranunculaceae family. It grows widely in many Middle Eastern countries. Its seed is black in colour and bitter in taste. Nigella sativa has many different chemical ingredients including thymoquinones(30-48%), flavonoids, anthocyanins, alkaloids and essential fatty acids, particularly linoleic and oleic acid. It has been traditionally used for the treatment of different diseases such as respiratory and digestive disorders, kidney and liver dysfunction and rheumatism in different forms[9,10]. Thymoquinone has demonstrated some protective roles in relation to oxidative status, such as superoxide anion scavenger, direct cytoprotective effects and indirect antioxidant and androgen activities, it may protect sperm and semen against a testicular toxin[11]. The present study is conduted to see the effect of thymoquinone on testosterone and LH levels in diabetic induced male albino rats.

MATERIALS AND METHODS

Study design - This work is conducted as part of Ph.D work under Department of Physiology, Shri BM patil Medical College, BLDE University, Bijapur. University ethical committee and Inistitution Animal Ethical committee are approved the work according to CPCSEA Rules. 18 rats were selected for this study and divided in to 3 groups each contains 6 rats, one group served as negative control, one group served as Diabetic control and one groups served as Treatment group with Thymoquinone(4mg/kg BW), at the end of 45th day blood was collated and measured serum Testosterone and LH by kit method.

Thymoquinone – Thymoquinone purchaged from Sigma-Aldrich, Bangalore and administrated to rats through intraperitoneal injections(4mg/body Kg weight).

Streptozotocine – Induced diabetes -The rats were given Streptozotocine intraperitoneal injection 50mg/BW, Streptozotocine dissolved in ice-cold citrate buffer(PH 4.5). The diabetes was confirmed by measuring glucose by Code free Glucometer, the glucose level above 250mg/dl considered as diabetes, glucose levels were checked at regular periodical periods.

Results -Testosterone(ng/dl) level of Normal Control rats was 82.78±8.26, Diabetic rats was 41.62±7.28 and treated with Thymoquinone(4mg/KgBW) rats was 76.30±8.23. LH(mIU/L) level of Normal Control rats was 0.46±0.12, Diabetic rats was 0.20±0.06 and treated with Thymoquinone(4mg/KgBW) rats was 0.28±0.10(Table 1).

DISCUSSION

The testes, epididymis and other reproductive organs are structurally and physiologically dependent upon the testosterone and other androgens. Testosterone stimulates growth and secretary activity of the reproductive organs so a significant increase of these hormones could increase the number and function of somatic and germinal cells of testis and in results increase the testis and epididymis weight[12]. Thymoquinone is the major active component derived from Nigella sativa and many of the pharmacodynamic effects reported above for N. sativa are due to Thymoquinone [13]. Gokce et al [14] has been confirmed that Thymoquinone treatment has protective effects on testicular parameters. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and indirectly stimulates spermatogenesis via testosterone. In our study the testosterone was decreased in diabetes induced rats and at the same time LH was also decreased, in thymoquinone treated rats the testosterone levels are increased significantly but LH levels were not significant change. Our results are in agreement with studies of Mukhalad AM et, al.[15], Gokçe A et.al.[16], Rahmatollah Parandin[17]. Thymoquinone, the major active constituent of Nigella sativa, could lead to decreased total antioxidant capacity and could prevent the increase in the myeloperoxidase Activity[14].

According to singh etal Thymoquinone can improve dyslipidaemia and antioxidant defence[18]. Wafai et al.'s study reported that Thymoquinone could suppress cyclooxygenase-2 enzyme expression and lipid peroxidation, and raise SOD levels in diabetic rats [19]. Zohra et al treated diabetic rats by adding 2% NS seeds to their diet for 30 days. This addition improved testosterone levels and testis tissue, semen quantity and mobility, and it reduced blood glucose and oxidative stress parameters[20]. According to studies of Sultan et al and Ahmad, reported that the antioxidant characteristics of Thymoquinone led to an improved antioxidant status in diabetic rats[21,22].

Nigella sativa and its main constituent, thymoquinone can improve sperm parameters, semen,Leydig cells, reproductive organs and sexual hormones in animal studies. The main potential mechanism is the antioxidant properties of Nigella sativa which play a key role in free radical scavenging. Although the previous studies findings suggest that Nigella sativa and thymoquinone are good candidates for male infertility treatment, to date there is insufficient evidence to make recommendations for its use as an adjunct therapy in infertile men. Based on the positive effects of thymoquinone of our study conclude that further research is to be done on humans for finding beneficial reproductive effect of thymoquinone in diabetic human individuals.

Table 1. One way results of Testosterone(ng/dl)and LH (mIU/L)

Parameter	Group 1 Normal Rats– Control	Group 2 Diabetic Rats– Control	Group 3 Diabetic Rats – Thymoquinone(4mg/KgBW)	F	P
Testosterone (ng/dl)	82.78 ±8.26[a]	41.62±7.28 [b]	76.30±8.23 [a,c]	33.625	.000
LH(mIU/L)	0.46±0.12 [a]	0.20±0.06 [b]	0.28±0.10 [a,b]	7.088	.000

The difference between groups P<0.05 considered as significant.

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Original Research Article

Effect of Nigella Sativa seed and Thymoquinone on Reproductive Parameters in Sterptozotocine Induced Diabetic and Normal Male Albino Rats

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ABSTRACT

Introduction: In diabetes an increase in free radicals, DNA damage and lipid peroxidation in human sperm may occur. Antioxidant components in medicinal herbs such as Nigella sativa have been indicated to improve spermatogenesis and steroidogenesis. Thymoquinone and unsaturated fatty acids are the main antioxidant components of Nigella Sativa. Nigella Sativa seeds has been used in traditional Iranian medicine as a natural remedy for promotes females menstruation, galactagogue, carminative, laxative and anti-parasitic properties. The present study is conducted to find out effect of Nigella sativa seed and Thymoquinone on testosterone and LH levels in streptozotocine induced diabetic and normal male albino rats.

Materials and Methods: 36 rats were selected for this study and divided in to 6 groups each contains 6 rats, one group served as normal control, one group served as normal rats received the nigella sativa seed powder(300mg/Kg BW), one groups served as normal rats received the Thymoquinone(4mg/kg BW), one group served as Streptozotocine(50mg/kg BW) induced diabetic control rats, one group served as diabetic rats received the nigella sativa seed powder(300mg/Kg BW) and one groups served as diabetic rats received the Thymoquinone(4mg/kg BW).Testosterone and LH were estimated by Chemiluminosis(CLIA).

Results: There is no change in Testosterone and LH levels in normal rats treated with nigella sativa seed powder and thymoqinone. The levels testosterone and LH are decreased significantly in diabetic rats compared with normal rats, when the diabetic groups treated with nigella sativa seed powder and thymoqinone the Testosterone levels are normalised significantly($p<0.05$).

Conclusion: Compared with normal rats the level of testosterone was decreased in diabetic rats, when it is treated with Nigella sativa seed and Thymoquinone the levels of testosterone increased significantly. Compared with normal rats the level of LH was decreased significantly in diabetic rats, when it is treated with Nigella sativa seed and 'Thymoquinone the levels of LH not significant with other groups.

KEY WORDS: Testosterone, LH, STZ, Diabetes, Thymoquinone, Nigella Sativa seed.

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INTRODUCTION

Seeds of Nigella sativa have been employed for thousands of years as a spice and food preservative. The oil and the seed constituents, in particular thymoquinone, have shown potential medicinal properties in traditional medicine. The nigella sativa seed used as a laxative, diuretic, treating infectious fever and local treatment for skin infections and wounds. Nigella sativa seed also used for hair full treatment, headache, ears pain, parturition diseases, toothache, digestive system disturbances, glands diseases, fraction healing, liver, spleen, and eye diseases [1-3]. The insufficient vitamins intake can cause deleterious effects on spermatogenesis and production of normal sperm [4], the sufficient consumption of vitamins and natural antioxidants can protect sperm DNA from oxidative stress and improve male fertility [5].

In diabetes the production of ROS is more common, Reactive oxygen species (ROS) that belong to the class of free radicals are highly reactive oxidizing agents. Production of ROS in various tissues like testis is a common event; however, the abnormal increase in its synthesis could stimulate the DNA damage and oxidation of many cells. The sperm plasma membrane contains a high level of unsaturated fatty acids, this is the reason sperm plasma membrane is more liable to peroxidative damage. Lipid peroxidation could lead to the damage of lipid matrix structure in spermatozoal membranes, and this damage may lead to impaired motility.

Antioxidants are compounds which scavenge and decrease the synthesis of ROS and lipid peroxidation. Biological antioxidants include glutathione (GSH), catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide-dismutase (SOD) that have a very crucial role in scavenging of free radicals. ROS scavenger's application is likely to improve sperm quality [6,7].

Many cellular metabolites can cause an increase of the concentration of electrophilic radicals, which can react with oxygen giving rise to ROS. The main sources of free radicals include singlet-oxygen (1O2), hydroxyl radical (OH) and H2O2. ROS are normally generated in many vital metabolic processes for living cells including the spermatozoa; however, marked generation of ROS produced by spermatozoa[7] or by the combinations of xenobiotics and immunosuppressive agents can induce the production of toxic lipid peroxides [8,9].

Cells exhibit defensive pathways using various antioxidants. The main detoxifying systems for peroxides are GSH and CAT. CAT is an antioxidant enzyme that destroys H2O2 which can synthesize a highly reactive OH. On participation of the glutathione redox cycle, GSH together with GSH-Px converts H2O2 and lipid peroxides to non-harmful products [9]. Phenolic compounds derived from pomegranate [10]. ROS are highly reactive molecules that can react with many intra-cellular structures, mainly unsaturated fatty acids and trans-membrane proteins. The oxidation of these molecules can produce disturbance in cellular membrane permeability. Spermatozoa are highly susceptible to peroxidative damage due to existence of high concentration poly-unsaturated fatty acids, which are responsible for regulation of sperm maturation, capacitation, acrosome reaction, spermatogenesis and membrane fusion, with low antioxidant capacity. Moreover, sperm lipid peroxidation could destroy the structure of the lipid matrix in spermatozoal membranes, accompanied with a rapid decrease of intracellular ATP that leads to decreased sperm viability, axonemal damage and increased mid-piece morphological defects, and it could dramatically finally inhibit spermatogenesis [11].

In previous studies Thymoquinone has demonstrated some protective roles in relation to oxidative status, such as superoxide anion scavenger, direct cytoprotective effects and indirect antioxidant and androgen activities, it may protect sperm and semen against a testicular toxin[12-16]. The present study is conduted to see the effect of Nigella Sativa seed powder and thymoquinone on testosterone and LH levels in diabetic induced male albino rats. The aim of present study is to observe the effect of Nigella Sativa seed and Thymoquinone effect on reproductive hormones in normal and streptozotocine induced diabetic rats.

MATERIALS AND METHODS

Study design: This work is conducted as part of Ph.D work under Department of Anatomy, Shri BM patil Medical College, BLDE University, Bijapur. University ethical committee and Inistitution Animal Ethical committee are approved the work according to CPCSEA Rules (BLDEU/Dept of pharmacology 603/13). The 36 rats were selected for this study and divided in to 6 groups each contains 6 rats, 3 groups are normal rats out of that one group served as normal control rats, one group was treted with nigella sativa seed powder, one group was treated with thymoquinone. Other three groups were induced diabetic by streptozotocine out of that one group served as diabetic control, one group as diabetic treated with nigella sativa and one group served as diabetic rats treated with thymoquinone, at the end of 45th day blood was collated and measured serum testosterone and LH by manual method.

Plant material: Nigella sativa seeds were purchased from Safa honey & Co, Bangalore and grinded in to fine powder [16] with piston and mortal with help of Bapuji pharmacy college, Davangere. Nigella sativa powder administrated orally according to study of M. Murugesan[17].

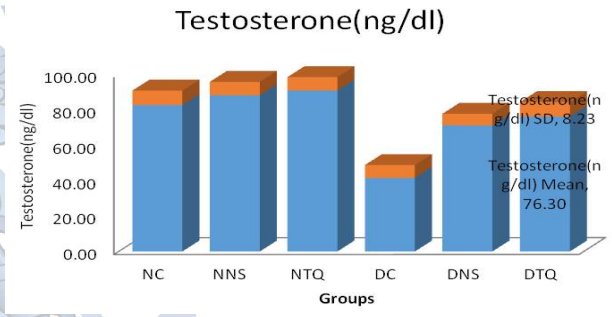
Thymoquinone: Thymoquinone purchaged from Sigma-Aldrich, Bangalore and administrated to rats through intraperitoneal injections (4mg/ body Kg weight).

Streptozotocine – Induced diabetis: The rats were given Streptozotocine intraperitoneal injection 50mg/BW, Streptozotocine dissolved in icecold citrate buffer (PH 4.5). The diabetes was confirmed by measuring glucose by Code free Glucometer, the glucose level above 250mg/dl considered as diabetes, glucose levels were checked at every day morning.

RESULTS

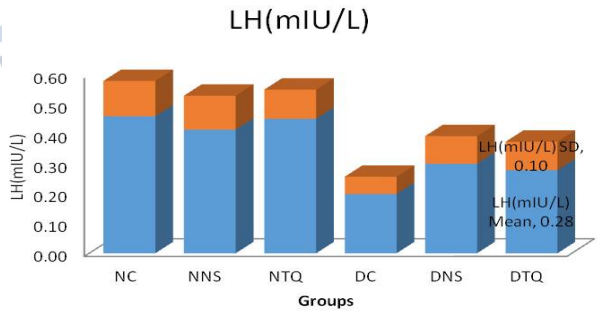
Testosterone (ng/dl) level of Normal Control rats was 82.78±8.26, normal rat treated with Thymoquinone rats was 91.08±7.51, diabetic rat treated with nigella sativa rats was 71.34±6.58. Diabetic control rats was 41.62±7.28, diabetic rat treated with Thymoquinone rats was 76.30±8.23, diabetic rat treated with nigella sativa rats was 71.34±6.58. LH(mIU/L) level of Normal Control rats was 0.46±0.12, normal rat treated with Thymoquinone rats was 0.45±0.10, diabetic rat treated with nigella sativa rats was 0.30±0.09. Diabetic control rats was 0.20±0.06, diabetic rat treated with Thymoquinone rats was 0.28±0.10, diabetic rat treated with nigella sativa rats was 0.42±0.11(Table 1).

Graph 1: Testosterone(ng/dl).



NC- Normal Control Rats, NNS- Normal rats treated with NIgella Sativa, NTQ- Normal rats Treated with Thymoquinone, DC- Diabetic Control Rats, DNS- Diabetic rats treated with NIgella Sativa, DTQ- Diabetic rats Treated with Thymoquinone.

Graph 2: LH(mIU/L).



NC- Normal Control Rats, NNS- Normal rats treated with NIgella Sativa, NTQ- Normal rats Treated with Thymoquinone, DC- Diabetic Control Rats, DNS- Diabetic rats treated with NIgella Sativa, DTQ- Diabetic rats Treated with Thymoquinone.

Table 1: One Way ANOVA Results of Reproductive Hormonal Profile (Testosterone, Progesterone, Oestrogen, FSH, LH).

Parameter	Groups							
	Group A Normal Rats- Control	Group B Normal Rats - Nigella Sativa Seed Powder	Group C Normal Rats - Thymoquinone	Group D Diabetic Rats - Control	Group E Diabetic Rats - Nigella sativa seed powder	Group F Diabetic - Thymoquinone	F	P
Testosterone (ng/dl)	82.78±8.26^{a}	88.35±7.66^{a}	91.08±7.57^{a}	41.62±7.28^{b}	0.28±6.58^{a,c}	76.30±8.23^{a,c}	33.625	0
LH (mIU/L)	0.46±0.12^{a}	0.42±0.11^{a}	0.45±0.10^{a}	0.20±0.06^{b}	0.30±0.09^{b}	0.28±0.10^{b}	7.088	0

Values with superscripts in each row among various groups are statistically significant with each other (P<0.05)

DISCUSSION

In our present study the testosterone and LH serum levels in normal rats treated with nigella sativa seed powder and thymoquinone not shown any significant change compared to normal control rats, in diabetic rats the testosterone and LH levels are decreased significantly compared with normal rats, when treated diabetic rats with Nigella sativa seed powder and thymoquinone the testosterone levels are normalised and there is no significant change in LH. In study of Rahmatollah Parandin found that Nigella sativa having enhancing effect on reproductive hormone profile [18]. In study of E.A. Datau, also observed that nigella sativa increased testosterone levels in treated groups [19].

In study of Farooq T found same results [20]. In previous studies showed that Nigella sativa treated rats are not shown any toxicity and shown significant change in body weight [21, 22, 23]. In a previous study shown that Nigella sativa increased the number of Leydig cells and its diameter nuclear in rat testes [24]. Testosterone has direct effect on the growth of testes, epididymis and other reproductive organ, structurally and physiologically dependent upon the testosterone. Testosterone stimulates growth and secretary activity of the reproductive organs it is proved that our study shows that testosterone levels are increased and testicular somatic index, germinal cells of testis and in results increase the testis and epididymis weight.

In previous studies it is well confirmed that, the reproductive hormone parameters in mammals are regulated by the two Gonadotropins, LH and FSH. FSH binds with receptors in the sertoli cells and directly stimulates spermatogenesis. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and indirectly stimulates spermatogenesis via testosterone [25-27].

Therefore, a significant decrease in LH hormone concentration in diabetic group lead to decrease in testosterone level. Our study found that the FSH levels remained unaltered in diabetic treated rat. It is possible that testis seminiferous tubules induced directly by the N. sativa extract or indirectly by testosterone and stimulated sperm counts. The present study shows that Nigella sativa and thymoquinone having the ability to increase number of Leydig cells in testis and increased testosterone levels.

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