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Ameliorative Effects of Supplemented Tocotrienol on Some Physiological Parameters of Induced Obesity in Laboratory Male Rats

A Dissertation

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by

Eman Hanash Rahi

B.Sc. (2002)- M.Sc. biology (2018)

Supervisor:

Prof. Dr. Nameer Abdulkareem Khudhair

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بسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

﴿وَقُلِ اعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ

وَالْمُؤْمِنُونَ وَسَتُرَدُّونَ إِلَى عَالِمِ الْغَيْبِ وَالشَّهَادَةِ

فَيُنَبِّئُكُمْ بِمَا كُنْتُمْ تَعْمَلُونَ﴾

صدق الله العلي العظيم

التوبة: 105

Dedication

To the pure souls who left me and whose prayers still surround me... my mother and father, may God have mercy on them.

My support and beloved, my dear husband.

To those whose presence illuminates my life my sons and daughter.

To those who stood out for their loyalty and were the finest.... My beloved brothers and sisters.....…

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List of abbreviations

Abbreviation	Full term
ACACA	Acetyl-CoA carboxylase-1
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AnTT	Annatto tocotrienol
AST	Aspartate aminotransferase
AT	Adipose tissue
BAT	Brown adipose tissue
BMI	Body mass index
CAF	Cafeteria diets
CPT1A	Carnitine palmitoyltransferase1A
CRD	Chronic renal dysfunction
CRP	C reactive protein
DIO	Diet-induced obesity
DN	Diabetic Nephropathy
FASN	Fatty acid synthase
FBG	Fasting blood glucose
FNDC5	Fibronectin type III domain containing 5
GRP78	Glucose-regulated protein-78
GTT	Glucose tolerance test
HFD	High-fat diet
HFDT	High-fat diet plus tocotrienol
HSCs	Hepatic stellate cells
HSD	High sugar diet
IR	Insulin resistance
ITT	Insulin tolerance test
LXR	Liver X receptors
MetS	Metabolic Syndrome
MGSO	Muscadine Grape Seed Oil
MMP	Matrix metalloproteinase
NAFLD	Nonalcoholic fatty liver disease
NEFAs	Non-esterified fatty acids

NF-B	Nuclear factor-kappa B
OS	Oxidative stress
OVA	Ovalbumin-challenged
PCOS	Polycystic ovarian syndrome
PO-TRF	Palm oil tocotrienol rich fraction
PPARs	Peroxisome proliferator-activated receptors
RBO-TRF	Rice bran oil-tocotrienol rich fraction
ROS	Reactive oxygen species
SFO	Sunflower oil
STZ	Streptozotocin (STZ)
T3	Tocotrienol
TG	Triglycerides
TG	Triglyceride
TNF-α	Tumour Necrosis Factor alpha
TRF	Tocotrienol-rich fraction
UACR	Urine albumin:creatinine ratio
UCP1	Uncoupling protein 1
VLDL	Low-density lipoprotein
WAT	White adipose tissue
WD	Western diet
WHO	World Health Organizations

Summary

This study was conducted at the College of Veterinary Medicine - University of Basrah to evaluate the protective and therapeutic role of tocotrienol supplements against the harmful effects resulting from eating a high-fat diet and risks of obesity inducement in male laboratory rats.

Forty-four adult male rats aged around 8 weeks and weighting about 80± 25g were used. The animals were distributed along three experiments of the present study. The first experiment (protective) includes 18 male rats (6 for each group) divided into control group that fed on low fat diet and drench with olive oil (1ml/kg BW), HFD group represent rats fed high fat diet and drench with olive oil (1ml/kg BW) while the protective group fed its rats by HFD and drench by tocotrienol (60 mg/kg BW) dissolved in olive oil along 12 weeks. The second experiment (therapeutic) involved 18 male rats (6 for each group) were divided into control group, obese group (obese induced by fed rats HFD for 12 weeks), and therapeutic group that induced obesity and treated by drench tocotrienol (60 mg/kg BW) dissolved in (1ml/kg BW) olive oil for about 8 weeks and all group fed low fat diet. In the third experiment which was considered as reproductive experiment, 8 males that were extracted from protective and therapeutic experiments and 16 females were used, randomly divided into 4 groups (2 males and 4 females). The first group was normal male rats mated with normal females. The second group was male rats fed a high-fat diet for 12 weeks and mated with normal females. The third group was male rats fed a high-fat diet plus tocotrienol for 12 weeks. The fourth group was male rats fed a high-fat diet for 12 weeks than they were given tocotrienols for 8 weeks and a low-fat diet. Animal weight and feed consumption were recorded during the experimental period. Blood samples were collected for hematological examination, and serum were separative for Lipid profile, liver enzyme, kidney functions, total and fractional protein, C reactive protein and glucose level were measured.

Also, some metabolic hormones such as leptin, adiponectin, insulin and irisin hormones were measured. In addition, sex hormones and Evaluation of sperm vitality were recorded. Histological changes were observed in the body's organs (liver, kidneys, and testicles). and gene expression (quantitative real-time PCR) in the liver and adipose tissue were examined.

The effect of tocotrienols drench to laboratory rats showed significant decrease in body weight after the fifth week compared with rats fed HFD in protective trial. Although there was no significant difference in therapeutic trial along eight weeks. These in turn impaired on body wight gain of HFD and HFDT groups despite the tocotrienol reduced the values of body mass index BMI, lee index and adiposity index AI but still higher than their values in control group. Feed consumption of the studied animals recorded non-significant difference in protective experiment, whenever therapeutic experiment appeared significant decrease in food consumption for the rats of obese group.

The results of the present study revealed that there was no specific indication for the significant difference of the hematological indices. Whatever, total WBC and neutrophil recorded significant decrease in their values for rats fed HFD for 12 weeks to induced obesity and drench with tocotrienol compared with another studied groups. Administration of tocotrienol enhanced liver function and reduced the harmful lipid indicators (triglyceride, LDL-c and VLDL-c) and improved HDL-c values in rats treated with tocotrienol for the protective experiment.

The effect of tocotrienol supplement on HFD led to significantly reduce in total protein and globulin levels and failed to enhance the concentration of C-reactive protein and glucose too appeared unlike their values in control group. In contrast, the therapeutic additive of tocotrienol to obese laboratory rats to enhance values of albumin and glucose when compared with laboratory rats suffered from obesity risks. The effect of tocotrienol

administration on some metabolic hormones showed significant decrease in leptin concentration and improved irisin and insulin concentration in protective dose compared to HFD and obese rats in both experiments. In contrast, tocotrienol enhanced LH concentration and reduced the harmful effect of HFD on sperm viability in protective and therapeutic experiments. The results above were confirmed by histopathological examination of liver, kidney and testes. While tocotrienol up regulation gene expression of the cpt1b gene and down regulation the gene expression of the IL-1β gene. The use of tocotrienols improved reproductive capabilities. In light of the current study, we conclude that tocotrienols may have vital roles in reducing metabolic risk of HFD and obesity and enhance fertility performance.

Chapter One

Introduction

1.1 Introduction

Obesity has emerged as a prominent health problem across the world, and its prevalence has been significantly growing among an important portion of the global population over the last several years (Petelin *et al*., 2019). Researchers expected that a third of the world's population is now classified as overweight or obese and by 2025 worldwide obesity prevalence will reach eighteen percent in men and over twenty-one percent in women (Chooi *et al*., 2019).

Obesity is a complicated illness with various aetiologias, each with its own set of severe symptoms and complications(Mouton *et al*., 2020). It has been recognized as the most important factor of risk for the development of diabetes (Ng *et al*., 2021), and an increased risk of cardiovascular disease, liver disease, and some types of cancer(Marcelin *et al*., 2019). According to statistics, obesity was responsible for the greatest share of metabolic disease-related mortality (Chew *et al*., 2023).The metabolic effect of obesity represented by lipid deposition in insulin-sensitive organs, such as the liver and the muscles of the skeletal system is related to fatty acid derivates' ability to block ingredients of the insulin signaling pathway (Gepstein & Weiss, 2019). The adiponectin profile and reduce insulin sensitivity due to obesity are both independent determinants of the metabolic syndrome's (Gołacki *et al*., 2022). The Metabolic Syndrome (MetS) is described by the World Health Organizations (WHO) as the collection of metabolic changes that result in the development of obesity, hypertension, and diabetes mellitus…etc.(Sollano-Mendieta *et al*., 2023).The process by which metabolic disorder start is excess nutrients in metabolic cells may lead to the chronic low-grade inflammation associated with obesity, known as metaflammation. These metabolic cells'

inflammatory signaling eventually activates specialized immune cells, which result in an ongoing inflammatory response in the adipose tissue (Aryaie *et al*., 2020), lowered adiponectin release, reduced insulin sensitivity, and activated pro-inflammatory pathways in adipose tissue, the liver, and muscles of the skeleton (Kyrou *et al*., 2018). On the other hand, leptin levels increase in people with high body mass indexes compared to those with normal weights in spite of its role in lipolysis (Al-Hejaj *et al*., 2021).

Irisin is important a myokine and adipokine that has lately received a lot of interest due to its modes of action. Recent studies found that body mass index is negatively related to irisin levels in the blood (Mahmoodnia *et al*., 2017; Huerta-Delgado *et al*., 2020), Also, Dietary intervention significantly decreased irisin mRNA protein levels in adipose tissue and skeletal muscle of obese male mice (Li *et al*., 2022).

Obesity management entails making behavioral and lifestyle changes, such as increasing physical activity and decreasing calorie consumption, to rectify the calorie imbalance. However, several medicines, such as Tirzepatide, semaglutide, phentermine, lorcaserin, and orlistat, have been used to help in weight reduction despite their negative effects (Pang & Chin, 2019; Tan *et al*., 2023). The usefulness of bariatric surgery in weight control is becoming more well recognized, and it is the preferred therapy when other approaches fail (Pang & Chin, 2019). As a result, researchers are increasingly interested in investigations of alternatives to obesity therapy, such as functional foods and bioactive chemicals (de Moura e Dias et al., 2021). Bioactive food substances such omega-3 polyunsaturated acids (Belza *et al*., 2007), caffeine (Belza *et al*., 2007), astragaloside II (Xu *et al*., 2009), and vitamin E (Aggarwal *et al*., 2010; Peh *et al*., 2016) are used as dietary treatments. Tocopherols and

tocotrienols are two groups of physiologically active compounds found in vitamin E. Each class has four isoforms (alphaa (α), betaa (β), gammaa (γ), and deltaa (δ), each with a distinct biological function (Sen *et al*.,2006; Aggarwal *et al*., 2010).

Tocotrienols (T3s) are among the compounds that had metabolic effects belong to its nutritional properties as a food supplement. It is largely considered safe at low doses for alleviation of pathophysiology by animal models and ongoing human trials(Ranasinghe *et al*., 2022). Reports evidence recorded that gamma tocotrienol reduce high-fat diet induced obesity and insulin resistancce (Zhao *et al*., 2015). While, delta-tocotrienol improved lipid metabolism (Allen *et al*., 2017), and had the ability to modulating regulatory effect of leptin and adiponectin hormones in lipid metabolism in rats diagnosed with the metabolic disorder produced by high-carbohydrate and diets that are high in fat after three months of therapy (Wong *et al*. 2018). However, another study indicated no effect of tocotrienols on leptin and adiponectin levels in rats that suffered from Androgen Deficiency Induced by Buserelin (Mohamad *et al*., 2019).

1.2 Aims of the study

studying the role of tocotrienol supplementation on regulate obesity risks by modulation of irisin, leptin, adiponectin and insulin hormones and ameliorate the physiological complications result from it. Also, focusing on deleterious effect of obesity on reproduction and identifying the role of tocotrienol to ameliorate the obesity risks on some reproductive hormones and efficiency.

The study includes:

1. Inducing obesity in laboratory rats by high fat diet.
2. Studying the effect of tocotrienol supplementation on irisin, leptin, adiponectin, and insulin hormones as well as growth, physiological and biochemical parameters.
3. Study of reproductive efficiency for obese male rats followed administration of tocotrienol.

Chapter Two

Literatures Review

2.Literature Review

2.1. Obesity as metabolic disorder, causes and consequences:

Obesity is an important public health concern (phenomenon) among all age groups in the world (Castellini *et al.*, 2017).It is anxiety source in both developed and developing countries that acquired confession as the number one public health issue in the world due to the large number of death each year (Ali *et al.*, 2022). Being overweight or obese increases the risk of developing a number of chronic illnesses, such as heart disease and stroke, which are among the world's leading causes of death. Being overweight or obese also increases the risk of developing diabetes and other related disorders, which can result in blindness, amputations, and the need for dialysis (Piché *et al.*, 2020). It can lead to musculoskeletal disorders including osteoporosis (Korkmaz & Özkan, 2022). Also, Obesity was linked to some types of cancer, including cancer of the endometrium, breast, ovary, prostate, liver, gallbladder, kidney, and colon (Font-Burgada *et al.*, 2016). The risk of growing these metabolic diseases increases even when a person is slightly overweight and increases with a higher body mass index (BMI), According to a research, obesity-related diseases claim the lives of more than four million individuals each year (WHO, 2022). According to the global burden of illness, the prevalence of overweight or obesity doubled globally between 1975 and 2016, and it is now a significant societal issue everywhere (Kato *et al*., 2022). The definitions of overweight and obesity include excessive or abnormal fat accumulation that poses a health risk (WHO, 2022). Body weight may increase as a result of an imbalance between energy from meals and energy used, with the majority of surplus energy being stored as fat (Di Meo *et al*., 2017). Excess energy storage can cause fat cells to either multiply (hyperplasia) or enlarge (hypertrophy).

Hypertrophy of adipocytes can be stopped by consuming less food, while hyperplasia is more resistant to alteration (Otto & Lane, 2005).

Several variables, including genetic, endocrine, metabolic, neurological, pharmaceutical, environmental, and nutritional factors, impact both types of obesity (Sun *et al*., 2011).

Although changes in the environment have undoubtedly led to the rapid increase in prevalence, obesity is caused by an interaction between environmental factors and innate biological factors (Loos & Yeo, 2022b). Environmental factors such as prolonged work, the role of social networks, and peers also increase the prevalence of obesity (Omar, 2020). Obesity was classically divided into two types depending on the number of genes responsible for obesity in individuals, the so-called monogenic obesity, which is inherited in a Mendelian pattern, rarely, early and severe onset and involving either small or large chromosomal deletions or monogenic defects; and obesity Polygenic (also known as common obesity), which is the result of hundreds of polymorphisms each with a small effect. (Chami *et al.*, 2020) Crucially, there is a strong genetic component that underlies the large interindividual variance in body weight that determines people's response to this "obesity" environment (Loos & Yeo, 2022a). Studies on twin, family, and adoption have estimated the heritability of obesity to be concluding that it s percent ranges between 40% and 70% (Elks *et al.*, 2012). It can be said that among the factors led to the spread of obesity is the genetic variation among individuals, as more than 360 genes were identified that led to the development of obesity (Nadulska *et al.*, 2017). Depression, anxiety, and stress are among the causes of obesity (Gomez-de-Regil *et al.*, 2020). As well as, medical conditions such as polycystic ovary syndrome also contribute in weight gain (Behboudi-Gandevani *et al.*, 2017) hypo -thyroidism, Cushing’s

disease and certain medications such as anti-depressants, anti-diabetic and anti-hypertensives are contributing to weight gain (Wharton *et al.*, 2018). Studies on humans have shown that diet-induced weight loss is associated with elevated levels of hormones that promote appetite (such as ghrelin) and lowered levels of hunger-suppressing hormones (such as leptin). After the weight loss, these hormonal changes continue for at least a year (Gutyon and Hall.,2022).

Pathogenesis of obesity growing may include insulin resistance, adipose tissue inflammation, and lipogenesis constitute a pro-obesity mechanism (Wen *et al.*, 2022). The potential mechanisms for obesity development have brain networks that include nutrients (fatty acids, triglycerides (TG), and glucose), circulatory variables (insulin, leptin), and cytokines associated with inflammation (Grzęda *et al.*, 2022).

According to Suleiman *et al.* (2020), an overabundance of adipocytes has detrimental effects on the pancreas, liver, the kidneys, the brain, heart, organs for reproduction, muscle tissue, and joints. Type 2 diabetes becomes inevitable when pro-inflammatory cytokines are stimulated by the production of adipokines. This leads to an impairment of insulin in the pancreas and inflammation (Al-Goblan *et al.*, 2014).

Any individual who is fat or overweight has some level of insulin resistance, and when they don't take in enough insulin to equal their level of insulin resistance, they get diabetes. Even if these individuals have high levels of insulin, their blood sugar cannot be brought back to normal (Røder *et al.*, 1998). Obese individuals' adipose tissue secretes non-esterified fatty acids (NEFAs), which might support the theory that insulin resistance and cell dysfunction are probably connected (Kahn et al., 2006) Obesity-related

metabolic disorders are frequently linked to cellular illnesses such mitochondrial malfunction and/or lipotoxic stress on the endoplasmic reticulum or systemic processes like metabolic inflammation (Kleinert *et al*., 2018). Liptoxicity effect on liver tissues leads to liver disease. Similarly, an increase in adipocytes yielding mechanical stress on the joints, muscles, and kidneys results in mechanical stress on the joints and renal stress, respectively, which eventually causes kidney failure and osteoporosis (Heymsfield & Wadden, 2017). Increasing insulin resistance has an adverse effect on the brain by boosting the neurotransmitter Insulin stimulates leptin's ability to inflame neurons, which eventually leads to neurodegeneration in the hippocampus and memory impairment (Bloemer *et al.,* 2014). Fat builds up in the heart muscle as a result of growing obesity's negative effects on the cardiovascular system (Ortega-Loubon *et al.*, 2019) . Dyslipidemia is brought on by this mechanism, which speeds up the breakdown of triglycerides into free fatty acids. This ultimately results in coronary heart disease (Van Gaal, 2010). In addition to the above, the accumulation of fat affects the reproductive system through the production of reactive oxygen species, which leads to a decrease in sexual behavior, performance and fertility (Suleiman *et al.*, 2020) .

The pathophysiological background of obesity and its complications are determined by white adipose tissue (AT) mass and influenced by AT dysfunction, body fat distribution, and disease stage (Goossens, 2008; Blüher, 2013;Goossens & Blaak, 2015; Goossens, 2017; Frühbeck *et al.*, 2019).

Adipocyte-derived fatty acids and cytokines seep into the bloodstream when an individual is obese because their adipocytes' capacity to store fat is surpassed (Guilherme *et al.,* 2008). After then, the toxic lipids build up in the tissues and produce localized inflammation, which has a role in the development of insulin resistance, non-alcoholic fatty liver disease, and

endothelial dysfunction (Unger, 2003; Feldstein *et al.,* 2004). Impaired metabolism in other tissues, such as the liver or pancreas, is also a result of altered adipokine or lipokine production (Fasshauer & Blüher, 2015 ;Hotamisligil & Bernlohr, 2015).

2. 2 Obesity inducement methods:

Incidence of diabetic and hyperlipidemia with their relation to coronary heart disease in animals and human has attracted the researcher's attention. Research conducted on many animal species, particularly mice, rats, and sheep, facilitates comprehension of the mechanisms behind the emergence of obesity and facilitates the exploration of potential intervention approaches (Grzęda *et al.,* 2022). Scientists suggest various methods for obesity inducement in animals, it can be induced chemically, surgically, genetically, or through diet (Barrett *et al.,* 2016; Pinheiro-Castro *et al.,* 2019). Although obesity is regarded as multifactorial etiology, some scientists focused on unhealthy eating patterns (such as consuming fast food), whereas, others showed that the increased incidence of obesity belong to environmental factors (Grzęda *et al.,* 2022). Diet is considered as a major factor contributing to obesity Epidemic, not genetic changes (Malik *et al.,* 2013). For this reason instead of monogenic or polygenic models, animal models of diet-induced obesity have been favored used (Marques *et al.,* 2016). Therefore, scientists are interested in diet-induced obesity (DIO) as the main procedure for obesity inducement despite their time consuming and cost. However, the DIO model encapsulates a significant portion of the pathophysiology of obesity, including slow, steady weight increase and the emergence of secondary insulin resistance (Kleinert *et al.,* 2018).

The most prevalent models of obesity caused by diets are: high-fat diet (HFD), high sugar diet (HSD) (Sadowska & Bruszkowska, 2017), high-fat-high-sugar diet (Moreno-Fernández *et al*., 2018; Birulina Julia *et al*., 2020), cafeteria (CAF) diets (Lalanza & Snoeren, 2021) and Western diet (WD) (Bortolin *et al*., 2018).

In fact, food signaling maintain energetic homeostasis through vagus and humoral pathways that significantly interact with the brain immune pathways (Gomez-Smith *et al*., 2016). Chronic exposure to conditions of excessive caloric intake leads to abnormal feeding patterns and abnormal immune responses (Gómez-Pinilla, 2008). Diet-induced obesity (DIO), When rats were administered HFD, they developed obesity, but diet-resistant rats developed body weights similar to rats in the control group when fed a low-energy diet. DIO in rats becomes less responsive to leptin's hypophagic activity between 4 and 5 weeks of life, while they are thin and before their body weight begins to diverge (Willett and Leibel, 2002). It is clear that animals exposed to HFD acquire obesity and have lower levels of insulin and leptin sensitivity (Hariri and Thibault, 2010).

High-fat diets are frequently used to cause obesity in animals (Picklo *et al.*,2017; Bortolin *et al.*,2018) because they generate harmful metabolism effects, implying that diet is a major contributor to the obesity epidemic (Krishna *et al.*,2016).). The mechanisms associated with this model are excessive intake of HFD leading to reduced satiety, which in turn leads to storage of dietary fat in the body and modification of hormones required for energy balance (Garg *et al.*, 2022). This, in turn, led to lower inhibition of ghrelin production following ingestion of a the high-fat diet, also leptin and insulin resistance in HFD-induced hyperinsulinemia and hyperleptinemia

(Hariri and Thibault, 2010). Obesity frequently exhibits a noticeably elevated serum leptin level together with a leptin-resistant symptom (Friedman, 2002).

A high-fat (HF) diet can lead to obesity and metabolic disorders in rodents and dietary intervention is not standardized, and the phenotype induced by HF clearly differs between different studies (Buettner *et al.*, 2007). It can affect metabolic imbalances by decreasing energy intake and, as a consequence, increased body weights of laboratory animals (de Moura e Dias *et al.*, 2021). However, variances exist between rodent species and strains. Wistar and Sprague-Dawley rats can be utilized as models for HFD-induced obesity (Marques *et al.*, 2016). The HF diet increased weight, body fat mass, mesenteric adipocyte volume, plasma adiponectin and leptin levels, and decreased oral glucose tolerance in Wistar and SD rats. However, the majority of these effects was more pronounced or was detected earlier in Wistar rats. (Marques *et al.*, 2016), Many studies have used high-fat diets with varying compositions and fat content. Animals (lard, tallow), plants (olive oil, sunflower, maize, coconut), and fish can all be sources of fat (Hariri & Thibault, 2010). Another crucial component is the content of fat in the diet, which typically ranges from 30% to 60% (de Moura e Dias *et al.*, 2021). The feeding time varied between research, often ranging from 4 to 16 weeks (de Moura e Dias *et al.*, 2021).

A sugar-rich diet also contributed to obesity in experimental mice. In experimental animals, sucrose was provided separately from conventional feed, as a superfood addition, or combined with drinking water (Pinheiro-Castro *et al*., 2019). HSD impairs glycemic control in rodents. These effects were comparable to those reported following HFD diet (Grzęda *et al*., 2022). Another study had given rats high-fat, high-carbohydrate diet rich in lard (17%) and fructose (17%) and drank 20% fructose solution, after 12 weeks of

feeding on the respective diet the final body weight did not differ between control and experimental groups. At the same time, adipose tissue/body weight proportion of experimental rats, including mesenteric, epididymal and retroperitoneal fat increased for more than 2 times, this was an important Indicator to the presence of obesity in experimental mice group (Birulina Julia *et al.*, 2020). Daffalla *et al.*, (2015) showed that the body weight in rats fed with the high carbohydrate was increased when compared with the normal, high fat diet and high fat plus carbohydrate diet rats. Rats fed a diet high in carbohydrates and fats developed increased blood pressure, cardiac fibrosis, and increased hardening of the heart and endothelium. Dysfunction, impaired glucose tolerance, increased abdominal fat deposition, altered plasma lipid level, cirrhosis and increased plasma liver enzymes with increased plasma markers of oxidative stress and inflammation as well as increased inflammatory cell leakage due to high carbohydrate and fat intake (Poudyal *et al.*, 2010).

However, body weight growth and adiposity in HSD-fed mice were lower than those fed an HFD (Kleinert *et al.*, 2018). Mice fed the HFD gained more weight than the mice fed HFHS despite having a similar amount of energy (Omar *et al.*, 2012). Exposing mice to the HS diet increases visceral adipose tissue without increasing or decreasing body weight or glucose disposal rates. Increased glucose in the liver binds to plasma glucose and free fatty acids in the serum (Cao *et al*., 2012). This model of Lipidosis and hepatic mitochondrial swelling was found also in HS diet when fed to rats (Cao *et al.*, 2012). It also led to the emergence of differential effects on the development of insulin resistance and beta cell adaptation between HFD and HFHS diet models (Omar *et al.*, 2012).

The CAF diet is effective in inducing obesity and metabolic disorders, as evidenced by increased body weight and obesity index, hyperlipidemia, hyperglycemia, glucose intolerance and insulin sensitivity (dos Reis Costa *et al.*, 2022). The cafeteria diet is a clear imitation of Foods paradigms that cause human obesity. Excessive eating causes increases in energy expenditure as a result of sympathetic stimulation of brown adipocytes. Excessive consumption of diet meals in the cafeteria indicates an increase in frequency and/or average meal size. These diets give the animals with a mix of sugar and salt, as well as rich fats from solid meals (La Fleur *et al.*, 2010; Cook *et al.*, 2017). The CAF diet is used in laboratory settings, where animals are fed a combination of high-fat and high-sugar foods that people commonly consume (e.g., cake, biscuits, crisps, processed meat, peanut butter, chocolate, cheese, dried fruit) (Lutz & Woods, 2012; Small *et al*., 2018; Pinheiro-Castro *et al*., 2019). The components of the CAF diet are high in energy and extremely tasty, which enhances the tendency of animals to overeat and gain weight (Lalanza & Snoeren, 2021). These led to increases fat pad mass and pre-diabetic parameters such as glucose and insulin intolerance.(Sampey *et al.*, 2011), However, the diet is imbalanced due to differences in product quantity, kind, and energy value (Grzęda *et al*., 2022). Cafeteria food contains food additives and is deficient in vitamins and minerals, which may alter the makeup of the gut flora, leading to obesity and other metabolic abnormalities (Bortolin *et al*., 2018). As a result, it is difficult to tell whether the metabolic issue is caused by the excessive fat content or the food additive, because the low level of micronutrients may affect it, making it impossible to analyze the real impact of nutrients on the development of obesity (Bortolin *et al.*, 2018).

Western diet (WD) a composition similar to Western food has also been used as similar as possible to the human diet (i.e. a diet high in fat, sugar and

salt and low in fiber content), but nutritionally adequate and free of additives and does not show dietary heterogeneity. WD design was intended to combine the optimal properties of HFDs and CAFs and thus create a highly suitable and reproducible diet (Bortolin *et al.*, 2018).

Other considerations to take into account are that mice and rats respond differently to diet; and strain, sex, and age, influence responses to diet, with younger animals and males being more susceptible to comorbidities associated with obesity (de Moura e Dias *et al.*, 2021). Compared to vegetable fats, dietary lipids derived from animals have a more noticeable impact on obesity and insulin resistance (Kubant *et al.*, 2015). Minor modifications in food composition such as changes in proportion saturated to unsaturated fatty acids (Timmers *et al.* 2011), As well as the form of the food, whether solid or liquid leads to various DIO outcome (Kleinert *et al.*, 2018) .

Rats fed a high-fat, high-carb diet for 12 weeks showed an increase in body weight/adipose tissue ratio as well as elevated blood pressure. An oral glucose tolerance test (GTT) and an insulin tolerance test (ITT) have shown hyperglycemia, poor glucose tolerance, and insulin resistance in mice with MS. While total plasma cholesterol (TC) and HDL-Ch did not differ from the controls, a greater plasma TAG level was noted in the experimental group. Mice with MS had increased levels of TAG and Ch in their livers, as well as a body weight to liver ratio (Birulina Julia *et al.*, 2020).

The combination of genetic and dietary impacts on the animal is included in the diet-induced obesity paradigm; it can also be a rapid technique to generate obesity, which then leads to insulin resistance. The model is economical and exhibits a lot of similarities to human obesity (Suleiman *et al.*, 2020).

2.3- Tocotrienols a subclass of vitamin E:

A potent and important component of the human diet is vitamin E. The existence of this powerful fat-soluble antioxidant is essential for the maintenance of the body cells. The advantages of this vitamin, which is quite widespread in nature, come from its potent antioxidant properties (Devasagayam *et al.*, 2004). Due to its fat-soluble nature, it can easily react with deleterious oxidizing substances and pass through cellular membranes, exploiting its weak hydrogen bonds to prevent possible oxidative damage (Ravisankar *et al.*, 2015). Two subclasses of vitamin E are found: Tocopherol and Tocotrienols (TT), which are further divided into four isomers alpha (α), beta(β), gamma (γ) and delta (δ) (Wong *et al.*, 2017a). These isoforms have modest molecular variances in composition despite tocotrienol sharing the same primary structure with tocopherols by consisting of a 6-chromanol ring and a hydrophobic carbon side chain (Pang & Chin, 2019), Comparing tocotrienol to the fully saturated side chain of tocopherol, it is recognize from the unsaturated phytyl side chain at the positions of 3, 7, and 11 of the side chains. This tocotrienol property enables more effective tissue penetration, which causes variations in their bioavailability, performance and functioning (Chin *et al.*, 2016). TT is a lipophilic substance that is associated with cellular membranes, fat deposits, and lipoproteins (Suzuki *et al*, 1993 ; Meydani,1995). Common plant-based oils including canola, soybean, and maize oils are among these sources. Low-used sources with strong antioxidant properties, such as palm and rice bran oils, as well as the annatto plant, can contain significant amounts of tocotrienols. Rice bran, oats, wheat germ, palm oil, and annatto oil are the main food sources for TT(Mesri Alamdari *et al.*, 2020). Tocotrienol obtained from annatto *(Bixa orellana)* bean is unusual in

that it mostly includes δ-tocotrienol (about 90%) and γ-tocotrienol (roughly 10%), with no α-tocopherol (figure 2.1)(Qureshi *et al*., 2015 a). It is acknowledge as a functional dietary ingredient with antioxidant and anti-inflammatory properties (Pervez *et al*., 2020).

Tocotrienols are transported throughout the body's tissues and important organs, including the skin, liver, heart, spinal cord, skeletal muscles, lung, adipose tissue, and brain (Patel *et al*., 2006). TT is absorbed in the small intestine and enters the systemic circulation as chylomicrons before disintegrating and attaching to circulating lipoprotein vectors for transmission (Napolitano *et al*., 2019). A typical way to improve fat solubility vitamin absorption provided in the form of an emulsifier, and eaten tocotrienols create an emulsion with the gastro-intestinal fluids as soon as they enter the intestines (Yap and Yuen, 2004).

Tocotrienol intake has a significant downside represented by quickness of metabolism and easiness of removal from the body (Lodge *et al.*, 2001). It is either removed after being dissolved in bile acids and absorbed in feces or it is evacuated through urine after being made more water-soluble by shortening the side chains (Liu and Jiang, 2020). When tocotrienol concentration in the blood reaches its highest, the bioavailability is only of brief duration and is not sustained in plasma for an appropriate amount of time, lasting no longer than 3.5–4 h. After 24 hours, it totally vanishes from blood (Jaafar *et al.*, 2018) .

Previous preclinical studies showed that tocotrienol, particularly delta tocotrienol, have a range of physiological effects, including remarkable antioxidant (Vasanthi *et al* 2012), anti-inflammatory and anti-obesity (Wong *et al.* 2017b), anti-insulin resistance (Shen *et al.*,2018), anti-hepatosteatosis (Allen *et al.*,2017), and antihypercholesterolemic properties (Zaiden *et al.*,

2010). An TT produced from annatto had high concentration of TT and almost no TF (figure 2.1)(Qureshi *et al*., 2015 a). TT supplementation enhances bone health in rats deficient in testosterone by promoting bone formation (Chin & Ima-Nirwana, 2014). Among the eight vitamin E isomers, δ-TT and γ-TT are regarded as the most effective isomers compared to alpha and beta isomer of TT because they have antioxidant and mevalonate-suppressing properties (Shen *et al.*, 2018). A study on Tocotrienol rich fraction (TRF) therapy revealed various effects, including the up-regulation of gene expression for cell division, transcription, G protein-coupled receptor signaling, which is multi-cellular organismal growth, protein- Kinase activity, cell surface receptor signaling, and response to glucocorticoids, as well as a decrease in the expression of aging-related genes when therapy was continued for three months (Ghani *et al.*, 2019). Other study for supplementation with tocotrienol-rich fraction (TRF) found it reduced overall DNA damage more in older people (>50 y) than in younger adults (35-49 y) (Chin *et al.*, 2008). Additionally, older people fed with TRF showed improved blood lipid profiles, levels of vitamins E and C, as well as decreased levels of protein and lipid damage (Chin *et al.*, 2011).

In NAFLD patients, miRNAs related in hepatic steatosis, inflammation, and apoptosis are downregulated by T3 and TF. Meanwhile, miR-375 and miR-34a, which are connected to the control of inflammation and apoptosis, are decreased by T3 more significantly than by TF(Pervez *et al.*, 2023) .

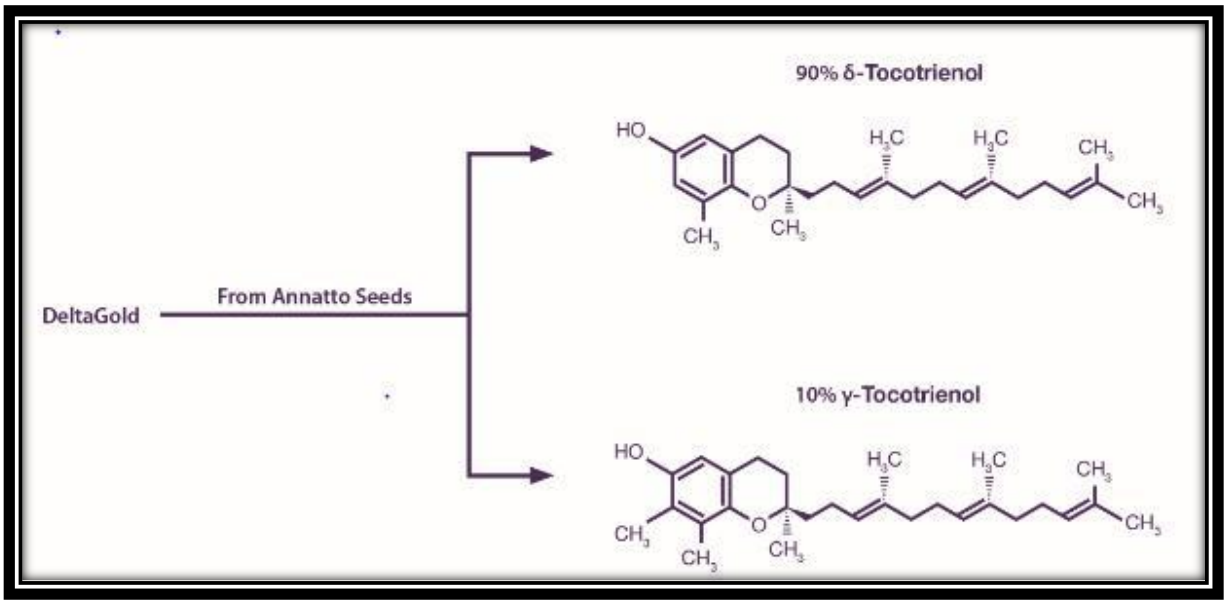


Figure 2.1 Chemical structures of annatto tocotrienols (90% δ-tocotrienol + 10% γ)-tocotrienol) (Qureshi *et al*., 2015 a)

2.4- Effect of Tocotrienol Supplement on body weight and weight gain

Body weight is one of the main indicators of obesity, Wong and his colleagues observed that giving Wistar rats HFD for 8 weeks 120 mg/kg/day of TRF had no influence on their overall weight increase, but it reduced body weight gain in high carbohydrate diet supplemented with TT fed rats group compared with other groups of the study (Wong *et al.*, 2012). Chin and Ima Nirwana, (2014) evaluated the antiosteoporotic effects of annatto derived tocotrienol (AnTT) 60 mg/kg/day given orally for 8 weeks using a testosterone deficient osteoporotic rat model. The results showed that The percent increase in body weight was significantly less in the testosterone group than in AnTT groups. Adding to HFD with 0.05 percent T3 for four weeks reduced the

obesity caused by the HF diet in young mice (Zhao *et al.*, 2015a). In diet-induced obese rats, δ - tocotrienols administration has demonstrated to decrease total body fat mass, abdominal circumference, and visceral adiposity index(Wong *et al.*, 2017). The average body and fat pad weights of the treatment groups in different doses with supplementation delta tocotrienol (δT3) at 400 mg/kg and 1600mg/kg for 14 weeks revealed non-significant difference from that of high fat diet fed mice(Allen *et al.*, 2017). A previous study indicated that the use of tocotrienol-rich fractions TRF for 8 weeks in obese rats did not lead to a significant decrease in weight after the treatments for a period experiment(Mesri Alamdari *et al.*, 2020). It has been reported that combined treatment with HFD and T3s in male mice (50 mg/100 g diet) for 8 weeks significantly inhibited body weight gain compared to HFD without T3 supplement group , while no significant difference was found between the control groups and tocotrienol treatment group (Kato *et al.*, 2021).

Shen *et al.*, (2021) discovered that there were no variations in body weight between the HFD group and the HFD+TT800 group during the course of the 14-week trial in C57BL/6Jmice fed either a high-fat diet (HFD control) or HFD supplemented with 800 mg annatto extracted TT/kg .

In other study, using a HFD increased body weight compared to HFD with additive tocotrienols (50 mg/100 g diet; α-:β-:γ-:δ- = 33.4:4.4:46.7:15.2, After 13 weeks, a significant reduction in body weight was noticed the buildup of perirenal fat reduced in comparison to the high-fat diet-treated Mice group(Kato *et al.*, 2022). The T3s-treated obese mice had a greater end body weight than the T3s-treated control animals, despite the fact that T3s therapy inhibited body weight gain in obese mice compared to the untreated group. The inhibitory impact of T3s on body weight slowed the HFD-induced weight

gain.T3s undoubtedly inhibit the body weight gain produced by the HFD (Kato *et al.*, 2022).

Palm oil-derived tocotrienol-rich fraction has caused greater weight gain compared to vehicle-treated diabetic rats when TRF oral treatment for 12 weeks. Although there was no significant difference between the two groups till the fifth week of experiment. However, subsequently, rats in Tocotrienols showed significantly greater weight gain compared to vehicle-treated diabetic rats and the difference amounted to 1.8-fold at week 12 post-streptozotocin STZ administration for diabetic inducement ($p<0.05$).(Abdul Ghani *et al.*, 2023).

Brain, heart, liver, kidneys and testes weights significantly increased in Tocotrienol group (3%) of a preparation in powdered diet (Nakamura *et al.*, 2001).Whereas in the other study, γT3 treatment considerably reduced the weight of the liver, total epididymal fat, and mesenteric fat in mice, but it had little effect on the weights of other tissues like perirenal fat or brown adipose tissue (Zhao *et al.*, 2015).

2.5- Effect of Tocotrienols supplementation on feed intake of laboratory rats: -

Estimation of feed quantity consumed from the experimental animals had a relation with the general health and body weight status for these animal. Also, it is an indicator for the right preparation of formula through follow-up the daily consumption. In case of obesity, there were many studies that used HFD for induction and investigation of the good palatability from the animals (Marques *et al.*, 2016; de Moura e Dias *et al.*, 2021), but still there was a difference when compared with tocotrienol addition to feed.

Budin *et al*., (2009) reported Throughout the trial, palm oil tocotrienol-rich fractions TRF supplementation had a significant impact on water and food intake, but the statistical outcomes were unaffected. Palm tocotrienol-rich fraction (TRF) 120 mg/kg treatment in high fat diet rats increased food intake compared with high fat diet rats, but it reduced food intake in rats fed a high carbohydrate for 8 weeks compared with control rat (Wong *et al.*, 2012). Conversely, another study has reported that young C57BL/6J mice fed a high-fat (HF) diet supplemented with 0.05% γT3 for 4 weeks showed no significant impact on food intake (Zhao *et al*., 2015). Separate groups from high fat diet were supplemented with either α-, γ-, δ-tocotrienol or α-tocopherol (85 mg/kg/day) for the final 8 of the 16 weeks. Treatment with α-tocopherol or individual tocotrienols did not change food or water intake compared with their respective controls except CγT3 which had lower food intake compared with control rats(Wong *et al.*, 2017b) . Shen *et al.*, (2021) experiment14 weeks of annatto-TT (90% δ-TT+10% γ-TT) supplementation and found there were no variations in food intake or water consumption between the HFD group and the HFD+TT800 group along the duration of the study.

Administration of Delta-Tocotrienol and Alpha-Tocopherol in different animal groups showed non- significant differences between these two groups, patients with NAFLD were randomized to either receive T3 (n = 50) 300 mg or TF (n = 50) 268 mg twice/day for 48 weeks in terms of dietary intake at baseline ($p > 0.05$) (Pervez *et al.*, 2023).

2.6-Effect of Tocotrienols Supplementation on hematological indices

Hematological examinations are very important tools for clinicians and physiologists. Blood analysis for complete blood count could support diagnosis through their indices that revealed general health of animals, immunity status, nutritional status and others like infections or inflammation. Therefore, it is important to include hematological indices in the study although little researchers have included it in their studies on obesity on tocotrienol.

Nakamura *et al*. (2001)conducted a 13-week oral toxicity study in Fischer 344 rats of both sexes at different dose levels of 0 (group 1), 0.19 (group 2), 0.75 (group 3), and 3% (group 4) of a preparation tocotrienol, it had the following composition: a-tocotrienol 21.4%, b-tocotrienol 3.5%, g-tocotrienol 36.5%, d- tocotrienol 8.6%, a-tocopherol 20.5%, b-tocopherol 0.7%, g-tocopherol 1.0% and d-tocopherol 0.5%. in powdered diet. Results revealed significant decrease in mean corpuscular volume (MCV) in all treated males. The results demonstrated a substantial decrease in mean corpuscular volume (MCV) in all treated males. Platelets were dramatically reduced in group 3 and 4 of male rats, whereas hemoglobin concentration, MCV, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were significantly lowered in group 3 and 4 females.

In another study conducted by Tasaki *et al.* performed a 52-week as chronic study by using Wistar Hannover rats of both sexes to reports regarding toxicological effects of long-term exposure of tocotrienol, that given the compound of tocotrienol at doses of 0, 0.08, 0.4 or 2% in powdered basal diet The male rats showed significant decrease in HTC and WBC values at the

dose of 0.8%. A significant decrease was also found in MCV at 0.4% of tocotrienol supplemented in diet. Whereas, females revealed significant decrease in Hb, HTC, MCV and MCH at the highest dose compared with other studied dose *(*Tasaki *et al.*, 2008).

Large doses of tocotrienol were administration for Fischer 344/slc (F344) rats that composed of 98.7% T3 (2.5% α-T3, 92.0% γ-T3, and 4.2% δ-T3) and 1.0% γ - tocopherol (wt/wt) was respectively 4.7, 14.6, and 42.2 (mg/rat. day) was prepared from rice bran for 13 weeks, tocotrienol did not affect the Red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), and white blood cells (WBC) levels (Shibata *et al.*, 2012) .

A human clinical trial including 31 individuals with metabolic syndrome was done to evaluate the tolerance and safety of tocotrienols T3 supplementation. The individuals were given tocotrienol-rich fraction (TRF) 200 mg , while other received placebo capsules twice a day for two weeks, the study investigated that T3 supplementation had no significant impact on the red blood cell (RBC), white blood cell (WBC), and platelet counts between TRF and placebo treatments. (Lin *et al*., 2016) .

Rats treated with palm oil tocotrienol-rich fraction TRF (30 mg/kg body weight) had considerably less white blood cells Compared to asthmatic rats. Significantly, palm oil TRF more successfully decreased the neutrophil, lymphocyte, and eosinophil cell count(Zainal *et al.*, 2019).

2.7-Effect of Tocotrienols Supplementation on liver and kidney function

Liver is a crucial organ that manages obesity by regulating the body's lipid metabolism. T3s are crucial in reducing the fatty liver caused by a high-fat diet (Tres *et al.*, 2012). on the other hand, palm tocotrienol-rich fraction (TRF) has shown to diminish hepatic triglyceride buildup caused by high-fat diets and enhanced liver structure and function with lower plasma liver enzymes, inflammatory cell infiltration, fat vacuoles, and balloon hepatocytes when taken as a dietary supplement for 16 weeks in rats (Wong *et al.*, 2012). T3 supplementation reduces inflammatory cell infiltration in the liver caused by high fat diet (Yachi *et al.*, 2010). According to Burdeos *et al.*, T3 at 10-15 ? lowers the mRNA and protein expression of fatty acid synthase while increasing the mRNA expression of genes related to oxidation, such as CPT-1 and CYP3A4 (Burdeos *et al.*, 2012). Muto *et al.* have repeatedly demonstrated that T3 administration in rat primary hepatocytes reduces the expression of genes related to hepatic steatosis, such as Chop and SREBP-1c, lessening ER stress and inflammation in the fatty liver (Muto *et al.*, 2013). Feeding a high-fat diet and a T3 supplement have been reported to increase the expression of genes related to fatty acid oxidation in the livers of mice (Zhao *et al.*, 2015). Li *et al.* discovered that 0.2% TRF supplement in diet reduces atherosclerosis in mice and this is done by increasing liver X receptors LXR and cholesterol transporters such ABCA1 in the liver(Li *et al.*, 2010).

Nakamura *et al.*, (2001)reported that the administration of a 3.0% T3 diet to F344 rats for 13 weeks elevated of alkaline phosphatase and induced hepatocellular hypertrophy.

According to Tasaki *et al.*, (2008)feeding Wistar Hannover rats a 2.0% Tocotrienols diet for 52 weeks caused an increase in blood ALT, ALP and the formation of nodular liver lesions. However Shibata *et al.* (2012) 's study found there were no differences in the AST or ALT levels for any of the groups treated with tocotrienol. Another human study used the mixed tocotrienols 61.5 mg, 112.8 mg and 25.7 mg for alpha-, gamma- and delta-tocotrienol, respectively and 61.1 mg of alpha-tocopherol, 400 mg daily where they showed a hepatoprotective effect in hypercholesteremic adults (Magosso *et al.*, 2013).Gamma-tocotrienol, but not alpha-tocopherol, has been demonstrated to be able to reduce triglyceride buildup by controlling fatty acid syntase and carnitine palmitoyltransferase , which reduces hepatic inflammation and endoplasmic reticulum stress (Burdeos *et al.*, 2013).

Measures of serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and albumin did not differ between 31 subjects with metabolic syndrome supplementation with tocotrienols (T3) at the dosage of 400 mg per day for 14 days and placebo interventions (Lin *et al.*, 2016). Treatment with vitamin E homologues for 8 weeks attenuated the degree of liver injury in treated rats groups, as demonstrated by lower plasma ALT and AST activities, less infiltration of inflammatory cells and decreased lipid droplets(Wong *et al.*, 2017). The δ-tocotrienol supplementation significantly improved lipid profile and liver functions (ALT, AST) in the patients with nonalcoholic fatty liver disease (NAFLD) were randomly assigned to receive δ-tocotrienol 300 mg twice daily or placebo for 24 weeks, compared with placebo (Pervez *et al.*, 2020), as well as this finding is consistent with the observation in animal studies, where palm tocotrienol-rich fraction (TRF)(Wong *et al.*, 2012), tocotrienol enriched palm olein(Wong *et al.*, 2017) and annatto-derived tocotrienols (90 % δ- and 10 % γ-tocotrienol) (Allen *et*

al., 2017) have been shown to significantly reduced ALT, AST, TG and fatty acid levels in high fat diet-fed rodents.

High-fat diet has already been shown to develop lipid droplets in the liver, and T3s block this process (Kato *et al*., 2021). While diet-induced obesity exacerbated liver damage, the antioxidant action of tocotrienols may have reduced the HFD impact. (Kato *et al*, 2022).

Effect of tocotrienol on renal function also investigated by some researches. In general, proteinuria, or other renal lesions were sensitive indicators of renal damage. They can be derived from glomerular or tubular damage also serum creatinine increased whereas creatinine clearance decreased indicating a decreased glomerular filtration rate. Renal function of type 1 diabetic rats were considerably improved by therapy with both PO-TRF palm tocotrienol rich fraction and RBO-TRF rice bran oil-tocotrienol rich fraction, Nevertheless, PO-TRF was more effective at a given dose than RBO-TRF(Siddiqui *et al.*, 2010).

Tan *et al.*, (2018) found that 2 months of tocotrienol-rich vitamin E supplementation as Tocovid 200 mg twice a day while the control group received placebo twice a day significantly reduced serum creatinine in individuals with Diabetic Nephropathy DN, but not Hemoglobin A1c, urine albumin:creatinine ratio (UACR).

placebo-controlled clinical trial, 54 patients with diabetic nephropathy were randomized to receive Tocovid 200 mg or placebo for 12 weeks has found Tocovid significantly decreased serum creatinine levels and significantly increase eGFR compared with placebo. There were no significant changes in HbA1c, blood pressure, and other parameters (Tan *et al.*, 2019).

The treatment with s tocotrienol-rich fraction (TRF) from palm oil (100 mg/kg b.w; orally) significantly reduced dyslipidemia and inhibited the development of chronic renal dysfunction (CRD) caused by atherogenic factors. These findings show that low-dose treatment of TRF may provide significant health benefits in the prevention of lipid induced CRD (Rashid Khan *et al.*, 2015) .

Khan *et al.,* (2010) have reported that tocotrienol-rich fraction (TRF) from palm oil (200 mg/kg, bw, orally, once daily for 21 days) attenuates potassium dichromate K2Cr2O7-induced acute renal injury in rats by protection the integrity of membrane by inhibiting lipid peroxidation and augmenting the activity of antioxidant enzymes in the kidneys of diabetic rats

2.8-Effect of tocotrienols supplement on total protein, albumin, globulin and C reactive protein

Albumin and globulin are two key blood protein components that have been linked to systemic inflammation. Low serum albumin levels indicate malnutrition, liver and renal malfunction, and have been found to be an independent predictor of poor survival in critically ill patients(Feketea & Vlacha, 2020).

Lin *et al*., (2016) showed that no significant difference was noted between TRF and placebo interventions on total protein, albumin, globulin, total bilirubin levels and the ratio of albumin to globulin. A total of 28 male Sprague-Dawley rats were supplemented with TRF (200,mg/kg) was 30 minutes prior to fenitrothion FNT (20 mg/kg) administration, both orally for 28 consecutive days. The result showed that TRF administration significantly

decreased the total protein level compared as fenitrothion-treated rats group (Jayusman *et al.,* 2014).

The liver produces CRP, an essential inflammation-sensitive plasma protein, which serves as an inflammatory marker (Taubes, 2002). CRP levels rise in response to inflammation in tandem with the release of IL-6 by macrophages and T cells (Michihara *et al.,* 2010). Tocotrienols have better anti-CRP effects than tocopherol. Tocotrienols inhibit the production of inflammatory mediators, with -tocotrienol being the most effective(Prasad, 2011).It has been demonstrated that consuming grape seed oil alongside high TT levels reduces serum levels of tumor necrosis factor- and high-sensitivity C-reactive protein hs- CRP and enhances insulin resistance in obese and overweight women(Irandoost *et al.,* 2013). Microalbuminuria and high-sensitivity C-reactive protein (hs-CRP), markers of acute inflammation, were decreased in type 2 diabetic patients by TT supplementation (34.6% α-TT+43.5% γ-TT) (Haghighat *et al.,* 2014) . Supplementation with tocotrienol-rich fraction (78 % tocotrienol and 22 % tocopherol, 150 mg/day) or placebo capsules for 6 months, lowered the plasma levels of C-reactive protein (hs-CRP) precursor in subjects were divided into two age groups—32 ± 2 (young) and 52 ± 2 (old) years old(Heng *et al.,* 2013). Plasma C-reactive protein CRP level was decreased by 44% in rats treated with palm oil tocotrienol-rich fraction TRF (30 mg/kg body weight) compared with that in ovalbumin-challenged (OVA) asthmatic rat model group (Zainal *et al.,* 2019).

Daud *et al.* (2013) did not observe any significant change in inflammatory biomarkers (C-reactive protein and interleukin 6) , in chronic hemodialysis patients were provided daily with capsules containing either vitamin E tocotrienol-rich fraction (TRF) (180 mg tocotrienols,40 mg tocopherols after 12 and 16 weeks of intervention when compared with the baseline within the

group or when compared with the placebo group(0.48 mg tocotrienols, 0.88 mg tocopherols). Also. Measurements of soluble inflammatory (hs-CRP) did not show notable changes throughout the interventions with palm-based tocotrienols and tocopherol mixture supplementation PTT (400 mg/d) in subjects with metabolic syndrome (Gan *et al.*, 2017).

δ-tocotrienol 300 mg twice daily significantly reduced high sensitivity C-reactive protein (hs-CRP), Compared with placebo for 24 weeks in patients with nonalcoholic fatty liver disease (Pervez *et al.*, 2020) .while Despite strong mechanistic evidence for the anti-inflammatory properties of tocotrienols inflammatory markers, specifically serum hsCRP, TNFa, IL-6 or adiponectin, were not affected when examined the effects of palm-tocotrienols (420 mg/day tocotrienol plus 132 mg/day tocopherol), palm-carotene (21 mg/day carotenes), or placebo (palm olein) supplements for 8 weeks on vascular function and cardiovascular disease risk factors in persons at elevated risk of impaired vascular function (Stonehouse *et al.*, 2016).

2.9- Effect of Tocotrienols Supplementation on lipid profile and glucose

Cholesterol and triglycerides are two types of lipids. Both of which are essential for life because triglycerides supply a large portion of the energy required for cell function (Shi & Cheng, 2009), while cholesterol is needed to construct cell membranes and produce a number of vital hormones (Zaiden *et al*., 2010) According to physiological principles, they must interact with lipoproteins in order to disintegrate and circulate in the blood (VLDL, LDL, HDL and chylomicrons). LDL/HDL and VLDL are the two main lipoproteins

in charge of transporting cholesterol and triglycerides from the endogenous pathway (hepatic production), respectively, in addition to chylomicrons, which largely carry triglycerides from the exogenous pathway (intestinal absorption) (Levy *et al*.,1971).

In a previous clinical experience, it was shown that supplementing with a tocotrienol mixture for eight weeks with 60 mg of each δ- and γ-tocotrienol significantly decreased TG and chylomicron levels in hypercholesterolemic patients (Zaiden *et al*., 2010).Also led to decreased plasma concentrations of free fatty acids, triglycerides, and cholesterol(Chou *et al*., 2009; Das *et al*., 2012) , and enhanced glucose and insulin tolerance in laboratory animal (Budin *et al*., 2009;Burdeos *et al*.,2012; Allen *et al*., 2017). Tasaki *et al*., (2008) were regarding toxicological effects of long-term exposure to tocotrienol for 1 year TO WHAT?, they performed a 52-week chronic study using Wistar Hannover rats of both sexes given the Tocotrienol at doses of 0, 0.08, 0.4 or 2% in powdered basal diet ,they found there was no changes in serum cholesterol levels among the groups under experimental condition. Budin *et al*. (2009) Palm oil tocotrienol-rich fractions (200 mg/kg body weight) were administered to streptozotocin-induced diabetic rats daily for eight weeks glycemic status significantly improved by reducing fasting blood glucose and glycohemoglobin HbA1c levels.

TRF supplementation prevented a rise in TC, LDL-C, and triglyceride levels, and significantly higher levels of HDL-C than the non-TRF group (Budin *et al*., 2009). Administration of Tocotrienol rich fraction (TRF) from palm oil (PO) and rice bran oil (RBO) for 8 weeks post induction of diabetes reduced the fasting blood glucose FBG and glycosylated hemoglobin HbA1c level significantly compared to the diabetic untreated group , the reduction offered by PO-TRF was found to be greater as compared to RBO-TRF,

indicating PO-TRF as a better hypoglycemic agent than RBO-TRF(Siddiqui *et al*., 2010). Fang *et al*., (2010) showed the tocotrienol-rich fraction of palm oil TRF composed of 23.54% a-tocotrienol, 43.16% g-tocotrienol, 9.83% d-tocotrienol, and 23.5% a-tocopherol showed enhanced insulin sensitivity and whole-body glucose utilization in diabetic Db/Db mice were treated daily with 50 mg/kg TRF by gavage for 2 weeks , by selectively regulating PPAR target genes. Palm tocotrienol-rich fraction TRF reduced triglyceride and non-esterified fatty acids (NEFA) but not total cholesterol concentrations (Wong *et al*., 2012 b). Other research showed Supplementing obese women with grape seed oil (rich in tocotrienols) lowered FPG, FSI, and insulin resistance. HOMA-IR when compared with control group as sunflower oil "SFO" (consuming 15% of energy from SFO) through a weight loss diet for 8 weeks (Irandoost et al., 2013).

Previous study showed a link between dietary δ-tocotrienol intake and lower serum levels of total cholesterol and lipoproteins in hypercholesterolemic subjects took increasing doses of δ -tocotrienol (125, 250, 500,750 mg/d) plus American Heart Association Step-1 diet for four weeks during the30 weeks study period by the dose-dependent inhibition/activation properties of δ -tocotrienol contribute to its ability to control inflammation (b Qureshi *et al*., 2015).

For the last 8 weeks of a 16-week study, rats on a high fat diet were given 85 mg/kg/day of α-, γ-, δ-tocotrienol, or α-tocopherol. They improved glucose tolerance, insulin sensitivity, and lipid profiles (Wong *et al*., 2017b). This improvement in insulin sensitivity and glucose uptake contributes to a decreased pro-inflammatory microenvironment and, as a result, to a decrease in visceral adipose tissue and total body fat mass (Allen *et al*., 2017). δ-tocotrienol effectively improved descriptors of glycemic control and insulin

resistance in NAFLD patients by increasing expression of PPAR-γ, PPAR-γ activation increases insulin sensitivity by inducing genes implicated in the insulin signaling cascade (Pervez *et al*., 2020). Serum cholesterol levels of the high fat diet plus tocotrienols group were improved compared to those treatment with HFD induced serum lipid metabolism dysfunction in high-fat diet-treated mice, also high-density lipoprotein levels were showed non significantly different in the presence or absence of T3s (Kato *et al*, 2022).

In vitro and in vivo studies have shown that T3s inhibit the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase, a key enzyme in cholesterol biosynthesis, hence lowering hepatic cholesterol production. Furthermore, the T3-rich fraction (TRF) of palm oil reduces triglyceride, apolipoprotein B, total cholesterol, and LDL-cholesterol levels in human blood (Black *et al*., 2000; Nafeeza *et al*., 2001; Baliarsingh *et al*., 2005; Li *et al*., 2010). Prasad reviewed that Tocotrienols T3s have anti-sclerotic and hypocholesterolemic impacts on humans, rats, and mice (Prasad, 2011) Palm oil's tocotrienol-rich fraction reduces blood lipids and increases HDL-C levels. TRF-treated rats exhibited improvement in blood sugar levels compared to vehicle-treated diabetic rats, but the blood glucose levels in TRF-treated rats were remained considerably higher than the normal control group, indicating that a hyperglycemic condition was maintained throughout the trial period (Abdul Ghani *et al*., 2023).

The administration of delta-tocotrienol along with oral glycemic therapy in a type-2 diabetes clinical study resulted in significant improvements in glycemic control, plasma insulin levels, and insulin resistance, as well as a reduction in the levels of markers for oxidative stress and inflammation (Mahjabeen *et al*., 2021).

Das *et al*.demonstrated that the two isomers of tocotrienols, α -tocotrienol, γ -tocotrienol, render the hypercholesterolemic hearts resistant to ischemic reperfusion injury by lowering several hypercholesterolemic proteins including matrix metalloproteinase (MMP) MMP2, MMP9, endothelin-1 ET-1, and SPOT 14 (linked with hypercholesterolemia) and upregulating TGF-b, when rabbits were kept on cholesterol diet for 60 days supplemented with α -tocotrienol, δ-tocotrienol and γ -tocotrienol for the last 30 days (Das *et al*., 2012). For five weeks, rats with streptozotocin/nicotinamide-induced type 2 diabetes mellitus (T2DM) showed reduced lipid metabolism and insulin resistance when given rice bran oil (RBO), which contained 5.25 g γ-oryzanol and 0.9 g γ-tocotrienol/150 g oil in contrast to the group under control (Chou *et al.,* 2009).

2.10-Effect of tocotrienol supplement on adiponectin, leptin and irisin hormones

Adipose tissue is a complex network of endocrine organs that has been divided into white adipose tissue (WAT) and brown adipose tissue (BAT)(Al-Suhaimi & Shehzad, 2013) . Unlike white adipose tissue (WAT), which is the principal location of surplus energy, brown adipose tissue is a major site for adaptive thermogenesis. Brown adipocytes' ability to thermogeneize is mostly due to their high concentration of mitochondria and strong expression of Uncoupling protein 1 (UCP1). When it is active, it mediates the waste of chemical energy by isolating the oxidation of mitochondrial substrates, which produces heat, from the synthesis of ATP (Spiegelman, 2015; Lapa *et al.*, 2017 Cohen &). So, from a therapeutic standpoint, Brown fat tissue function and activation have great potential. Its crucial role in the management of obesity is also remarkable (Mesri Alamdari *et al.,* 2020).

Adipose tissue secretes polypeptide hormones such as adiponectin and leptin, which contribute to the progression of obesity-related diseases such as hypertension, atherosclerosis, and type 2 diabetes (Zahorska, 2006). Adiponectin controls glucose and lipid metabolism in insulin-sensitive tissues (Diez, 2003). It improves fatty acid oxidation and glucose absorption in the muscle while decreasing glucose production in the liver. Pro-inflammatory cytokines inhibit its secretion, implying that inflammation may play a role in hypoadiponectinemia in insulin-resistant and obese individuals. Adiponectin insufficiency causes insulin resistance, obesity, type 2 diabetes, and atherosclerosis (Lago *et al*., 2007). Adiponectin has strong anti-diabetic, antiatherogenic, and anti-inflammatory properties (Kershaw & Flier 2004). In clinical investigations, plasma adiponectin levels decline as obesity increases (Arita *et al.*, 1999), dyslipidemia (Matsubara *et al.*, 2002), diabetes (Rothenbacher *et al.*, 2005) and cardiovascular disease (Xydakis *et al.*,2004). Adipocyte dysfunction in Metabolic Syndrome is associated with an accumulation of macrophages inside the adipose tissue, which enhances the release of cytokines that are pro-inflammatory and induces systemic inflammation (Srikanthan *et al.*, 2016). In the Metabolic Syndrome, there is a low level of plasma adiponectin (Sader *et al*., 2003). On the other hand, leptin plays a crucial role in the development of MS and cardiovascular disease (Ahima, 2000). Leptin, one of the most significant adipose-derived hormones and among the best-known hormone indicators for obesity, is generated and released by the fat cells in adipose tissue (Kivrak *et al.*, 2013). Leptin was discovered in 1994 by Fiedman and his colleagues (Büyükokuroğlu *et al.*, 1999). It is a multipurpose hormone that regulates body weight and energy exchange (Steinberger *et al.*, 2003). Leptin levels in the bloodstream are increased by an increase in body fat, and this regulates the need for food and

lowers body weight. Conversely, a decrease in body fat causes leptin levels to drop and body weight to rise. In addition, it affects reproduction, sexual development, and metabolism (Hekimoğlu, 2006). It is a crucial hormone that controls the amount of body fat stored. This peptide stimulates energy consumption and reduces food intake to regulate energy metabolism at the hypothalamic level (Koerner *et al.*, 2005). Leptin levels are higher in lean people compared to obese people (Büyükokurolu *et al.*, 1999). they play a role in the regulation of insulin levels in the liver as well as the control of alimentation, thermogenesis, the immune system, reproduction, bone density, brain development, and hemodynamics, because its receptors are found in peripheral and brain tissues (Hekimoğlu, 2006). High level of circulated leptin was reported in obese people due to leptin resistance (Guerre-Millo, 2003). Insulin resistance is mostly influenced by hyperleptinemia and leptin resistance (El-Atat *et al.*, 2004). Visceral fat causes lipolysis in hyperlipidemia, which accumulates free fatty acids and prevents pancreatic β-cells from releasing insulin (El-Atat *et al.*, 2004).

Leptin inhibits lipogenesis while triggering the liver's -oxidation of fatty acids (Stern *et al.*, 2016). The liver contains many leptin receptors, and leptin treatment and brief fasting both increase leptin receptor expression in the liver (Trak-Smayra *et al.*, 2011). To investigate the effects of leptin signaling on the liver, Huynh and colleagues created a liver-specific leptin receptor knockout mouse that exhibits decreased circulating levels of apolipoprotein-B and increased levels of low-density lipoprotein (VLDL) triglycerides along with an increase in hepatic lipoprotein lipase activity (Huynh *et al.*, 2013). Adiponectin levels in the blood are decreased in humans and animals with type 2 diabetes who are obese and insulin-resistant (Scherer, 2006). Excessive fatty acid exposure causes adipocyte exhaustion, resulting in adiponectin

suppression and leptin stimulation. Leptin stimulates the activation of hepatic stellate cells (HSCs), which increases inflammation and fibrogenesis in the liver. However, adiponectin reduces inflammation by inhibiting the expression of nuclear factor-kappa B (NF-B) and TNF-α (Abe *et al*., 2021). In animal models of obesity, overexpression of adiponectin decreases the buildup of hepatic lipids caused by a high-fat diet, pointing to a probable causal role for this reduced adiponectin in insulin resistance of obesity (Kim *et al.*, 2007). Increased adiponectin production implies an activation of AMPK and increased protection against cell stress (Fazakerley *et al.*, 2018). Moreover, genetically removing adiponectin worsens hepatosteatosis in leptin-deficient obese mice (Holland *et al.*, 2013). A combination of the changes in leptin and adiponectin suggests an improved insulin sensitivity primarily in adipose tissue (Stern *et al.*, 2016).

Both delta-tocotrienol and alfa-tocopherol have shown to have similar positive effects on leptin and adiponectin levels in individuals with non-alcoholic fatty liver disease (NAFLD). They were randomized to receive either γ-tocopherol 268 mg twice daily for 48 weeks or δ-tocotrienol 300 mg (δT3 was naturally obtained (annatto beans), and each capsule contained 90% δ- and 10% γ-tocotrienol and was devoid of αTF). Additionally, δ-tocotrienol was more powerful than α tocopherol (Pervez *et al.*, 2022).

Heng *et al.*, (2015) reported The 16-week mixed tocotrienols supplementation 400 mg/ day exerted potential beneficial effects on cytokines such as leptin and adiponectin in adults with metabolic syndrome. Wong *et al.*, (2018) have reported treatment of natural tocotrienol derived from annatto (Bixa orellana) beans, including 84% δ-tocotrienol and 16% γ-tocotrienol, decreased leptin, elevated adiponectin, and restored the inflammatory response in mice with metabolic syndrome generated by a high-carbohydrate, high-fat diet. When δ-

tocotrienol and α-tocopherol were used to treat patients with non-alcoholic fatty liver disease (NAFLD), Pervez *et al*., (2020) found that tocotrienol supplementation had a statistically significant effect on hepatic steatosis markers by lowering leptin (δ-tocotrienol) and raising adiponectin concentrations when γ-tocotrienol and placebo were used.

Delta Tocotrienol had an effect on leptin protein concentration, which was considerably reduced in the T400 but not the T1600 group when compared to high fat fed mice, but there were no changes in serum anti-inflammatory adipokine adiponectin among any of the groups (Allen *et al.*, 2017).

Kok-Yong has determined the effects of annatto tocotrienols on serum adiponectin, leptin levels in male rats treatment supplemented orally with AnTT at 60 or 100 mg/kg (n = 8/group) After treatment of 12 weeks, with buserelin, AnTT do not influence adiponectin and leptin levels in male rats(Kok-Yong, 2019).

Irisin is myokine/adipokine, first described in 2012 by Boström *et al.* (Boström *et al.*, 2012), it is produced by the fibronectin type III domain containing 5 (FNDC5) gene and controlled by the peroxisome proliferator activated receptor-coactivator-1-alpha (PGC-1) (Mazur-Biay *et al.*, 2017; Varela-Rodriguez *et al.*, 2016). White adipose tissue (WAT) is regarded as the second most significant source of irisin after skeletal muscle (Perakakis *et al.*, 2017). In mice, FNDC5/irisin is released mostly by adipocytes of the subcutaneous adipose tissue (SAT) and in smaller amounts from adipocytes of the visceral adipose tissue (VAT)(Roca-Rivada *et al.*, 2013). Its most prominent effect is to drive brown-fat-like conversion of white adipose tissues, and therefore has been suggested to improve metabolic and glucose homeostasis (Boström *et al.*, 2012), Since its discovery, irisin has been

associated with favorable effects on metabolic illnesses, including obesity, type 2 diabetes mellitus (T2DM), lipid metabolism and cardiovascular disease (CVD), nonalcoholic fatty liver disease (NAFLD), polycystic ovarian syndrome (PCOS), and metabolic bone diseases(Polyzos *et al.*, 2018). Although skeletal muscle accounts for approximatively 72% of the total amount of irisin in the circulation (Boström *et al.*, 2012), several studies suggest that irisin can also be produced by the pancreatic islets (Aydin *et al.*, 2014), thus emerging as a new potential intra-islet hormone (Marrano *et al.*, 2021). Liu *et al.* suggest that irisin may have applications in the prevention and treatment of T2DM because of its protective effect on the secretion of pancreatic β cells (Liu *et al.*, 2017). Irisin's primary target is adipose tissue, and its effects vary based on the species (rodents, humans), type of adipocytes (premature or mature), and location/type of adipose tissue (SAT, VAT, BAT) (Polyzos *et al.*, 2018). It has been demonstrated that irisin functions as a crucial metabolic regulator and benefits problems associated with obesity (Eslampour *et al.*, 2019). Injecting FNDC5-expressing adenoviral particles intravenously or intraperitoneally with recombinant human irisin improves glucose metabolism in obese mice while having only a minimal effect on body weight (Zhang *et al.*, 2014). Circulating irisin has been shown to positively correlate with weight and body mass index (BMI) (Perakakis *et al.*, 2017). Even in phenotypes with extremely high BMI, this correlation is still favorable. Individuals with anorexia nervosa have circulation irisin levels that are 15% lower than those of normal weight people and 30% lower than those of severely obese people (Stengel *et al.*, 2013). Irisin levels have been found to correlate favorably with leptin (Palacios-González *et al.*, 2015) and negatively with adiponectin (Nigro *et al*., 2017). A direct connection between leptin and irisin is regarded to be implausible, because leptin treatment in people does not

change circulating irisin levels (Gavrieli *et al.*, 2016).in 136 obese patients who followed an eight-week hypocaloric diet (30% reduced energy expenditure) to lose weight and were reevaluated four or six months after treatment., weight loss reduces irisin levels, which are restored after getting back the lost weight (Crujeiras *et al.*, 2014).

The relation of using tocotrienol with irisin is still unknown; this subject is far away from the consideration of researchers and student. ButIrandoost *et al.* have demonstrated no effect of tocotrienol-rich fraction (TRF) contained α-tocotrienol (12%), β-tocotrienol (2%), γ-tocotrienol (19.3%) and δ-tocotrienol (5.5%) together with α- tocopherol (11.9%). With dose about 85/kg/day on irisin in obese Wistar rats for a period of 8-week: also, TRF consumption reversed the HFD-induced inflammation independent of irisin in the obese rats (Irandoost *et al.*, 2020).

2.11-Impaired role of obesity on Reproductive function:

Obesity can result in hypothalamic-pituitary-gonadal (HPG) axis dysfunction in both sexes (Tena-Sempere, 2013). Obese male may experience diminished libido, erectile dysfunction, subfertility, and, in rare cases, hypogonadism (Michalakis *et al.*, 2013). Although research though that the function of adipose tissue in several neuro-endocrine networks has advanced recently, the pathogenetic pathways that connect excessive fat accumulation to HPG dysfunction are still not completely understood. Moreover, increased sex steroid metabolism in adipose tissue depots might result in aberrant androgen and estrogen plasma levels, which may alter the reproductive axis in obesity (Fischer-Posovszky *et al.*, 2007). Researches have been focused on the effects of obesity on the reproductive health of women. It is also conceivable that the harmful effects of obesity on male fertility have been overestimated (Tsatsanis

et al., 2015). In fact, there were some evidence suggest that obesity can considerably harm male reproductive health, resulting in lower libido, erectile dysfunction, and subfertility/infertility (Tsatsanis *et al.*, 2015). Hormonal Changes of Hypothalamic-Pituitary-Gonadal (HPG) Axis Dysfunction in Male Patients with Obesity (Kyrou *et al.*, 2018). Zhong *et al.* focused on the correlation between sperm parameters and diabetes and obesity (Zhong *et al.*, 2021). There are several ways that obesity affects the quality of semen, The first is male endocrine dysfunction, which may be the primary factor reducing semen volume and total sperm concentration (Abbasihormozi *et al.*, 2023). Obese men often have lower testosterone levels than lean men and this may reduce semen volume and sperm concentration (Esmaeili *et al.*, 2022). The second is the seminiferous epithelium of the testicular tissue which is harmed by inflammatory substances, which in turn causes damage to the spermatogenesis process in obese males (Wang *et al.*, 2021). Finally, as oxidative stress increases, sperm structure and function may be harmed because obese men may have higher amounts of reactive oxygen species (ROS) that can damage sperm mitochondria and nuclei (Condorelli *et al.*, 2018). The elevated temperature caused by the significant suprapubic and scrotal fat deposits may also affect spermatogenesis (Ferramosca *et al.*, 2016). Dyslipidemia may also affect semen quality and fertility since research in both humans and animals have linked changes in lipid profiles to male infertility (Hagiuda *et al.*, 2014).

Rats treated with a high-fat diet (HFD) that suffer from oxidative stress-induced pathogenic alterations which impact sperm concentration and motility together with reduced mitochondrial respiration efficiency in sperm (Ferramosca *et al.*, 2016, 2017). Long-term HFD eating can damage testicular structure, increase intracellular ROS, and promote testicular germ cell death

using the mitochondrial intrinsic route (Ghosh & Mukherjee, 2018). Jing *et al.* demonstrated that mice fed with HDF had increased ROS levels and decreased mitochondrial function, also indicated that they match the symptoms emerge in human semen samples taken from individuals in the overweight/obese group, these findings have physiological significance (Jing *et al.*, 2023). It has been found that in obesity, hyperinsulinemia and hyperleptinemia may have a deleterious effect on sperm quality and male reproductive function (Leisegang *et al.*, 2014). The persistent pro-inflammatory state in humans may also be significantly influenced by leptin. According to Zhao *et al.* (2014), an increase in adipose cytokines may inhibit testosterone release, which could be the cause of the decreased sperm motility (Meeker *et al.*, 2007).

2.12-Effect of tocotrienol supplement on reproductive function:

Vitamin E administration has been shown to improve epididymal number and mobility of sperm (Mahanem *et al*., 2006; Sönmez *et al*., 2007), as well as restore testicular weight in stressed rats (Banudevi *et al*., 2004). Tocotrienols are plentiful in palm oil (palm oil tocotrienol-rich fraction), which contains 76% tocotrienol (α-, β-, γ-, δ-tocotrienol) and 24% tocopherol(α-tocopherol). significantly reduce the spermatoxic effects shown in spermatozoa of rats exposed to organophosphate insecticides fenitrothion by significantly raising sperm counts, motility, and viability and decreasing abnormal sperm morphology (Taib *et al.*, 2014). According to Jegede *et al.* 's study it was found that using red palm oil rich in tocotrienol reduced lead acetate-induced testicular injury in adult male Sprague-Dawley rats (Jegede *et al.*, 2015). Lee *et al.* (2016) showed a significant increase ($p<0.05$) in sperm motility, viability and count in groups administered high dose of feeding tocotrienols from palm oil for 6 weeks to male (1500 mg/kg tocotrienol) but

testes weights were not significantly affected by treatment of any sort. A study found that administering palm tocotrienol-rich fraction (TRF) to male rats treated with corticosterone (CORT) for 7 days increased their reproductive organ weight and testosterone levels. TRF, which contains approximately 60% tocotrienol, prevented testicular germ cell degeneration and Leydig cell loss after stress exposure (Abd Aziz *et al.,* 2019).

When females were mated with Corticosterone (CORT) plus palm tocotrienol-rich fraction (TRF) adult male rats, there were considerably more implantation locations, live pups birthed, and their birth weights than in the CORT group. As a result, TRF reduces the risk of fetal loss in females that mate with CORT + TRF-treated males (Abd Aziz *et al.,* 2019). Although these researches investigated the ameliorative effect of tocotrienol on sperm and fertility, but still this subject need more researches and studies and the research regard scarce.

2.13- Effect of tocotrienols on the histopathological effects of the liver, kidney, and testes

Liver the largest glandular organ of the body and the key organ of metabolism has a pivotal and immense task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents. Hence this organ is subjected to a variety of diseases and disorders. In the absence of reliable hepatoprotective drugs in the allopathic (modern) medicinal system and the wide range of hepatic disorders, dietary antioxidants play an important role in the management of liver disorders (Ahsan *et al*., 2014).

Several animal studies have investigated the effects of tocotrienols on liver histology. In a study on male mice were fed a high-fat diet (HF) with or without supplementation of δT3 (HF+δT3) at 400 mg/kg and 1600 mg/kg for

14 weeks, tocotrienol supplementation was found to improve liver histology by reducing liver damage and inflammation (Allen et al.,2017). Also study on rats found that δ-tocotrienol improved liver structure and function by reduction in organ inflammation (Wong *et al*., 2017). Additionally, a one study on male mice are fed a diet containing high fat (45%) and cholesterol (0.2%) along with sucrose drink (HFCS) showed that γ T3 supplementation is effective in attenuating liver damage by reducing hepatic steatosis, inflammation, and oxidative stress (Kim *et al.*, 2018). Another study conducted on rats found that supplementation with palm- or annatto-derived tocotrienol improved liver histology by reducing hepatocyte ballooning, inflammation, and fibrosis in a HCHF diet-fed rat model of non-alcoholic fatty liver disease (NAFLD) (Wong *et al*., 2020).

Overall, these animal studies suggest that tocotrienols have potential protective effects on liver histology by reducing liver damage, inflammation, and oxidative stress.

Gupta and Chopra studies found that pretreatment with tocotrienol (50 mg/kg/day) for 7 days before Fe-NTA administration in rats protects the kidneys from ferric nitrilotriacetate (Fe-NTA) toxicity, a well-established nephrotoxic agent, by significantly lowering serum creatinine and BUN levels, reducing lipid peroxidation, and restoring kidney tissue histology (Gupta & Chopra, 2009). Siddiqui *et al.* (2010) demonstrated that administration of TRF for 8 weeks improved the GFR in diabetic rats significantly, implicating its nephroprotective action, indicating an effective protection offered by TRF against the nephrotoxic effect in diabetic nephropathy.

palm oil tocotrienol-rich fraction (TRF) have been reported to cause testicular oxidative damage under various pathological conditions, such as

exposure to organophosphates (fenitrothion), which that it has potential to reduce oxidative stress (Taib *et al*., 2015).

2.14- Effect of tocotrienols on gene expression

Previous studies identified a number of genes every investigation to study changes in their expression to gain more knowledge about metabolic pathways, lipogenesis, or genes with roles in fighting inflammation in obese individuals. T3 from rice bran extracts (Candiracci *et al*., 2014), muscadine grape seed oil as tocotrienol rich fraction (Zhao *et al.,* 2015b), annatto oil (Allen *et al*., 2017), and purified γT3 (Kim *et al.,* 2016; Matsunaga *et al.*, 2012; Wang & Jiang, 2013; Zhao *et al*., 2015a) Pro-inflammatory mediators such TNF-α, granulocyte colony-stimulating factor, CCAAT-enhancer-binding proteins β, leptin hormone, IL-1β, IL-6, IL-8, iNOS, and MCP-1 have been shown to be suppressed. Furthermore, the annatto oil T3 combination increased IL-10 (anti-inflammatory cytokine) mRNA levels in the adipose tissue of obese C57BL/6J mice (Allen *et al*., 2017). Annatto T3 (Allen *et al*., 2017) and γT3 supplement (0.05%) (Zhao *et al*., 2015a) It also reduced macrophage infiltration in the adipose tissue of obese mice. In addition, Ahn *et al*. (2007) found that γT3 inhibits NF-κB activation and downstream gene expression, including TGFβ-activated kinase 1, receptor-interacting protein, tumor necrosis factor, phorbol myristate acetate, okadaic acid, lipopolysaccharide, cigarette smoke condensate, IL-1β, and epidermal growth factor. Moreover, electrophoretic mobility shift experiment demonstrated that γT3 is the most powerful NF-κB inhibitor relative to other T3 isoforms (Ahn *et al*., 2007). The mechanistic study discovered that γT3 inhibited NF-κB activation by creating the degradation of tumor necrosis factor receptor-associated factor 6 (positive regulator of NF-κB) (Kim *et al*., 2016) and

inhibiting nuclear factor-κB inhibitor type α kinase (IKK) signaling, Akt signaling, and nuclear factor-κB inhibitor type α (IκBα) damage (Matsunaga *et al*., 2012; Zhao *et al*., 2015a). Furthermore, γT3 inhibits caspase-1 activation and interleukin-1β release via suppressing the NLRP3 inflammasome and AMPK activation (Kim *et al*., 2016).

Muscadine Grape Seed Oil MGSO's high levels of γ-tocotrienol (40.7-68.9 mg/100 g oil) and α-tocotrienol (30.1-48.1 mg/100 g oil) reduced mRNA expression of PPARγ and CEBPα, as well as downstream targets of adipogenesis. TRF derived from MGSO had a stronger effect than MGSO itself due to removing the compounding adipogenic effects from fatty acids in the oils (Zhao *et al*., 2015b).

Treatment groups of δT3 oil isolated from annatto fruit had higher expression of genes involved in fatty acid oxidation (mRNA levels of WAT beta-oxidation markers namely carnitine palmitoyltransferase1A (CPT1A), carnitine palmitoyltransferase 2 (CPT2), and Forkhead box A2 (FOXA2)) and lower expression of genes involved in fatty acid synthesis Fatty acid synthase (FASN) and acetyl-CoA carboxylase-1 (ACACA) mRNA) (Allen *et al*., 2017).

Previous study showed that a high-fat diet suppressed the expression of the peroxisome proliferator-activated receptors (PPARs)α _γ and in the liver and retroperitoneal white adipose tissue r WAT, that is the major regulation of fat metabolism and adipogenesis, and that a tocotrienol-rich fraction from palm oil did not reverse the suppression (Cheng *et al*., 2017).

Annatto-extracted tocotrienols have suppressed liver mRNA levels of inflammation markers including IL-2, IL-23, IFN-γ, MCP-1,TNF-α, ITGAX and F4/80(Shen *et al*., 2018). Irandoost *et al*. evaluated Endoplasmic reticulum (ER) stress causes adipose tissue dysfunction and chronic

inflammation in obesity in White adipose tissue WAT, brown adipose tissue BAT, and hypothalamus by measuring expression of the expression of TNF-α, MCP1 and assessed Glucose-regulated protein-78 (GRP78) because of its role in protein folding is considered as a key regulator of ER stress GRP78 following calorie restriction diet alone and together with Royal jelly and tocotrienol-rich fraction (Irandoost *et al*., 2020). Mesri Alamdari and his colleagues studies the Effects of Royal jelly, tocotrienol-rich fraction, and combined supplementation on critical thermoregulatory gene expression and they stated that TRF did not show any significant effects on the expression mentioned in their study thermoregulation genes and the brown fat-like phenotype (Mesri Alamdari *et al*., 2020).

Chapter Three

Materials and Methods

Materials and Methods

3.1 Chemical Materials:

The chemical materials and reagents that were used in this study are listed in table 3.1:

Table 3.1 The chemicals used in this study and their sources.

Chemicals	Suppliers
Adiponectin elisa kit	BT-laboratory- China
Annatto tocotrienol	American River Nutrition Inc.USA
Eosin stain	Scharlau – Spain
Ethanol 100%	Scharlau – Spain
Formalin	BDH – England
FSH elisa kit	BT-laboratory- China
hematoxylin stain	Scharlau – Spain
Irsin elisa kit	BT-laboratory- China
Ketamine	Alfasan -Holland
Leptin elisa kit	BT-laboratory- China
LH elisa kit	BT-laboratory- China
Negrosin	Merk - Germany
Olive oil	Spanish
Paraffin wax	Scharlau – Spain
Physiological saline	Pioneer- Iraq
qPCR kits	Promega-USA
Sodium citrate	fluka - Switzerland
Testosterone elisa kit	BT-laboratory- China
Xylazine	VMD -Belgium
Xyline	Scharlau – Spain

3.2 The Instruments and Apparatus

The following laboratory instruments and apparatus were used in this study as shown in table 3.2:

Table 3.2 The laboratory Instruments and apparatus were used in this study

Instruments	Suppliers
Centrifuge	Hettich -Germany
cobas c 311	Genex - Germany
Curve scissor	Pakistan
Digital camera	Sony, Japan
digital weighing scale	Turkey
Electric grinder	Turkey
Electronic balance	Mettler, Switzerland
ELISA system	Bioactive, Germany
Filter papers	Whatman No. 541, UK
Haemocytometer	Germany
hematological analysis	Genex - Germany
Hot plate	Alssco - India
Light microscope	Japan (Olympus)
Micropipette (10-100 µl)	Human, Germany
Oven	Binder -Germany
Real-Time PCR System	Thermo Fisher Scientific - USA
Rotary Microtome	American Optical/ UAS
Water bath	FANEM/Sao-Paulo/Brazil

3.3 Animals of the Study

The current investigation was conducted out at the College of Veterinary Medicine, University of Basrah, during the period extended from 1/2/2022 to 1/10/2022. Forty-four male rats weighting (80±25 gm) aged (2 months) and sixteen healthy adult fertility female rats weighting (200±25 gm) were used for the present study. The animals had been kept in the animal home for acclimation one week before the start of the experiments and maintained under optimum conditions (24±2°C) and

(12/12 hours light/dark) cycle throughout the study. The food and drinking water interduce *ad liabitum*, along the experimental period.

3.4. The Preparation of the tocotrienol

Annatto tocotrienol 70% concentrate, which contained 90% delta-tocotrienol and10% gammaa-tocotrienol produced by American River Nutrition Inc. (Hadley, MA, USA). The prepare a dose of 60 mg per kg according to Chin *et al.*, (2016) need to weight 85.71 mg of tocotrienol and diluted with olive oil to 1 ml.

The prepared substance was kept in a dark container, protected from light to use by drenched orally for rats of the study.

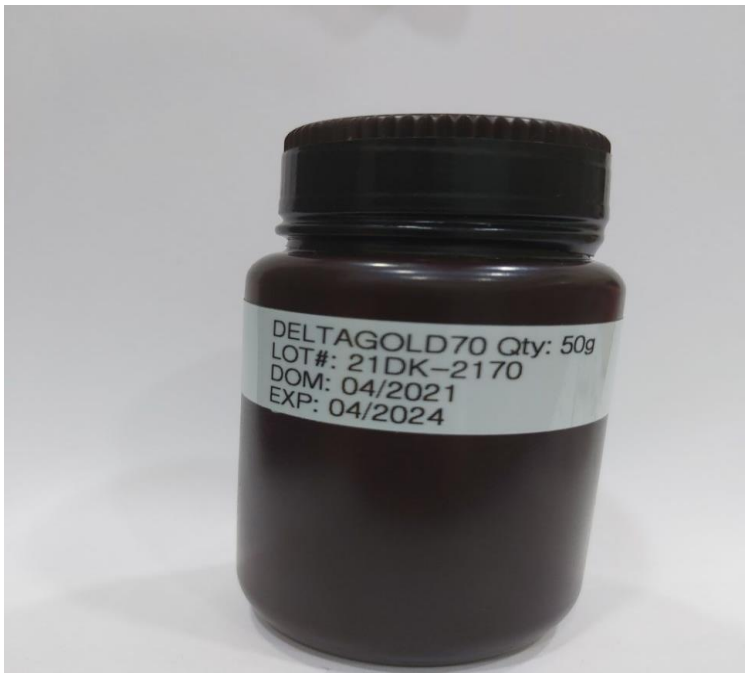


Figure 3.1 Tocotrienol

3.5 Obesity inducement:

The inducement of obesity in laboratory rats implemented by using Diet Induced Obesity (DIO) in rodents (HF 60 % fat) D14031902 while for control group interduce (LF 10 % fat) D14031901 were formulated according to the Research Diet INC. (2014).

Table 3.3 Diet formula were used in this study

Product	D14031901		D14031902	
%	gm	kcal	gm	Kcal
Protein	19	20	26	20
Carbohydrate	67	70	26	20
Fat	4	10	35	60
Total		100		100
kcal/gm	3.8		5.2	
Ingredient	gm	kcal	gm	Kcal
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	506.2	2025	0	0
Maltodextrin	125	500	125	500
Sucrose	68.8	275	68.8	275
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Beef fat	20	180	245	2205
Mineral&Vitamin Mix	20	40	20	40
Dicalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate	16.5	0	16.5	0
Choline Bitartrate	2	0	2	0
Total	1055.00	4057	773.80	4057

3.6 Diet preparation and ingredient mixing

The preparation of diet began by cleaning beef tallow and connective tissue removed. After that beef tallow cut into small pieces and melted by spreading it on a hot plate at 75°C. Then mixing the required dry quantities with each other well by food processor and adding the wet ingredients and mixing for 2 minutes at low speed, after those meals were shaped into a cylindrical form by rolling them manually. Diets were stored in plastic container at 4C°with tightly closing.

Figure (3.2) Diet preparation and form

3.7 Experimental design

Forty-four male rats aged around 8 weeks and weighting about 80± 25g and 16 female rats were used in all experiments. included 14 rats that represented the control group, and obesity was induced for a period of 12 weeks for 30 male rats, Figure (3-3) study as follows.

3.7.1 First experiment: - (inducement of obesity)

Eighteen male rats were used in this experiment and randomly divided into 3 groups as follows:

1. Group 1 (Control group): six rats were fed a low-fat diet and drench with olive oil 1 ml/ kg B.W. by gavage for 12 weeks.

2. Group 2 (high fat diet group HFD): six rats were fed a high-fat diet and drench with an oral dose of olive oil 1 ml/kg by gavage for 12 weeks.

3. Group 3 (protective group HFDT): six rats were fed a high-fat diet and drench with an oral dose of 60 mg/kg according to (Chin *et al*., 2016) . of tocotrienol supplement dissolved in olive oil (1ml/kg) by gavage for 12 weeks.

3.7.2 Second experiment: -

In this experiment, obesity was induced as in the first experiment, for 12 male rats along period of 12 weeks, with a control group of 6 male rats. At the end of the twelve-week period, the mean weight of high-fat diet provided rats increased considerably compared to normal chow diet consuming rats, demonstrating that the HFD-induced obesity model was achieved according to (Mesri Alamdari *et al*.,2020). After that, an 8-week therapeutic experiment began as follows, Figure (3-3) study.

1. Group 1 (Control group): It includes six rats were fed a low-fat diet, and drench with olive oil 1 ml/ kg by gavage for 8 weeks.
2. Group 2 (obesity group): It includes six rats were fed a low-fat diet and drench with an oral dose of olive oil 1 ml/kg by gavage for 8 weeks.
3. Group 3 (therapeutic group): includes six rats were fed a low-fat diet and give an oral dose of tocotrienol supplement (60 mg/kg) dissolved in olive oil (1 ml/kg) for 8 weeks.

3.7.3Third experiment (Fertility experiment)

3.7.3.1 study design

sixteen female and eight rats used in this experiment included rats were separated into four groups, two males and four females. Male rats are mating with the females as in figure(3,3) as follows:

1. Control group: Untreated male rats mated with untreated females
2. Group two (inducement obesity group): male rat fed a high-fat diet mated with untreated females.
3. Group Three (protective group): male rat fed a high-fat diet for 12 weeks, in addition to an oral dose of 60 mg/kg BW of tocotrienol supplement mated with untreated females.

4. Group Four (therapeutic group): male rat fed a high-fat diet for 12 weeks, ,then give an oral dose with 60 mg/kg BW of tocotrienol supplement mated with untreated females.

3.7.3.2 parameters of third study:

The fertility rate (number of pregnant females/number of females in each group multiplied by 100), mean birth number (number of births/number of females in each group), and average birth weights were reported.

3.8. Samples collection

3.8.1Collection of Blood Sample

After the treatment phase, the rats were fasted for 12–14 hours to conclude the experiment. They were anesthetized with 1.9% breathed diethyl ether, about 0.08 mL/L of container capacity (Aledani *et al.*, 2020). Blood samples (10 mL) were taken from each rat through the heart (cardiac puncture). About 8 ml of blood samples were deposited into the gel tube and then centrifuged at 3000 rpm for 15 minutes for serum separation. These serum samples were divided into four parts and stored in polyethylene Eppendorf tubes at -20 ^{o}C, which were used for hormonal and biochemical evaluation. The remaining 2 ml of blood was placed in the tube with the anticoagulant for hematological examination (RBC, Hb, MCV, MCH, MCHC, PCV, total and differential WBC, and PLT).

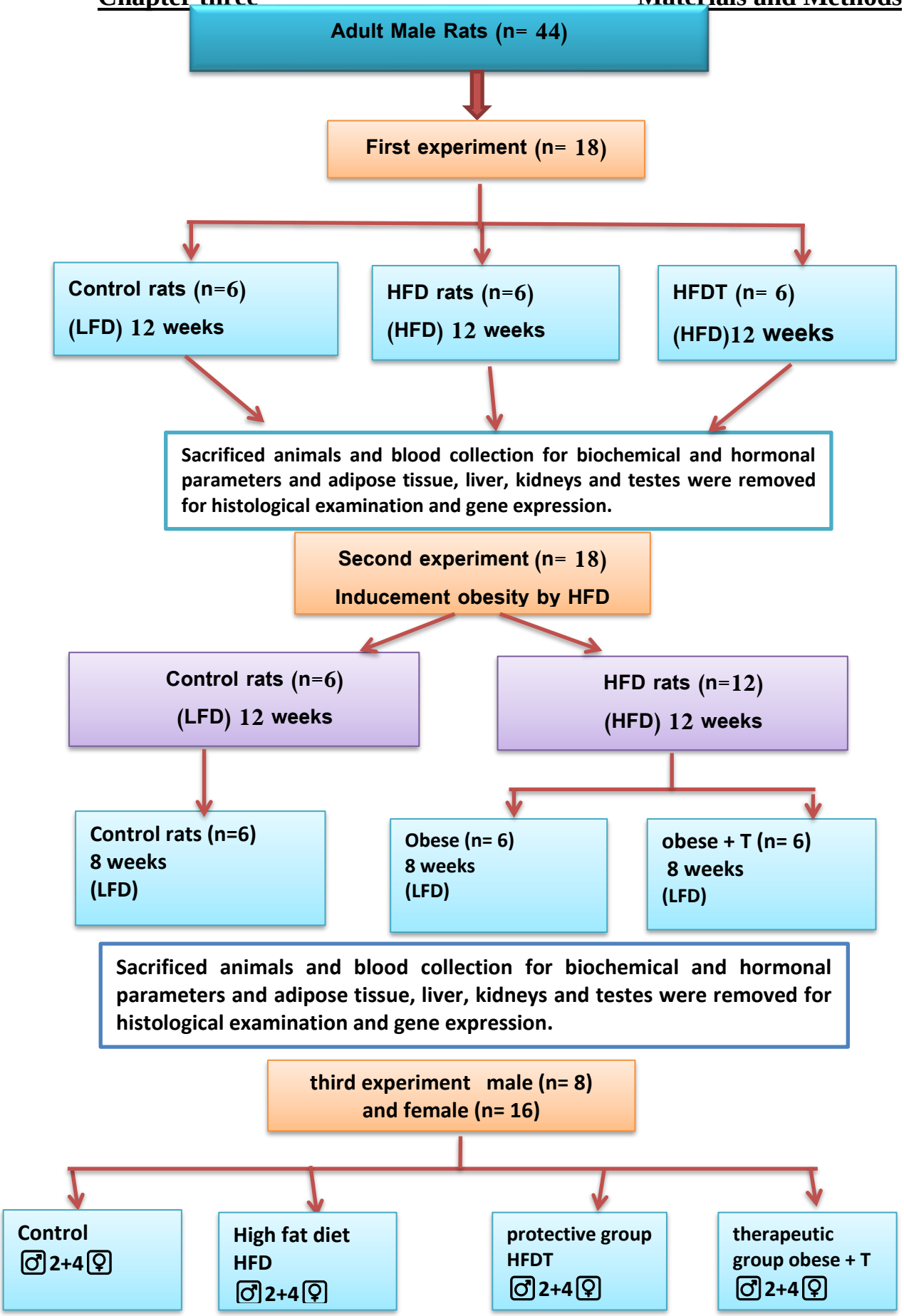


Figure (3-3) study

3.8.2 Body length and organs separation

Body length (nose - anus) and organ weights (liver, testes and kidneys) were recorded.

Retroperitoneal adipose tissue and peri-kidney fat, kidney, testes and liver were separated from each animal. Each tissue weighted by A digital weighting scale and stored at −20 °C.

3.9 Animals body weight, weight gain and relative organ weight of protective and therapeutic experiments:

body weight was weekly recorded to keep track of the weights during the experimental period using a digital scale balance. while weight gain of experimental rats was estimated according to the following mathematical equation:

Body weight gain (weekly) = final weight - initial weight (gm)

Each animal that was slaughtered had its organs (liver, kidneys, and testes) taken right away and weighed using an electronic scale; the weights were then standardized by body weight as the relative weights, as follows:

3.10 Body mass, adiposity and lee obesity indexes of protective and therapeutic experiments:

Body mass index was calculated according to Novelli *et al*., (2007) by using the following equation:

Body mass index = Body weight (gm) ÷ Length^2(cm).

Adiposity index was calculated according to Tayler & Phillips, (1996) depend on the following equation:

AI = [Weight of fat pads (gm) ÷ body weight (gm)] × 100

Obesity was determined by the Lee index at the end of experiment according to Bernardis ,(1970)who measured the obesity by using the following equation:

LOI= $\sqrt[3]{}$body weight (gm) ÷ naso-anal length (cm)

3.11 Food consumption of protective and therapeutic experiments:

All experimental rats were fed on standard ration as mentioned in table (3-3). Feed consumption was determined based on the feed residue of the ration during 24 hours of administration, by using the following mathematical equation:

Food consumption(g) = feed introduced (g) - feed residue (g)

3.12 Biochemical Parameters of protective and therapeutic experiments:

Frozen serum stored previously under -20ºc in Eppendorf tube for serological examination was thawed and prepared for biochemical analysis. The examination of creatinine, urea, triglycerides, cholesterol, HDL-cholesterol, LDL-cholesterol, aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), C reactive protein, total protein and glucose levels were measured by using cobas c 311 analyzers. It is a standalone system that was used to obtain clinical chemistry profile This analyzer has the capacity for ion-selective electrode (ISE) determination of biochemical parameters in serum, plasma, and urine.

3.13. Hormonal assay:

3.13.1. Leptin assay

The leptin hormone concentration was measured using RatLeptin Elisa kit BT-Laboratory/ China (Cat. No E0561Ra).

Principles of assay: An enzymne-linked immaunosorbent assay (ELISA) is what this kit is for. Rat LEP antibody has been pre-coated on the plate. After being introduced, the sample's LEP binds to the antibodies coated on the wells. After that, LEP in the sample is bound by the addition of biotinylated Rat LEP Antibody. Next, the biotinylated LEP antibody binds to streptavidin-HRP. During a washing phase, unbound streptavidin-HRP is

removed following incubation. After adding the substrate solution, the color changes in direct proportion to the concentration of Rat LEP. The addition of an acidic stop solution ends the process, and the absorbance is measured at 450 nm. (Appendix 1)

3.13.2 Insulin assay

The plasma rat insulin was assessed using the rat Insulin Elisa kit BT-Laboratory/China (Cat.No E0707Ra).The kit employs an ELISA to detect Rat INS antibodies in a sample. It contains INS, biotinylated INS, and streptavidin-HRP. The reaction is ended with an acidic stop solution, and absorbance is measured at 450 nm. (Appendix 2)

3.13.3 Adiponectin Elisa Test:

The plasma rat adiponectin level was determined using the rat adiponectin Elisa kit from bt-laboratory. Cat. No. E0758Ra.

Principles of the assay: This ELISA kit involves a pre-coated plate coated with Rat ADP antibody, followed by adding ADP from the sample, incubating with biotinylated Rat ADP Antibody, and adding Streptavidin-HRP. After washing, unbound Streptavidin-HRP is removed. Substrate solution is added, and color appears based on Rat ADP quantity. The addition of an acidic stop solution ends the process, and the absorbance is measured at 450 nm.(Appendix 3)

3.13.4 Irisin Elisa Test:

Plasma rat irisin was quantified using a rat irisin ELISA kit from BT Laboratory. Cat. No. E6281Ra.

principles: This ELISA kit use a pre-coated plate coated with Rat Irisin antibody. Irisin is introduced to the sample, which binds to the antibodies before being bound by biotinylated Rat Irisin Antibody. Streptavidin HRP binds to biotinylated Irisin antibodies. Following incubation, unbound Streptavidin HRP is washed away. The substrate solution is added, and the color develops in accordance to the quantity of Rat Irisin. The reaction is

terminated with an acidic stop solution, and the absorbance is measured at 450 nm.

3.13.5 FSH Elisa Test:

The plasma rat Follicle-stimulating Hormone was tested using the rat FSH ELISA Kit (bt-laboratory Cat.No: EA0015Ra).

The principles of the assay: This kit is an ELISA that requires transferring samples to a pre-coated plate, adding biotinylated antigen, and incubation with the capture antibody. Unbound antigen is washed away, and avidin-HRP is introduced. TMB Substrate is added, and color emerges. The reaction is terminated with an acidic stop solution, and the color shifts to yellow at 450 nm. The color intensity is inversely proportional to the FSH content in the sample. The FSH concentration is calculated by comparing the O.D. of the samples to the standard curve. (Appendix 5)

3.13.6 LH Elisa Test:

The plasma rat luteinizing hormone was tested using a rat LH ELISA Kit from bt-laboratory, Cat. No. EA0013Ra.

The principles of the assay: This kit is an ELISA that requires placing samples on a pre-coated plate, adding biotinylated antigen, and incubation with the capture antibody. The unbound antigen is rinsed away, then avidin-HRP is added. TMB Substrate is introduced, and color appears. The process is halted using an acidic stop solution, and the color shifts to yellow at 450 nm. The color intensity varies inversely with the concentration of LH in the sample. The concentration of LH is measured by comparing the samples' optical density to the standard curve. (Appendix 6)

3.13.7 Testosterone Elisa Test:

Using a rat testosterone ELISA kit from BT Laboratory (Cat. No. EA0023Ra), the amount of testosterone in the plasma was quantified.

The principles of the assay: With this ELISA kit, samples are placed on a plate that has already been coated, biotinylated antigen is added, and the capture antibody is then incubated. Avidin-HRP is added after the unbound

antigen has been removed. When TMB Substrate is introduced, color starts to emerge. When the acidic stop solution is used to halt the process, the color shifts to yellow at 450 nm. The amount of testosterone present in the sample is negatively correlated with the color's intensity. By comparing the O.D. of the samples to the standard curve, the concentration of testosterone is ascertained.(Appendix 7)

3.14 Epididymal sperm analysis:

Immediately following scarification, each animal's caudal epididymis was removed and placed in a sterile glass petri dish with 5 milliliters of warm normal saline (0.9% NaCl). In order to analyze the following features of epididymal sperm, the epididymal tissue is finely chopped using microsurgical scissors and then placed in a water bath at 37°C for five minutes. This allows the spermatozoa to swim out to the media.

3.14.1. Epididymal sperm concentration:

The sperm count was determined using the technique of Evans & Maxwell, (1987).by using Neubauer hemocytometer chamber which use for RBC and WBC count .

1.The epididymis was placed in a Petri dish with 5 milliliters of 0.9% normal saline.

2. A sharp scalpel was used to cut the epididymis into six to ten pieces.

3. A clean piece of gauze was used to filter the suspension that came from the previous stage and put it into a test tube.

4-A single drop of the filtrate was applied to the Neubauer chamber, which had been covered with a cover slide earlier.

5. The sperms on the five squares that are used to count the RBCS with the 40 X objective lens.

6. The number of sperm was determined in one millimeter cubic.

Sperms/cmm = n X 10000 where n is the number of sperm in each of the five squares.

3.14.2 Sperm motility percentage:

Using the graduation basis proposed by Chemineau *et al.* (1991), the motility of each epididymal sperms was assessed as follows:

1. A diluted epididymal sperm drop was placed on a clean, warm slide at 37°C, and the slide was covered with a cover slide.
2. A 40X power microscope was used to analyze the sperm.

The percentage is determined by measuring the strength and speed of the forward-moving sperm as they advance.

3.14.3 Sperm abnormality:

Using 200 sperms and a light microscope with a 100X power, the aberrant spermatozoa percentage was counted on the same slide that was used to assess the viability of epididymal sperm (Evans & Maxwell, 1987).

1. A heated, spotless slide was covered with diluted semen.
2. A glass rod was used to properly mix the semen after a drop of warm eosin-negrosin dye was applied.

A clean slide was placed angularly on top of the semen slide and pulled horizontally to create a smear.

3. We let the slide air dry.
4. The slide was inspected at 40 X power using a light microscope. The color of dead sperm was red, whereas the color of live sperm was white.

The components of the Eosin-Negrosin stain are

Eozin	1.67 gm
Negrosin	10 gm
Sodiium citrate	2.9 gm
And diistal water	100 ml

3.15 The histological preparation:

The animals were slaughtered at the conclusion of the trial, and the organs—the kidney, liver, and testes—were carefully removed, washed, and preserved in 10% buffered formalin for a full day.

After utilizing a graded series of ethanol to dehydrate the specimens, they are cleaned in two changes of xylene and then embedded in paraffin wax. A rotary microtome was used to cut the section's thickness of 5 µm. The sections were then placed on clean slides for histological inspection after being stained with hematoxylin and eosin (H&E), and the tissue structure was assessed under the light microscope (Mescher, 2010).

3.16 Molecular procedure

3.16.1 Samples:

RNA Stabilization Solution: RNAlater solution had been used to preserve tissue samples. RNAlater is an aqueous tissue storage solution that quickly enters tissues, stabilizing and protecting RNA in fresh specimens. Samples in the RNAlater solution can be held for a prolonged length of time to prevent RNA degradation. Tissue samples were placed in containers containing 5–10 volumes of RNAlater solution and stored at 4°C overnight (to allow the solution to thoroughly penetrate the tissue), and on the next day, the samples were moved to a -20° freezer until RNA extraction.

3.16.2 Primers

The following primers were used in Real-time PCR experiment

Table 3-4 Primers:

Cpt1b	**CTCCCGACAAGGTATGGCTC** **GCTTGGGCAGTGATGTTTGG**
IL-1β	**GGGATGATGACGACCTGCT** **CCACTTGTTGGCTTATGTTCTG**
B-actin-F **B-actin-R**	**GTATGGGTCAGAAGGACTCC** **GTTCAATGGGGTACTTCAGG**

3.16.3Tissue homogenization and RNA extraction

The RNA isolation was done using the GoScript™ SV Total Isolation System kit (Promega).

3.16.4 Preparation of Lysates from Small Tissue Samples

1. All studies utilized RNase-free pipettes and gloves to prevent RNase contamination.
2. The frozen samples were washed from the RNALATER solution with chilled RNAse free water.
3. Tubes containing the RNA Lysis Buffer were weighted and recorded for their weight.
4. We cut the tissue into small pieces with a sterile razor blade. Tissue samples were immersed the tissue with liquid nitrogen and ground using a mortar and pestle. Ground tissue powders were immediately transferred to a tube containing RNA Lysis Buffer. Samples were mixed thoroughly by inverting tubes a few times.
5. We calculated the tissue mass by subtracting the weight obtained in Step 2.
6. In general, we maintained the ratio of tissue mass to RNA Lysis Buffer to approximately 30 mg/175µl.
7. 350µl of RNA Dilution Buffer were added to 175µl of the lysate and mixed by inverting 3–4 times and centrifuged for 10 minutes at 12,000–14,000.
8. Add 200 µl of 95% ethanol to the cleaned lysate and stir 3-4 times using a pipette.
9. The mixture was transported to the spin column assembly and spun down for one minute.
10. Following, the samples underwent washing with washing buffer, DNAse treatment, and a final elution step using RNAse free water.

3.16.5 RNA concentration determination:

Using Nonodrop one, the concentration of RNA was measured for each sample. The concentration of all samples was diluted and adjusted to 100n/µl.

3.16.6 cDNA synthesis

1. We mixed the primers and total RNA to a **5µl** final volume as listed in the following table**.**

Table 3-5 cDNA synthesis:

Component
RNA (300 ng total) 3 µl
Random Primer (0.5µg/reaction) 1µl /reaction
Nuclease-Free Water 1µl
Final volume 5µl

2. The tubes were put into a warmed 70°C heat block for 5 minutes followed by chilling on ice and were kept on ice until the reverse transcription reaction mix is added.

3. We prepared the opposite transcriptase A combination of the GoScript™ Reverse Transcription System according to the concentration of each sample to a final volume of 15µl in a sterile microcentrifuge and then we kept it on of the ice before distributing into reaction tubes.

4. We combined 5µl of RNA and primer mix prepared in step one to the 15 µl reverse transcriptase mix prepared in step three to achieve Use a final reaction measurement of 20µl for tube.

Anneal: 25°C, for 5 minutes. First-strand synthesis reaction: 42°C for up to one hour.Inactivation: 70°C for 1 minute

3.16.7 QPCR reaction

The reaction mix was prepared by combining, Nuclease-Free Water and primers in the order listed.

Table 3-6 **QPCR reaction**

Component	**Volume**	**Final Concentration**
GoTaq® qPCR Master Mix (2X)	10µl	10µl 1X
Forward Primer (20X)	1µl	0.5 µM
Reverse Primer (20X)	1µl	0.5 µM
Supplemental CXR Reference Dye	0.2µl	300 nM
300nM Nuclease-Free Water	to a final volume of 16µl	
Final volume	**15 µl**	

3.16.8 Real Time qRT-PCR analysis

3.16.8. 1. ΔCT

To calculate the ratio of gene expression we utilized the values of Ct to calculate the relative gene expression according to the following equation: (Livak and Schmittgen, 2001).

ΔCT (test) = CT gene of interest (target, test) – CT internal control

3.16.8.2. ΔΔ CT

To compare the transcript levels between different samples the $2^{-\Delta\Delta Ct}$.

By calculating Calculate the difference in cycle threshold (Ct) values between the internal control gene and the gene of interest using the formula below:ΔCT (test) = CT gene of interest (test) – CT internal control

ΔCT (calibrator) = CT gene of interest (calibrator) – CT internal control. The calibrator was selected from the set of control samples.

The ΔCT of the test samples was normalized to the ΔCT of the calibrator:

ΔΔ CT was calculated according to the following equation:

ΔΔ CT=ΔCT (test)- ΔCT (calibrator)

Finally, the expression ratio was calculated according to the formula

$2^{-\Delta\Delta Ct}$ = Normalized expression ratio (Yang *et al*.,2014)

3.17 Statistical analysis

The data from the present three investigations were analyzed with univalent analysis of variance (ANOVA) in the computerized SPSS (Statistical Packages for the Social Sciences) V.23 program. $P<0.05$ was considered statistically significant. The data was reported as mean ± standard error. To compare groups, the least significant difference (LSD) test was used.

Chapter Four

Results

4- Results

4.1-Effect of Tocotrienols supplement on body weight of the male laboratory rats in protective experiment.

The effect of tocotrienols supplement on body weight in protective experiment is shown in table (4-1). It is normal for body weight results to appear insignificant during the first two weeks of the study. After that, there was significant increase ($P \leq 0.05$) in body weight of laboratory rats fed on HFD and HFDT when compared with body weight of laboratory rats of control group. However, the rats fed on HFDT showed significant decrease ($P \leq 0.05$) in their body weight after the fifth week compared with rats fed HFD, but still significantly higher ($P \leq 0.05$) than the body weight of rats in control group along the twelfth week of the experiment. ($P \leq 0.05$).

Table (4-1) Effect of Tocotrienols supplement on body weight (g) of male laboratory rats in protective experiment. (Mean ± SE)

Groups	Control	HFD	HFDT	Sign.
Week0	84.74±3.88	82.92±8.28	84.74±3.60	N.S
Week1	87.78±3.88	92.30±8.11	89.34±3.65	N.S
Week 2	93.94±3.52 b	106.65±8.26 ab	113.03±1.83a	*
Week3	99.27±3.06 b	117.79±8.39 ab	132.97±2.58 a	*
Week4	108.04±3.05 c	132.22±8.19 b	161.13±3.96 a	*
Week5	117.30±3.62 b	173.26±6.71 a	167.60±3.48 a	*
Week6	140.78±3.14 c	226.70±7.53 a	173.63±3.01b	*
Week7	146.87±3.05 c	231.05±7.47 a	179.29±3.04 b	*
Week8	156.70±2.70 c	238.09±7.47 a	185.38±2.40 b	*
Week9	157.20±2.65 c	242.17±7.30 a	193.72±4.22 b	*
Week10	159.10±2.56 c	259.56±7.63 a	208.24±6.31 b	*
Week11	160.24±2.70 c	263.92±7.63 a	218.98±5.50 b	*
Week 12	161.60±2.63 c	277.83±7.47 a	232.26 ±6.39 b	*

Small letter referred horizontally to significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet, HFDT: high fat diet plus tocotrienols.

4.2-Effect of tocotrienols supplement on body weight of the male laboratory rats in therapeutic experiment.

As illustrated in table 4–2, there was no significant difference (P≤0.05) between the therapeutic group and the obesity group along the eight weeks of the experiment period, while the two treatments (Obesity and obesity +tocotrienol) recorded significant increase (P≤0.05) in body weight of their rats comparing to body weight of control rats along the eight weeks of the experimental period.

Table (4-2) Effect of tocotrienols supplement on Body weight of male rats in therapeutic experiment. (Mean ± SE)

Groups	Control	Obese	Obese plus tocotrienol	Signif
Week1	171.6350 ± 9.03 b	283.9767 ± 7.96566 a	282.2683 ±11.52961 a	*
Week2	175.1333 ±8.98 b	289.7083±7.90702 a	288.0467±11.04827 a	*
Week3	176.6667± 9.27155 b	291.5517±7.81200 a	291.1417±11.05448 a	*
Week4	180.8883±9.47734 b	297.4317±7.89940 a	296.2350±10.88358 a	*
Week5	186.2167± 9.72685 b	302.8367±7.77500 a	301.5417±10.65095 a	*
Week6	193.2983±9.74651 b	310.3967±7.93780 a	308.0233±10.22584 a	*
Week7	199.7200±9.49856 b	316.9700±8.16467 a	311.6800±9.75794 a	*
Week8	205.6217±9.38023 b	323.0667±7.87030 a	316.2533±9.53987 a	*

Small letters referred to significant difference at (P≤0.05). N.S.=non significant. * = significant.

4.3-Effect of tocotrienols supplement on body weight gain of male rats in protective experiment.

The results of body weight gain in the protective experiment are presented in table 4–3. It shows that body weight gain fluctuated significantly (P≤0.05) among the experimental groups. In the first fourth weeks of the experiment (P≤0.05), the rats of HFDT group gained weight higher significantly (P≤0.05) than the rats of HFD and control groups. Nevertheless, the increase in body weight gain of rats significantly changed (P≤0.05) to became higher in HFD group when compared with

HFDT and control groups extending this increasing for the seventh week of the study. After that, the results of body weight gain of rats for all studied groups appeared closely in their values to each other although there was signification (P≤0.05) among groups. In general, the body weight gain of rats fed HFD and HFDT showed significant elevation (P≤0.05) in their values when compared with body weight gain of rats fed standard ration in control group.

Table(4-3) Effect of tocotrienols supplement on Body weight gain of male rats in protective experiment . (Mean ± SE)

Groups	Control	HFD	HFDT	Signi.
Week 1	3.04±0.23 c	9.38±0.33 a	4.59±0.30 b	*
Week 2	6.15±0.84 c	14.34±0.58 b	23.69±4.00 a	*
Week3	5.33±0.77 c	11.14±0.56 b	19.94±2.54 a	*
Week4	8.76±0.60 b	14.42±0.42b	28.15±3.66 a	*
Week5	9.26±0.81 b	41.04±8.85 a	6.47±1.13 b	*
Week6	23.48±1.13 b	53.43±10.46 a	6.02±0.60 b	*
Week7	6.08±0.35 a	4.35±0.32 b	5.6533±0.51 a	*
Week8	9.83±0.87 a	7.04±0.11 ab	6.09±1.72 b	*
Week9	0.49±0.14 b	4.08±0.30 ab	8.34±3.57 a	*
Week10	1.90±0.30 b	17.39±0.55 a	14.51±2.85 a	*
Week11	1.13±0.22	4.36±0.12	10.74±7.49	N.S
Week 12	1.36±0.24 b	13.90±0.17 a	13.28±1.13 a	*

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.4 -Effect of tocotrienols supplement on body weight gain of male rats in therapeutic experiment.

Table (4-4) shows the effect of tocotrienol on body weight gain in a therapeutic experiment. The increase in body weight gained in the first week was significant (P≤0.05) between the therapeutic group and the obese group compared to the control group at P≤0.05. But the results of body weight gain of rats for all studied groups appeared non- significant (P≤0.05) in their values along the experiment period after the second week of the study.

Table (4-4) Effect of tocotrienols supplement on Body weight gain of male rats in therapeutic experiment. (Mean ± SE)

Groups	Control	Obese	Therapeutic	Signi.
Week1	3.49±0.20 b	5.73±0.10 a	5.77±0.59 a	*
Week2	1.53±0.43	1.84±0.16	3.09±1.46	N.S
Week3	4.22±0.45	5.88±0.36	5.09±1.13	N.S
Week4	5.32±0.66	5.40±0.26	5.30±1.15	N.S
Week5	7.08±0.44	7.56±0.33	6.48±1.72	N.S
Week6	6.42±1.37	6.57±0.87	3.65±1.15	N.S
Week7	5.90±1.41	6.09±0.44	4.57±0.33	N.S

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant.

4.5- Effect of tocotrienols supplement on feed consumption of male rats in protective experiment

The results in Table (4-5) showed food consumption for male rats during two weeks along the study period in the protective experiment. The results were recorded non- significant (P≤ 0.05) difference among variable

feed content groups. While the last two weeks of the experiment referred to significant decrease (P≤ 0.05) in feed consumption of HFDT male rats when compare with feed consumption of laboratory rats of control and HFD groups.

Table (4-5) Effect of tocotrienols supplement on feed consumption (gm/rat/day) of male rats in protective experiment. (Mean ± SE)

Groups	Control gm/rat/day	HFD gm/rat/day	HFDT gm/rat/day	Sign.
2Weeks	14.57±0.64	11.72 ±1.49	11.68 ±1.04	N.S
4Weeks	15.21±0.73	15.54±1.07	14.55±0.62	N.S
6Weeks	15.11±0.23	14.15±0.75	13.67±0.59	N.S
8Weeks	14.44±0.53	12.72±0.639	12.78±0.87	N.S
10Weeks	17.03±0.91	16.67±0.86	16.72±0.491	N.S
12Weeks	14.23±0.61 a	12.57±0.56 ab	12.12±0.63 b	*

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.6-Effect of tocotrienols supplement on feed consumption of male rats in therapeutic experiment

Food consumption in the therapeutic experiment was calculated every two weeks as shown in Table (4-6). The results showed a significant (P≤ 0.05) increase in food consumption of rats feed on obese and tocotrienol groups compared with rats feed low fat diet in control group through the period between second and fourth week of the experiment. But the period between fourth and sixth week of the experiment there was significant reduce in food consumption for the therapeutic rats group compared with the obese group. On the other hand, in the eighth week, food consumption decreased significantly in the obese group compared with the other studied groups

Table (4-6) Effect of tocotrienols supplement on feed consumption (gm/rat/day) of male rats in therapeutic experiment. (Mean ± SE)

Groups	Control gm/rat/day	Obese gm/rat/day	Therapeutic group gm/rat/day	Sign.
2Weeks	14.46 ± 0.31	15.59 ±0.98	14.94 ±0.92	N.S
4Weeks	14.33 ±0.39 b	18.92 ±1.30 a	18.80 ±1.60 a	*
6Weeks	17.31 ±0.81 ab	18.15 ±0.39 a	16.07 ±0.57 b	*
8Weeks	15.08 ±0.76 a	12.97 ±0.16 b	15.85 ±0.86 a	*

small letter represent significant difference at (P≤0.05). N.S.=non significant. * = significant.

4.7-Effect of tocotrienols supplement on BMI , Lee index and AI of male rats in protective and therapeutic experiments

The measurement of BMI, Lee index and AI recorded significant elevation (P≤ 0.05) in male rats fed on HFD when compared with the other two groups of protective experiment table (4-7). However, the supplement of tocotrienol for rats fed on HFDT enhance the values of BMI, Lee index and AI to appear less significantly (P≤ 0.05) when compared with HFD groups, although it remains significantly higher (P≤ 0.05) than control group.

In contrast, therapeutic experiment recorded the same manner of protective experiment, that recorded significant elevation (P≤ 0.05) for the HFD group, while addition of tocotrienol reduced the BMI, Lee index and AI values but still higher than (P≤ 0.05) control group.

Table (4-7) Effect of tocotrienols supplement on BMI , Lee index and AI of male rats in protective and therapeutic experiments. (Mean ± SE)

Groups	BMI g/cm^2	Lee index g/cm	AI %
control	0.481± 0.01 c	0.296 ± .004 c	4.083±0.12 c
HFD	0.733±0.03 a	0.334±.006 a	9.345±0.31 a
HFDT	0.610±0.01 b	0.314±.002 b	6.491±0.37 b
Signi.	*	*	*
control	0.520±0.01 c	0.296±.001 c	4.075±0.18 b
obese	0.733±0.02 a	0.326±.004 a	7.455±0.24 a
Therapeutic	0.662±0.01 b	0.311±.002 b	6.768±0.48 a
Signi.	*	*	*

Small letter referred to vertically significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.8-Effect of tocotrienols supplement on relative weight of liver, kidneys and testes of male rats in protective and therapeutic experiments

The relative weight of liver, kidney and testes on total body weight of animals illustrated in table (4-8). The protective experiment revealed a significant ($P \leq 0.05$) elevation ($P \leq 0.05$) in the relative weight of the liver in male rats treated with HFD and HFDT (protective groups) compared to the control. On the contrary, relative weight of testes impaired by HFD also when tocotrienol supplement in HFDT and showed significant decline ($P \leq 0.05$) when compared with relative weight of testes in animals of control group. whereas, the results recorded non- significant difference ($P \leq 0.05$) in the relative weight of kidney among the studied group.

On the other hand, there was significant elevation ($P \leq 0.05$) in relative weight of liver of HFD male rats compared with rats of the control and

HFDT groups. While testes and kidney recorded significant decline (P ≤ 0.05) in their relative weight for the rats fed HFD and HFDT groups when compared with control group.

Table (4-8) Effect of tocotrienols supplement as protective and therapeutic experiment on relative weight of liver, kidneys and testes of male rats. (Mean ± SE)

Groups	Liver weight/ body weight %	Kidneys weight/ body weight %	Testes weight/ body weight%
control	1.9±0.00 b	0.36±0. 02	0.53±0.01 a
HFD	3.3±0. 10 a	0.31±0. 01	0.39±0.03 b
HFDT	3.1±0. 20 a	0.33±0. 02	0.34±0.01 b
Signi.	*	N.S	*
control	2.2±0. 18 b	0.31±0. 02 a	0.48±.04 a
obese	3.5±0. 16 a	0.25±0.01 b	0.34±0.03 b
Theraputic	2.4±0. 12 b	0.28±0.00 ab	0.37±.02 b
Signi.	*	*	*

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.9-Effect of Tocotrienols supplement on some hematological indices of male rats in protective and therapeutic obesity experiments.

The estimation results of RBC, Hb, PCV%, MCV, MCH and MCHC were represented in table (4-9), Blood indices of protective experiment of obese and tocotrienol supplemented rats appeared non-significant (P≤ 0.05) in Hb, PCV, MCV, MCHC compared with control group. In contrast RBC and MCH showed significant difference (P≤ 0.05) among the studied groups of protective experiment.

In the therapeutic experiment, the estimation of Hb, PCV and MCHC recorded significant (P≤ 0.05) elevation in their values for rats of obese

and control groups. Also, RBC count reduce in obese and therapeutic groups compare with control group. Whilst results of MCV and MCH revealed significant (P≤ 0.05) decline for the control and therapeutic groups when compared with obese male rats in obese group.

Table (4-9) Effect of tocotrienols supplement on RBC Counts and RBC Indices of male rats in protective and therapeutic experiments. (Mean ± SE)

Groups	RBC × 10^6/µL	Hb g/dl	PCV %	MCV fl	MCH Pg	MCHC %
control	7.89±0.24 a	13.31±0.39	41.16±1.06	52.26±0.80	16.88±0.32 b	32.33±0.21
HFD	7.44±0.17 a	13.26±0.32	40.40±0.91	54.28±0.66	17.81±0.12 ab	32.83±0.28
HFDT	7.09±0.27 b	13.01±0.51	38.90±1.67	54.38±0.76	18.25±0.58 a	33.60±1.33
Signi.	*	N.S	N.S	N.S	*	N.S
control	8.25±0.10 a	13.86±0.26 a	42.60±0.62 a	51.61±0.62 b	16.76±0.24 b	32.55±0.16 a
obese	7.58±0.08 b	13.23±0.19 a	41.78±0.57 a	55.08±0.37 a	17.46±0.22 a	31.68±0.48 a
Therapeutic	7.33±0.30 b	12.00±0.46 b	38.23±1.12 b	52.35±0.92 b	16.38±0.22 b	31.33±0.35 b
Signi.	*	*	*	*	*	*

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.10-Effect of Tocotrienols supplement on total and differential WBC in protective and therapeutic obesity experiments

The total and differential WBC in protective obesity experiment represented in table 4-10 referred to significant elevation (P≤0.05) in total WBC count and neutrophils for rats fed on HFD, while the control group that had rats fed on LFD and rats fed on HFD supplemented with

tocotrienol appeared less total WBC and neutrophils number. The other types of WBC (Lymphocyte, monocyte, eosinophils, and basophils) appeared non-significant difference (P≤0.05) among all studied groups. Additionally, in the therapeutic experiment, the values of total WBC, neutrophils and lymphocytes revealed a significant (P≤0.05) reduced in their values in obese male rats treated with tocotrienol when compared with male rats in obese group, and appeared resemble (P≤0.05) of control group. In contrast there was no significant (P≤0.05) difference in basophils, eosinophils, and monocyte% in all studied groups .

Table (4-10) Effect of tocotrienols supplement on total and differential WBC of male rats in protective and therapeutic obesity experiments. (Mean ± SE)

Groups	WBC×103/µL	NEUT%	Lymph%	Mono%	Eosino%	% Baso
control	9.44±0.12 b	2.40±0.43 b	6.55±0.51	0.37±.06	0.07±.03	0.13±.09
HFD	13.72±1.16a	4.10±0.45 a	8.90±1.44	0.47±0.12	0.09±0.07	0.15±0.05
HFDT	9.32±0.12 b	1.84±0.20 b	7.26±0.08	0.20±0.12	0.07±0.02	0.11±0.06
Significant	*	*	N.S	N.S	N.S	N.S
control	9.67±0.14 b	2.318±0.17 b	6.97±0.19 b	0.34±0.05	0.08±0.03	0.21±0.11
obese	17.89±2.25a	3.80±0.53 a	13.15±1.94 a	0.60±0.13	0.17±0.09	0.15±0.03
Therapeutic	10.72±1.48 b	2.81±0.61 ab	7.02±1.11b	0.581±0.10	0.17±0.13	0.02±0.00
Significant	*	*	*	N.S	N.S	N.S

small letter represents significant difference at (P≤0.05). N.S.=non-significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.11-Effect of tocotrienols supplement on liver enzymes activity and kidney functions in protective and therapeutic obesity experiments

The effect of HFD administration to animals showed an obvious significant increase (P≤0.05) in the activity of liver enzymes (AST, ALT and ALP) when compared with animals of control group. In other hand, renal function recorded the deleterious effect of HFD administration to rats through significant elevation (P≤0.05) in urea levels for both groups fed HFD in spite of addition of tocotrienol for one of them, while animals of control group appeared less significant (P≤0.05) when compared with the HFD groups. But there was no change (P≤0.05) in creatinine levels among the studied groups.

The second experiment when tocotrienol additive after inducement of obesity, the liver enzymes showed similar concentration with control group, although there was significant increase in ALP enzyme activity in obese group. Conversely, renal function indicators (urea and creatinine) showed non-significant difference among all studied groups.

Table (4-11) Effect of tocotrienols supplement on liver enzymes and kidney functions in protective and therapeutic experiments. (Mean ± SE)

Groups	AST U/L	ALT U/L	ALP U/L	Urea mg/dl	Creatinine mg/dl
control	142.00 ±10.47 b	34.83 ±1.24 b	218.66 ±13.69 b	12.50 ±0.99 b	0.59 ±0.01
HFD	264.00 ±62.50 a	47.33 ±6.52 a	303.83 ±16.54 a	21.33 ±1.35 a	0.64 ±0.02
HFDT	161.50 ±11.898 ab	38.83 ±0.74 ab	265.66 ±19.81 ab	17.00 ±2.86 ab	0.60 ±0.01
Signi.	*	*	*	*	N.S
control	108.00 ±10.76	34.66 ±7.45	149.83 ±9.82 b	18.66 ±2.24	0.37 ±0.03
obese	129.16 ±21.05	42.33 ±6.87	224.16 ±15.46 a	19.83 ±1.99	0.43 ±0.04
Theraputic	111.66 ±12.51	33.50 ±7.38	168.00 ±16.39 b	18.00 ±2.70	0.41 ±0.02
Signi.	N.S	N.S	*	N.S	N.S

Different small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.12-Effect of tocotrienols supplement on total protein, Albumin, Globulin and C reactive protein in protective and therapeutic experiments

As shown in table (4-12), the effect of tocotrienol supplement on HFD led to significant reduction (P≤0.05) in total protein and globulin levels and failed to enhance the concentration of C-reactive protein and glucose to appeared unlike (P≤0.05) their values in control group while non-significant (P≤0.05) with HFD group. In contrast, the therapeutic additive of tocotrienol to obesity laboratory rats was able to enhance (P≤0.05)

values of albumin and glucose when compared with laboratory rats suffered from obesity risks. Although there was no significant difference (P≤0.05) in values of total protein, globulin and C- reactive protein.

Table (4-12) Effect of tocotrienols supplement on total protein, Albumin, Globulin, C reactive protein and glucose in protective and therapeutic experiments. (Mean ± SE)

Groups	Tprotein mg/dl	Albumin mg/dl	Globulin mg/dl	CRP mg/l	Glucose mg/dl
control	**54.16**±0.98 b	32.16±0.87	22.16±0.60 a	0.58±0.00 b	**133.16**±6.08 b
HFD	**58.83**±1.72 a	34.33±0.88	24.50±1.23 a	1.29±0.21 a	**230.33**±34.50 a
HFDT	**51.50**±1.176 b	31.83±1.16	18.83±0.47 b	1.06±0.11 a	**219.50**±25.13 a
Signi.	*	N.S	*	*	*
control	61.00±1.788	34.00±1.06 b	27.00±1.36	1.01±0.10	126.33±5.75 c
obese	64.50±1.310	37.16±0.70 a	27.33±0.95	1.23±0.09	212.50±6.69 a
Theraputic	62.00±1.437	34.16±0.94 b	27.83±1.07	1.050±0.08	176.66±11.76 b
Signi.	N.S	*	N.S	N.S	*

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.13-Effect of tocotrienols supplement on lipid profile in protective and therapeutic obesity experiments

The effects of tocotrienol supplementation on serum lipid profiles are represented in table (4-13). The beneficial effect of tocotrienol additive to HFD on male rats was clearly recognized, these appeared through lower significant levels of triglyceride, LDL-c and VLDL-c and high significant level of HDL-c in HFDT group than male rats fed on HFD without tocotrienol supplement, and get some results closely from male rats of control group. However, cholesterol levels showed non-significant difference among the groups of the study.

Also, when tocotrienol supplemented as therapeutic dose from obesity deleterious effect was able to reduce the values of triglyceride and LDL-c bur these reduce non-significant when compared with obese group. Regarding cholesterol, HDL-c and VLDL-c recorded non-significant effect among the studied groups.

Table (4-13) Effect of tocotrienols supplement on lipid profile of male rats in protective and therapeutic experiments. (Mean ± SE)

Groups	Cholesterol mg/dl	TG mg/dl	HDL-C mg/dl	LDL-C mg/dl	VLDL-C mg/dl
control	**59.83**±1.35	28.66±3.76 b	20.50±1.83 b	4.33±0.49 b	5.50±0.76 b
HFD	**66.16**±7.25	80.33±2.60 a	26.16±2.79 b	13.46±0.96 a	15.93±0.49 a
HFDT	**64.16**±5.30	33.16±3.91 b	38.50±3.59 a	10.83±1.712 a	6.66±0.80 b
Signi.	N.S	*	*	*	*
control	58.83±1.53	42.50±5.27 b	33.33± 1.58	9.33±1.28b	8.16±0.87
obese	56.50±2.69	67.33±8.01 a	32.50±1.72	15.33±2.99 a	13.00±1.54
Theraputic	60.00±6.92	61.83±7.95 ab	37.83±4.23	10.83±1.01 ab	14.33±3.29
Signi.	N.S	*	N.S	*	N.S

Different small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.14-Effect of tocotrienols supplement on irisin, leptin, Adiponectin and Insulin hormones of male rats in protective and therapeutic obesity experiments.

The effect of tocotrienol additives on metabolic hormones (Irisin, Leptin, Adiponectin and Insulin) is mentioned in table(4-14).In this table, results showed significant elevation in all metabolic hormones concentrations (Irisin, Leptin, Adiponectin and Insulin) in HFD group and unable

tocotrienol to improve their concentration when compared with their concentration in rats of control group, except for irisin and insulin hormones, tocotrienol supplement was able to reduce its values to appear non-significant with control group. In therapeutic experiment, the results of the effect of tocotrienols on the hormones irisin, adiponectin and insulin showed no significant differences (P≤0.05) among the groups of the study. However, the results showed a significant increase in leptin hormone concentration in obesity and therapeutic groups compared to control group, but between these two groups results did not record any significant difference at (P≤0.05).

Table (4-14) Effect of tocotrienols supplement on Irisin, Leptin, Adiponectin and Insulin of male rats in protective and therapeutic experiments. (Mean ± SE)

Groups	Irisin (ng/ml)	Leptin (ng/ml)	Adiponectin (ng/ml)	Insulin (ng/ml)
control	7.40±0.39 b	1.90±0.17 b	7.03±0.63 b	8.05±0.48 b
HFD	10.74±0.91 a	3.39±0.21 a	11.15±0.39 a	10.2923±0.44 a
HFDT	9.39±0.61 ab	3.13±0.23 a	11.75±0.47 a	8.74±0.28 b
signi	*	*	*	*
control	11.51±.91	3.27±0.26 b	13.15± 0.39	8.15± 0.53
obese	12.84±0.68	4.20±0.29 a	10.73±1.53	8.43±0.12
Theraputic	10.59±0.82	3.51±0.12 ab	11.51±1.65	8.89±0.55
signi	N.S	*	N.S	N.S

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.15-Effect of tocotrienols supplement on some sex hormones of male rats in protective and therapeutic Obesity experiments.

The effect of obesity on reproductive hormones and role of tocotrienol in improving the concentrations represented by table (4-15). The results investigated there was no significant effect of obesity on testosterone and FSH hormones concentration among the studied groups in protective experiment when tocotrienol added together with feed of animals with high fat diet, except for LH hormone that showed significant decrease in their concentration for HFD and HFDT groups when compared with control group. However, testosterone hormone reduces their concentration significantly in rats induced obesity and also in rats treated with tocotrienol compared with control group. Although the reduction in testosterone hormone concentration in therapeutic experiment, but FSH and LH showed non-significant differences among the groups of the present study.

Table (4-15) Effect of tocotrienols supplement on some sex hormones of male rats in protective and therapeutic experiments. (Mean ± SE)

Groups	Testosterone (ng/ml)	FSH (mlU/ml)	LH (mlU/ml)
control	0.94±0.14	0.73±0.05	2.14±0.23 a
HFD	0.90±0.15	0.60±0.02	1.25±0.05 b
HFDT	0.94±.14	0.69±0.05	1.48±0.13 b
Signi.	N.S	N.S	*
control	1.44±0.20 a	0.63±0.02	1.65±0.15
obese	0.85±0.06 b	0.59±0.04	1.37±0.11
Theraputic	0.97±0.13 b	0.65±0.04	1.37±0.20
Signi.	*	N.S	N.S

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.16- Effect of tocotrienols supplement on seminal analysis in protective and therapeutic obesity experiments.

At the end of the experiments, sperm viability was estimated in the scarified rats of protective and therapeutic experiments. As showed in table (4-16) sperm account and motility recorded sever significant reduction in their values for the rat's induced obesity, tocotrienol supplement introduced its ability to improve motility and account of sperm values whether in protective or therapeutic experiments, although there was significant difference between protective and therapeutic experiment, but still appeared significantly lower values of sperm motility and account than control group.

In contrast, dead and abnormal sperms in table (4-16) investigated the deleterious effect of obesity on sperm viability, it recorded high significant percent for dead and abnormal sperms when compared with all studied groups. While tocotrienol supplement ameliorated the deleterious effect of obesity in protective and therapeutic dose and appeared significantly less than control group.

Table (4-16) Effect of tocotrienols supplement on seminal analysis in third experiments. (Mean ± SE)

Groups	Spe.motility %	Spe.account x 10^6	Dead spe. %	Abnormal sperm%
control	83 ±1.35 a	178.66±1.49 a	7±0.73 d	13 ±1.35 b
HFD	61 ±2.27 d	157.16±2.03 c	14±0.87 a	28±1.75 a
HFDT (Protective)	76 ±2.08 b	179.33±4.34 a	9±0.30 c	17±1.80 b
Therapeutic	71±0.84 c	166±2.16 b	11±0.51 b	16±2.48 b
Signi.	*	*	*	*

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet, HFDT: high fat diet plus tocotrienols.

4.17 Fertility experiment

After feeding the rats with high fat diet for 12 weeks and supplemented with tocotrienol (protective group), while other group induced obesity and treated with tocotrienol) therapeutic group) for another 8 weeks. The male induced obesity and treated with tocotrienol mating with healthy female rats as mentioned in the paragraph (3-16). The reproductive efficiency represented in table (4-17). The effect of high fat diet that fed to the male rats for 12 weeks clearly showed reduction in fertility percent in comparison to tocotrienol treated groups and control. These results reflected on the number of birth percentage to appear non-significant among tocotrienol treated groups and control group. Birth weight was not affected significantly by high fat diet and tocotrienol supplement.

Table (4-17) Effect of tocotrienols supplement on the reproductive efficiency in male rats in protective and therapeutic experiments. (Mean ± SE)

Groups	Fertility percent%	Birth no.	Birth weight (g)
control	100% a	7.50 ±0.86 a	5.37±0.67
HFD	75% b	3.00±1.58 b	6.29±2.47
HFDT	100% a	6.25±0.47 a	5.83±0.60
Theraputic	100% a	7.75±0.75 a	5.26±0.32
Signi.	*	*	N.S

small letter represents significant difference at (P≤0.05). N.S.=non significant. * = significant. HFD: high fat diet ,HFDT: high fat diet plus tocotrienols.

4.18 Histopathological Examination

4.18.1 Histopathological changes in the liver

Figure 4-1 shown later represents the liver of control group. There were no histological changes may affect the liver function for each figure and the tissues appeared with normal structure, which consist of normal central vein, normal hepatocytes and normal sinusoids.

The histopathological examination of liver in rats fed high fat diet- induced obese for 12 weeks suffered from pathological lesions such as severe peri central vein vacuolation of hepatocytes (figure 4-2). In contrast, the histopathological examination of rat's liver that fed high fat diet and supplemented with tocotrienol for 12 weeks showed hepatic changes less than rats fed high fat diet alone such as early regenerated hepatocytes and normal sinusoids (figure 4-3). Obese rats in therapeutic experiment suffered from moderate peri central vein vacuolation of hepatocytes and dilation of sinusoids (figure 4-4). Histological section of liver of therapeutic group rats that fed high fat diet- induced obese for 12 weeks then supplemented with tocotrienol for 8 weeks and shows moderate vacuolation of hepatocytes, dilation of sinusoids (figure 4-5).

4.18.2 Histopathological changes in the kidney

As shown in figure 4-6, Kidneys of control group showed normal architecture of renal parenchyma, which consist of normal glomeruli and normal renal tubules.

Histological section of kidney of the rats fed high fat diet induced obese group showed moderate atrophy of glomeruli and dilation of renal tubules (figure 4-7). While histological changes in kidneys of rats fed high fat diet induced obese and supplement with tocotrienol for 12 weeks (protective group) appeared normal glomeruli and mild dilation of renal tubules (figure 4-8). Histological examination of kidney of obese group in therapeutic experiment exhibited moderate atrophy of glomeruli and dilation of renal

tubules (figure 4-9). Obese rat group in therapeutic experiment suffered from moderate atrophy of glomeruli; dilation of renal tubules (figure 4-10). In contrast, the histopathological examination of kidney in therapeutic group showed atrophy of glomeruli and dilation of renal tubules (figure 4-11).

4.18.3 Histopathological changes in the testes

The microscopic finding of testes in control rat showed normal structure of seminiferous tubules and spermatogenesis and supporting cells that arranged in the lining of testes (figure 4-12). The rats fed high fat diet exhibited histological changes in testes represented by suppression of spermatogenesis and vacuolation of seminiferous tubules (figure 4-12). The histopathological examination of testes of protective group that belong to rats fed high fat diet and supplemented with tocotrienol showed normal spermatogenesis (figure 4-13). Histological examination of testes of obese group in therapeutic experiment suppression of spermatogenesis and vacuolation of seminiferous tubules (figure 4-14). while histological examination of testis of therapeutic group showed mild suppression of spermatogenesis; vacuolation of seminiferous tubules (figure 4-15).

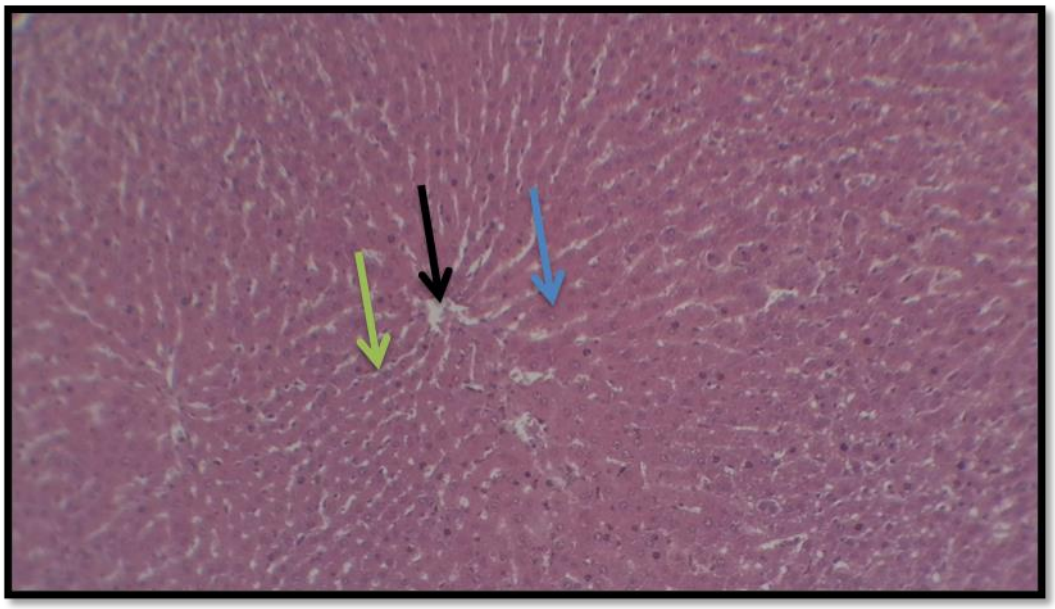


Figure (4-1): Histopathological section of liver of control group showed normal architecture of hepatic parenchyma, which consist of normal central vein (black arrow), normal hepatocytes (blue arrow), and normal sinusoids (green arrow). H&E stain. 100X

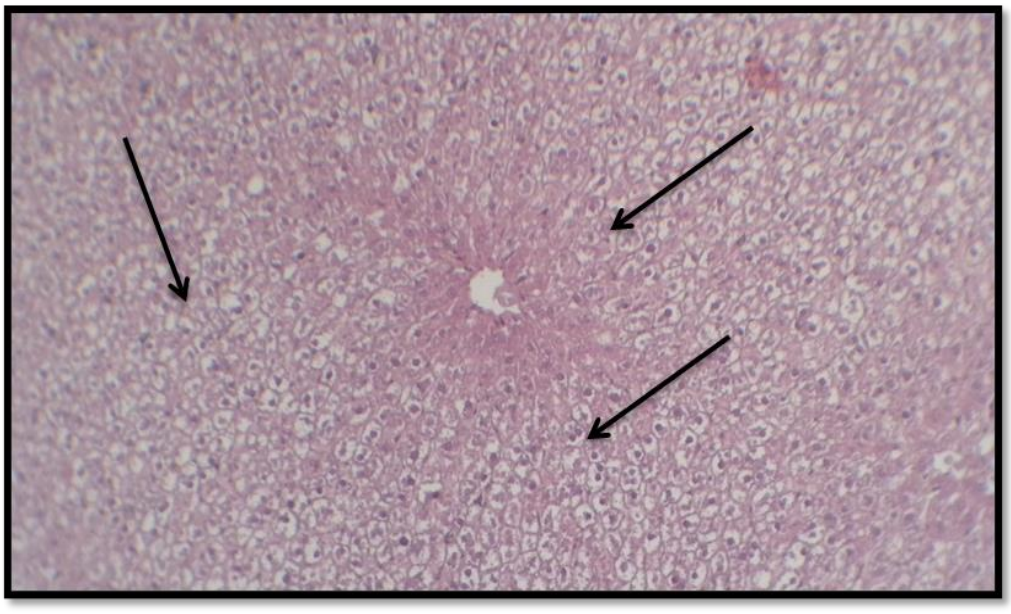


Figure (4-2): Histopathological section of liver of HFD group shows severe peri central vein vacuolation of hepatocytes (black arrow). H&E stain. 100X

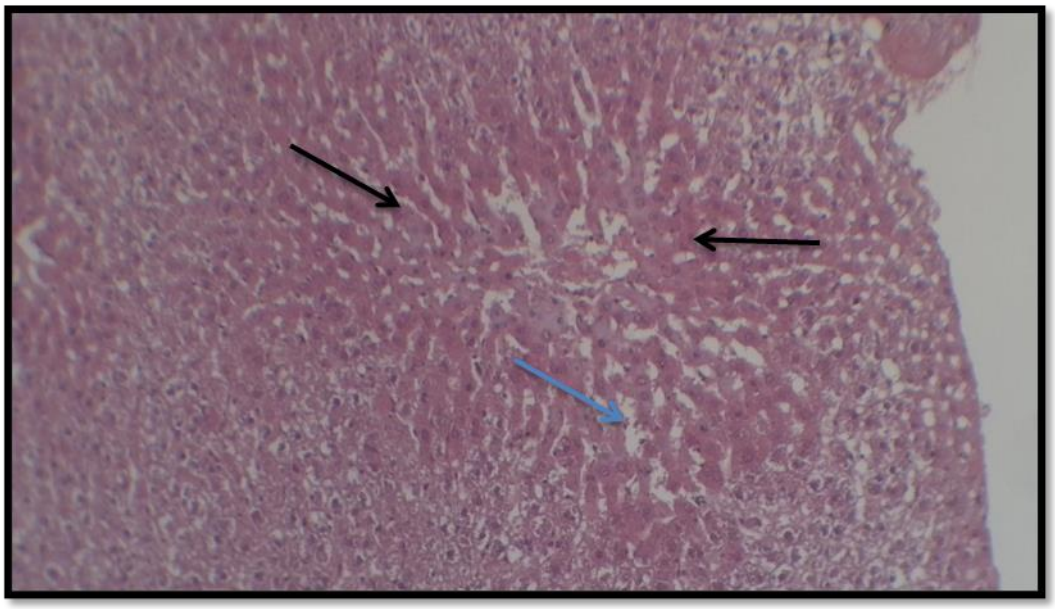


Figure (4-3): Histopathological section of liver of HFDT (protective) group shows early regenerated hepatocytes (black arrow), normal sinusoids (blue arrow). H&E stain. 100X

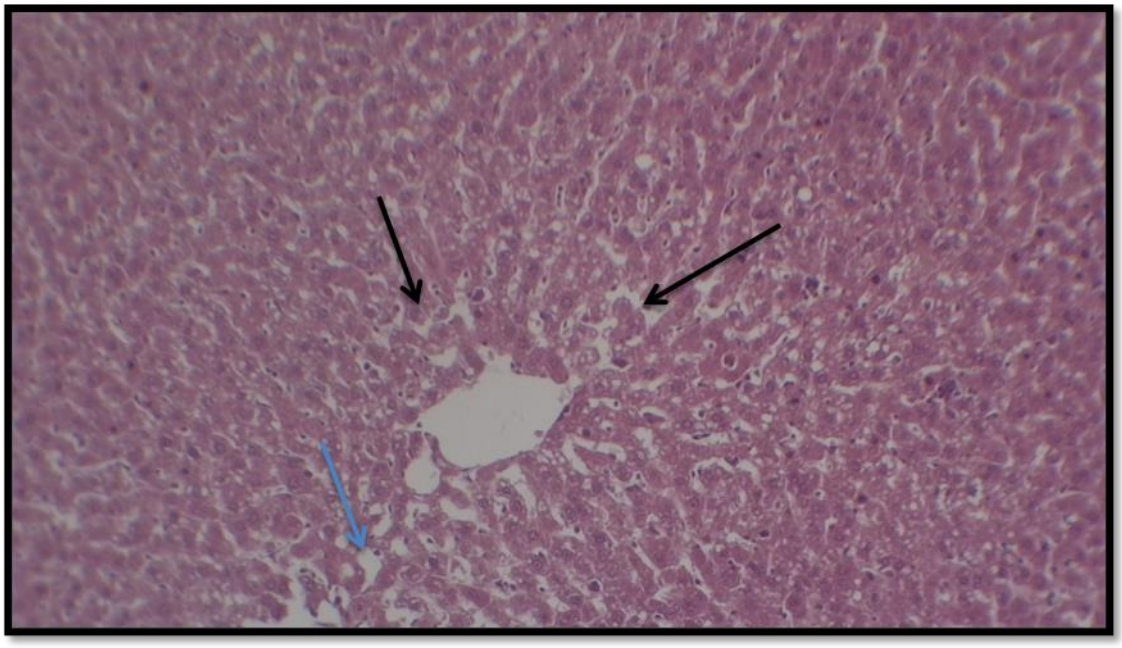


Figure (4-4): Histopathological section of liver of obese group shows moderate peri central vein vacuolation of hepatocytes (black arrow), dilation of sinusoids (blue arrow). H&E stain. 100X

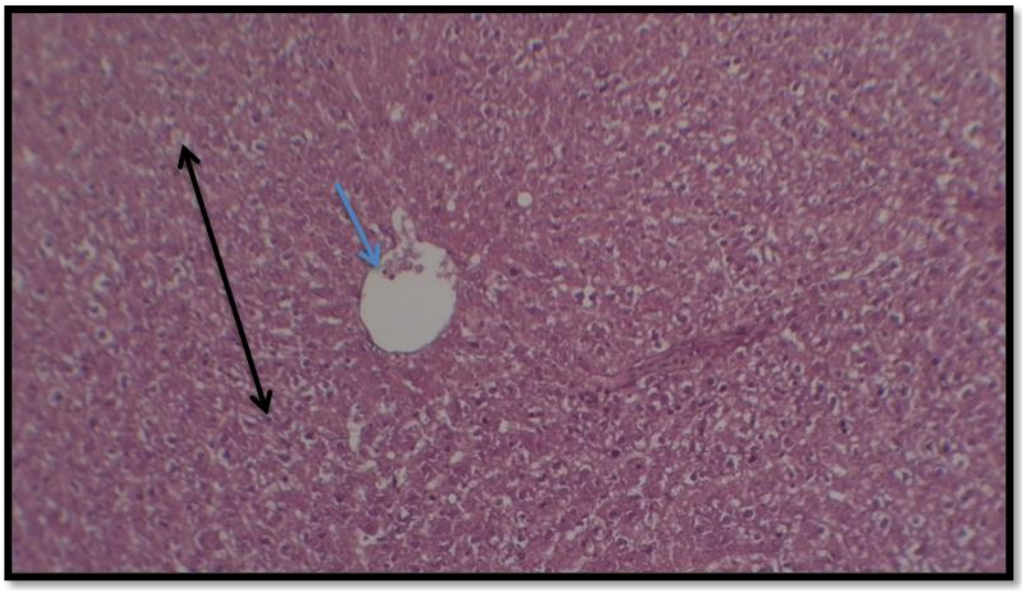


Figure (4-5): Histopathological section of liver of therapeutic group shows excessive vacuolation of hepatocytes (black arrow), dilation of sinusoids (blue arrow). H&E stain. 100X

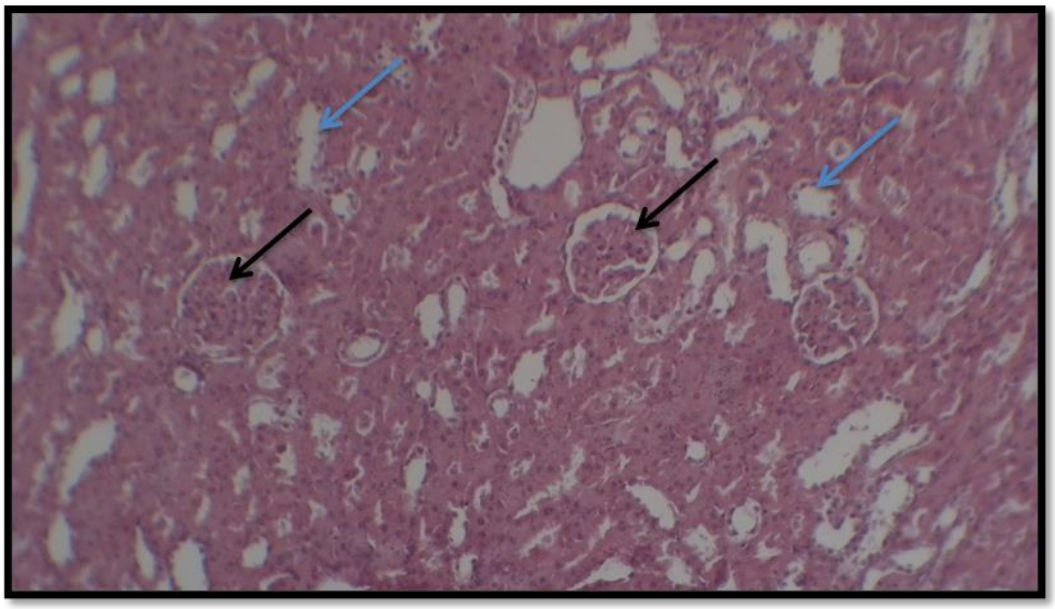


Figure (4-6): Histopathological section of kidney of control group showed normal architecture of renal parenchyma, which consist of normal glomeruli (black arrow), normal renal tubules (blue arrow). H&E

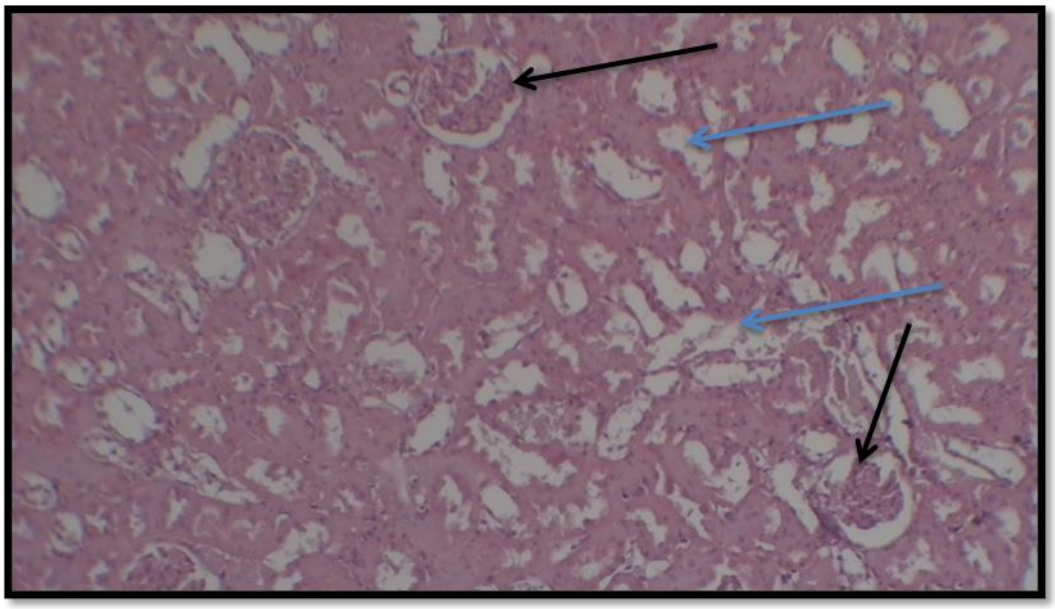


Figure (4-7): Histopathological section of kidney of HFD group moderate atrophy of glomeruli (black arrows); dilation of renal tubules (blue arrows). H&E stain. 100X

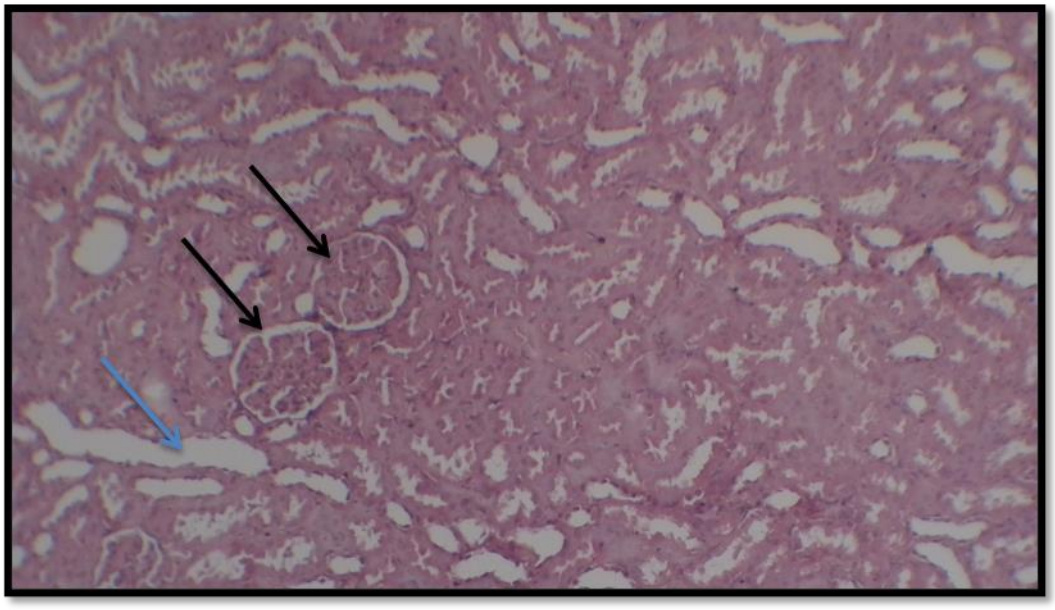


Figure (4-8): Histopathological section of kidney of HFDT (protective) group shows normal glomeruli (black arrows); moderate dilation of renal tubules (blue arrows). H&E stain. 100X

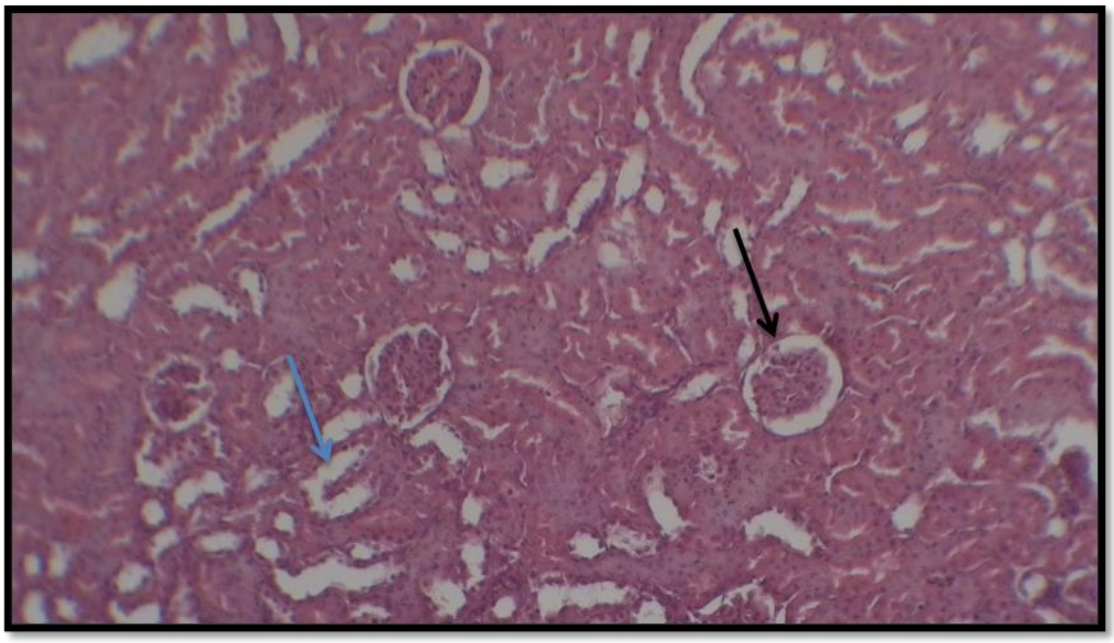


Figure (4-9): Histopathological section of kidney of obese group moderate atrophy of glomeruli (black arrows); dilation of renal tubules (blue arrows). H&E stain. 100X

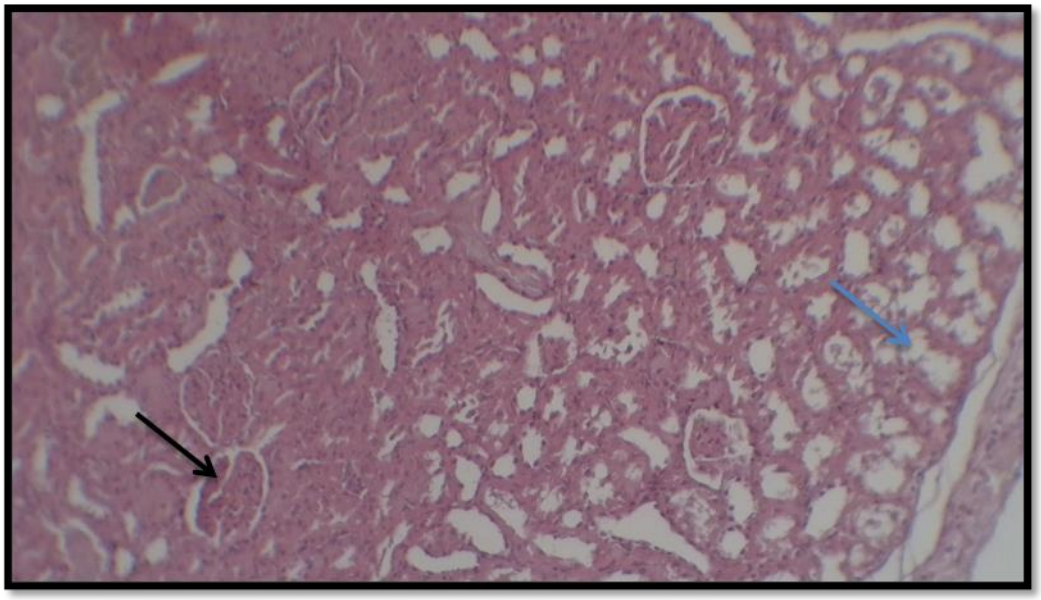


Figure (4-10): Histopathological section of kidney of therapeutic group atrophy of glomeruli (black arrows); dilation of renal tubules (blue arrows). Sever vacuolation .H&E stain. 100X

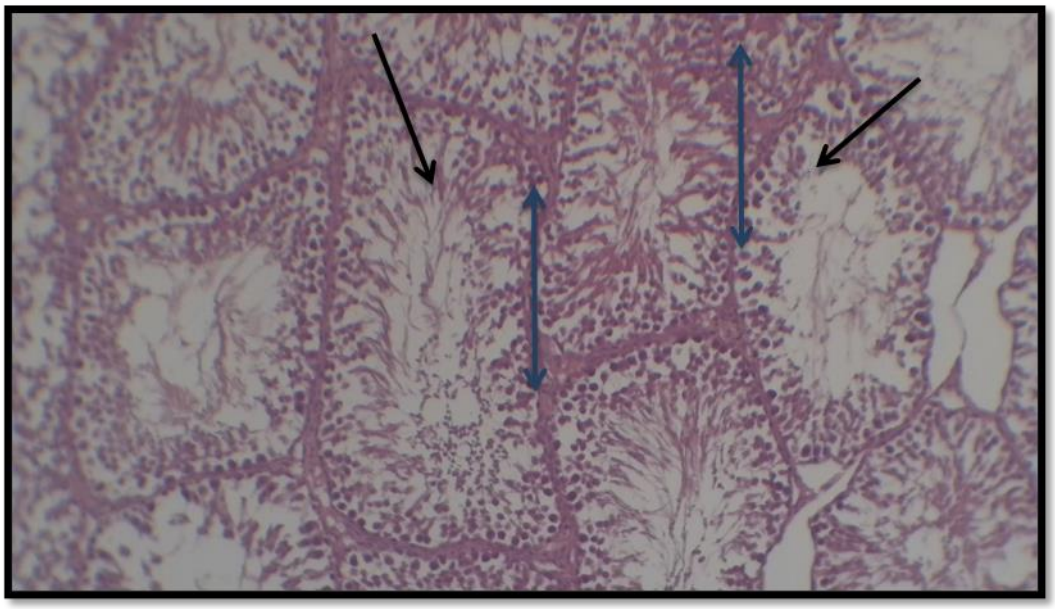


Figure (4-11): Histopathological section of testis of control group showed normal architecture of testis parenchyma, which consist of normal seminiferous tubules (black arrow), normal spermatogenesis (blue arrow). H&E stain. 100X

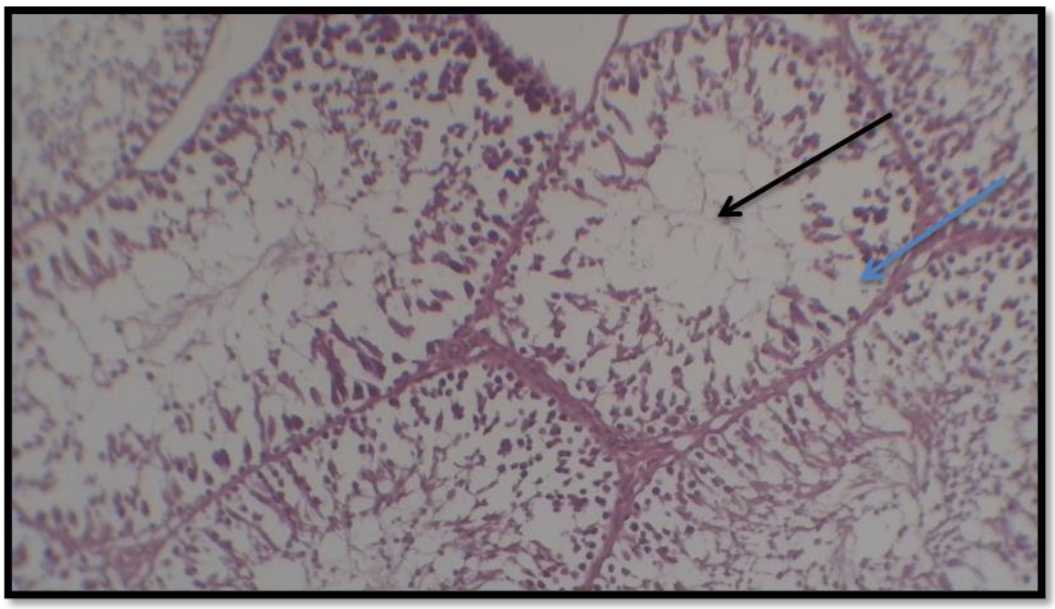


Figure (4-12): Histopathological section of testis of HFD group suppression of spermatogenesis (black arrows); vacuolation of seminiferous tubules (blue arrows). H&E stain. 100X

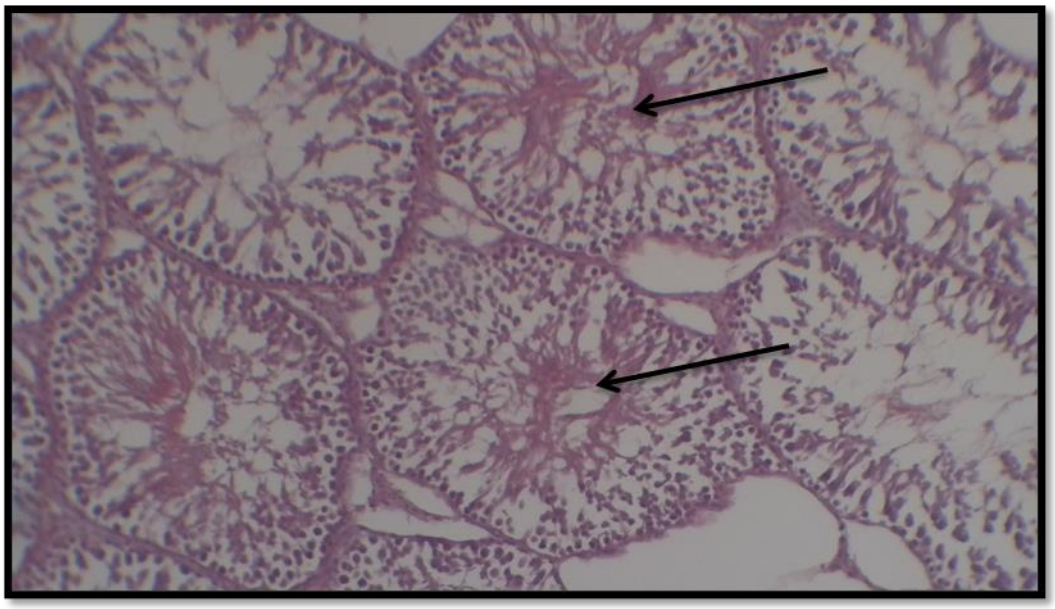


Figure (4-13): Histopathological section of testis of HFDT (protective) group normal spermatogenesis (black arrows). H&E stain. 100X

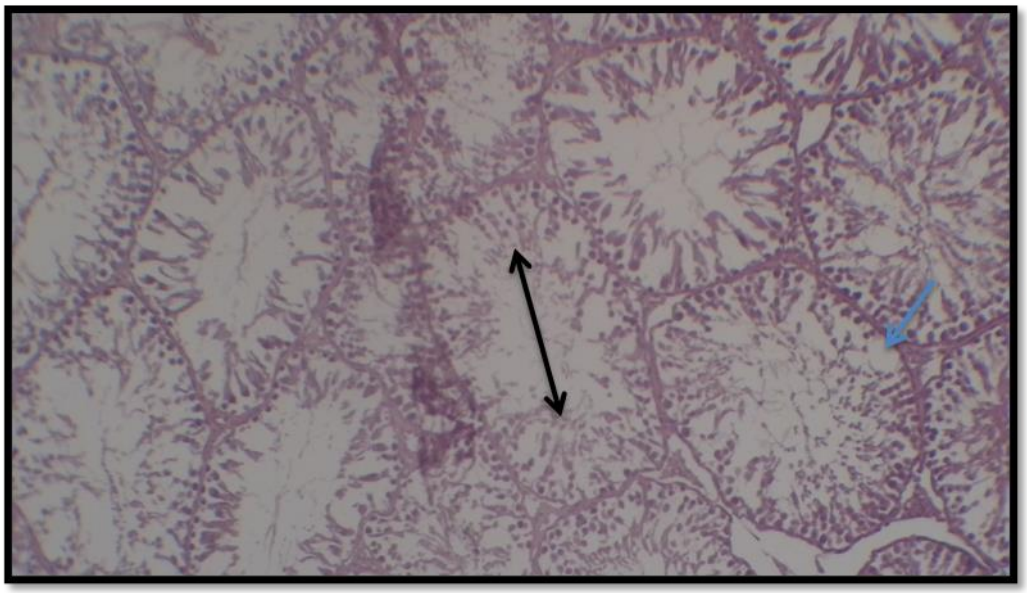


Figure (4-14): Histopathological section of testis of obese group suppression of spermatogenesis (black arrows); vacuolation of seminiferous tubules (blue arrows). H&E stain. 100X

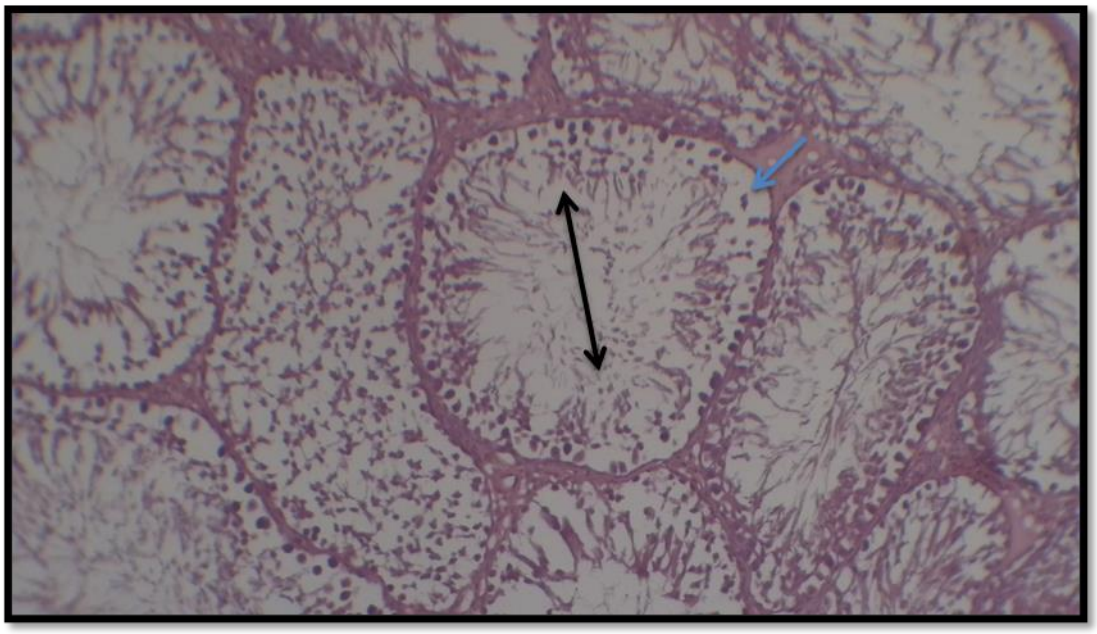


Figure (4-15): Histopathological section of testis of therapeutic group mild suppression of spermatogenesis (black arrows); vacuolation of seminiferous tubules (blue arrows). H&E stain. 100X

4.19 Gene Expression

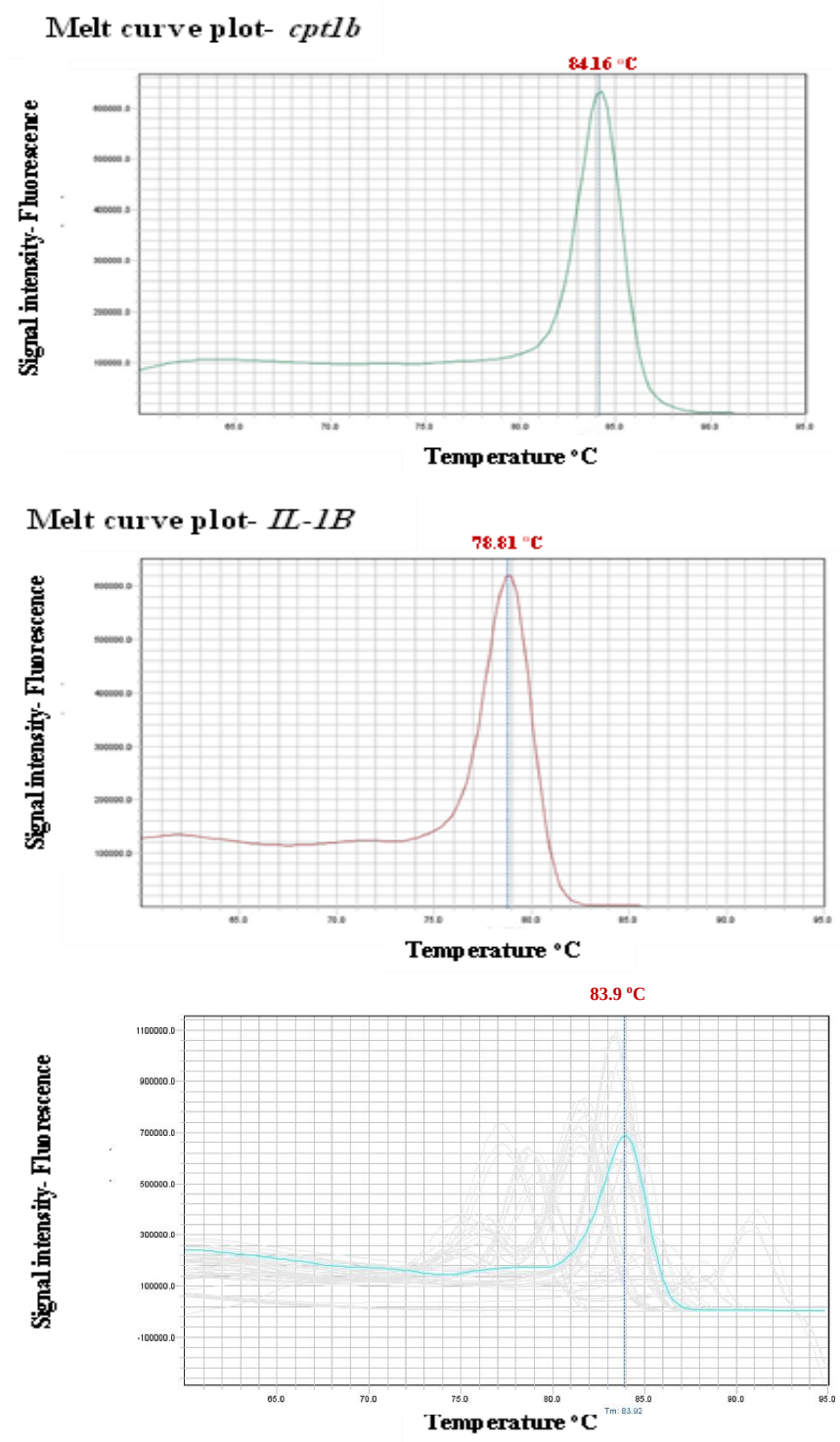
Real-time PCR, also known as quantitative reverse transcription PCR (RT-qPCR) and quantitative PCR (qPCR), is a very effective and sensitive technique used for gene analysis. Real-time PCR is used by scientists to objectively assess gene expression. Real-time PCR measures the amplification of PCR in real-time, allowing for the measurement of the starting amount of nucleic acid. During polymerase chain reaction (PCR), SYBR Green quantifies the amplified DNA fragment by specifically recognizing and attaching to the double-stranded DNA, while simultaneously measuring the emission of fluorescence.

When it is bound, it shows increased fluorescence. During the extension phase of PCR, SYBR Green binds to each new produced double-stranded DNA molecule as the targeted region is amplified. A fundamental

limitation of this method is its deficiency in specific binding. Therefore, we performed melt curve analysis to evaluate the integrity of amplification. In order to generate amplification plots, we plotted the fluorescent signal emitted by amplicons against the cycle number, which represents the accumulation of the product during the RT-qPCR experiment.

4.19.1 Amplification Curves and Specificity of Gene-Targeted Primers

The melt curve plot (also called a dissociation curve plot) displays data collected during a melt curve stage. Peaks in the melt curve can indicate the melting temperature of a target and can identify nonspecific PCR amplification. An analysis of melting curves was performed on all amplicons. Cpt1b, IL-1B, and beta-bactin, the control gene, and showed only one peak in all tested samples, as shown in Fig. (4-16). SYBR green emission produced a quantifiable fluorescence signal, which was measured from reaction tubes for each targeted gene.



Melt curve plot- *Beta actin*

Figure 4.16 displays the melt curves for the *cpt1b* , *Beta actin*, and *IL-1B* amplicons with each peaks temperature marked in red color at the top of the peak. A distinct, solitary amplicon was shown by the singular peak found for each amplicon. The melt curve program includes: a temperature rise to 95 °C for denaturation and a subsequent decrease to 60 °C for annealing. This is followed by a dissociation phase at 95 °C, during which data for the fluorescence signal is collected.

4.19.2 Relative Gene Expression

Relative quantitation of gene expression enables the quantification of differences in the expression level of a specific target (gene) between different samples. The data output quantified differences in expression levels as a fold-change or a fold-difference. The change in expression of Cpt1b, , IL-1B, and beta-bactin genes was monitored in treated versus untreated groups (high-fat diet and tocotrenol vs. control). After normalizing to the control values, we reported the data as the mean and standard deviation of the three technical replicates using the ΔΔCT method. Applied Biosystems™ QuantStudio™ 5 *Design and Analysis Software* v1.5.2 analysis generated the gene expression profile in each case. The thresholds were automatically adjusted for all genes, as shown in figures (4-17). Applied Biosystems™ QuantStudio™ 5 **Design and Analysis Software** v1.5.2 analysis generated the gene expression profile in each case. The thresholds baseline was automatically adjusted for all genes, as shown in figures (4-18).

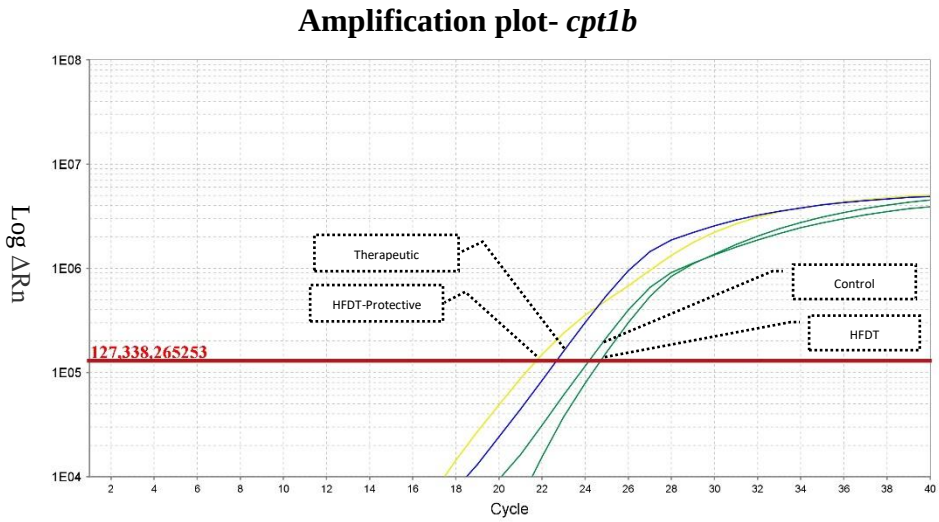


Figure 4.17 Amplification plot for *cpt1b* gene expression in the control, HFDT, HFDT-protective, and therapeutic groups. The Rn value, also known as the normalized reporter value, represents the fluorescent signal emitted by SYBR Green dye, which the instrument adjusts by dividing it by the signal of the passive reference dye in a specific reaction. The Log ΔRn value was obtained by subtracting the baseline signal provided by the instrument from the Rn value of an experimental response which was plotted against cycles of amplification.

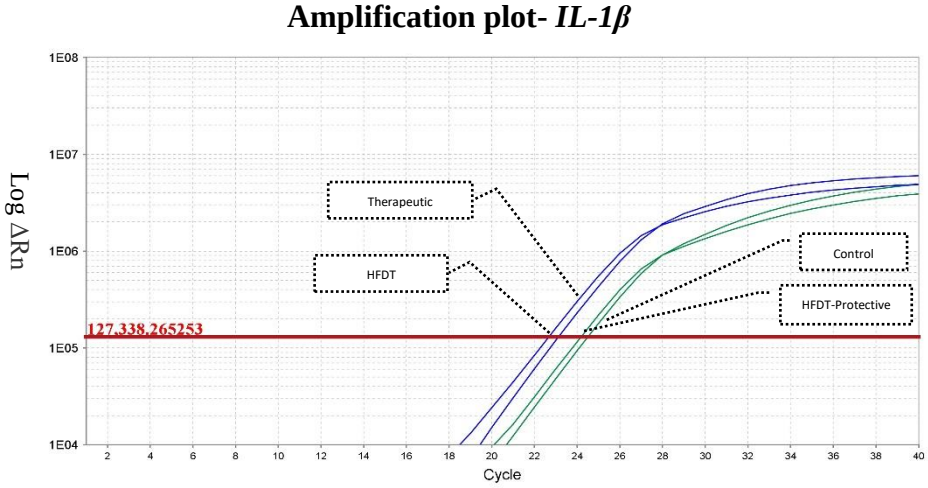


Figure 4.18 Amplification plot for ***IL-1β*** gene expression in the control, HFDT, HFDT-protective, and therapeutic. The Rn value, also known as the normalized reporter value, represents the fluorescent signal emitted by SYBR Green dye, which the instrument adjusts by dividing it by the signal of the passive reference dye in a specific reaction. The Log ΔRn value was obtained by subtracting the baseline signal provided by the instrument from the Rn value of an experimental response which was plotted against cycles of amplification.

4.19.3 Relative Gene Expression of Cpt1b

The gene expression of the cpt1b gene was 10.17-fold greater in the HFDT protective group and 2.54-fold higher in the therapeutic treatment group compared to the control group. A significant decrease of 0.78 fold was seen in the HFD group. To calculate the fold change, we standardized the results to the control groups, as seen in figure (4-19).

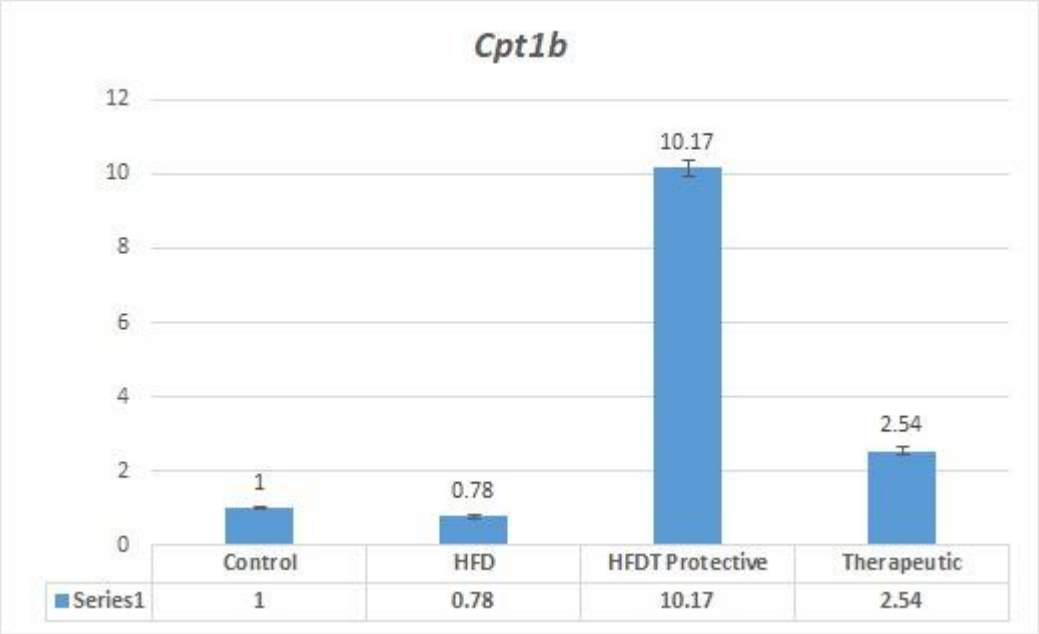


Figure 4.19

4.19.4 Relative Gene Expression of *IL-1β*

We measured IL-1β in all groups to evaluate the inflammatory profile associated with tocotrienol. The gene expression of the IL-1β gene was 8.85 fold greater in the HFD group, 2.67 fold higher in the HFDT protective group, and 5.09 fold higher in the HFDT therapeutic group compared to the control group. To calculate the fold change, we standardized the results to the control groups, as seen in figure (4-20).

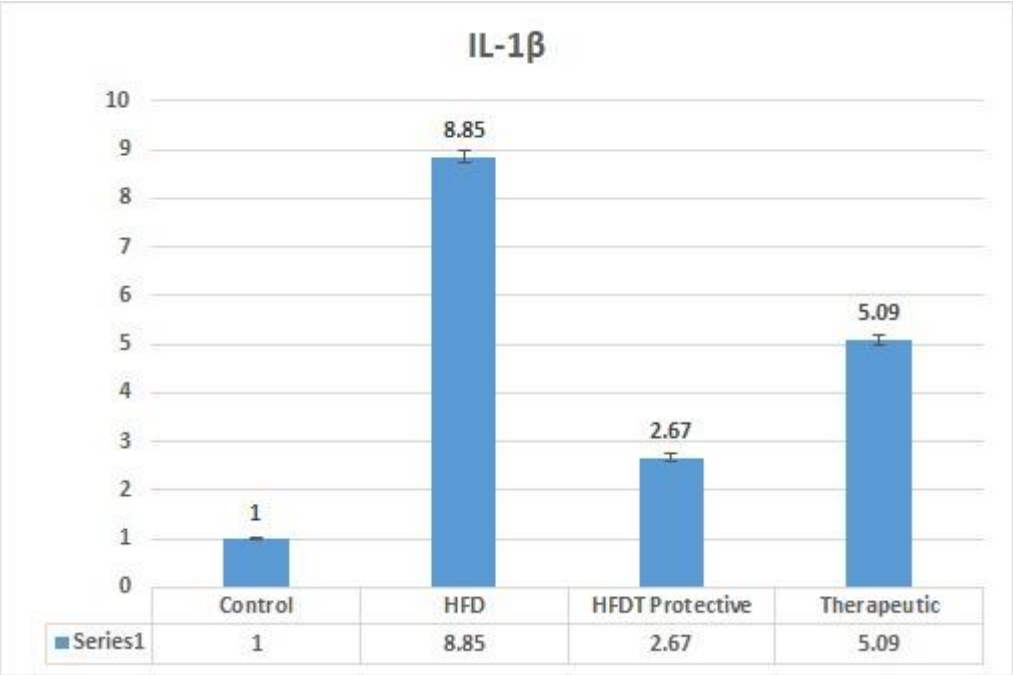


Figure4.20

Chapter Five

Discussion

Discussion

5.1-Effect of tocotrienol supplement on body weight, weight gain, and food consumption in obese male rats as protective and therapeutic .

The results of present study revealed no significant differences in the body weight of animals in all groups of the study through the first and second weeks of the experiment. This is normally appeared due to the random distribution of the experimental animals into the different groups. Otherwise, the experiment also showed an increase in body weight of the groups fed a high-fat diet compared to the control group as a result of eating high-fat foods with high energy content. These was consistent with several researches that investigated a diet heavy in fat causes obesity by increasing energy intake (Harrold *et al*., 2000; Ghibaudi *et al*.,2002; Woods *et al*., 2003). Also, Hariri and Thibault have attributed obesity induced by high-fat diets to their high food efficiency (Hariri and Thibault, 2010).

The results of this investigation were identical to those published by Buettner *et al.* (2007), who found that body weight disparities between control and HFD-fed rats may be noticed after at least two weeks of feeding, but became more visible after a minimum of four weeks of dieting. According to some researchers, animals fed a high-fat diet initially showed a large rise in adipose tissue buildup without any change in body weight However, after three weeks, there was an increase in body weight and adipocyte hypertrophy (Woods *et al*., 2003; La Fleur *et al*., 2010). Tocotrienols showed an anti-obesity effect starting from the sixth week until the end of the protective experiment. T3s definitely prevent the body weight increase caused by the HFD (Kato *et al*., 2022), when they attributed the significant reduction in body weight to reduce the buildup of fat in tocotrienol feeding group comparison to the high-fat diet-treated rats group.

The effect of Tocotrienol supplement absent when drainage for rats induced obesity and the weights of animals in the obesity and tocotrienol group increased significantly compared to the control group, despite the use of a low-fat diet for all groups (table 4-2). these may be attributed to the increased food intake for these two groups compared with control as indicated in the table (4-6). The result above agreed with previous study indicated that the use of tocotrienol-rich fractions TRF for 8 weeks in obese rats did not lead to a significant decrease in weight after the treatments for a period experiment (Mesri Alamdari *et al*., 2020).

Weight increase in comparison to the control group is commonly used to evaluate the effectiveness of an HFD intervention (Pinheiro-Castro *et al*., 2019). Obesity was defined as weight growth accompanied by increased adipose tissue mass due to increased fat cell number and size. Adipose tissue is a dynamic organ that regulates energy balance and mass in response to the needs of metabolism (Daffalla *et al.*, 2021). Diet-induced obesity (DIO) models in rodents have demonstrated that continuous consumption of obesogenic diets results in body weight gain and adipose tissue buildup, which leads to metabolic changes (Sáinz *et al*., 2015), According to our findings, rats fed a high-fat diet showed higher weight growth compared to control the rats. and that agreed with some study (Othman *et al*., 2019; Ayman *et al*., 2019;Madkhali, 2020), and used of tocotrienol reduced the body weight and this result agreed with the results of the Zhao study and his colleagues that indicated supplementing of HFD with 0.05% T3 for 4 weeks reduced the obesity caused by the HF diet in young C57BL/6 J mice (Zhao et al., 2015a). While the results of the current study were in disagreement with result of Wong and his colleagues who fed Wistar rats on HFD for 8 weeks 120 mg/kg/day of TRF and observed no effect on their body weight gain (Wong *et al*., 2012 b).

When energy expenditure was examined throughout the dark (the rodents' active phase) and light (the lower activity phase) cycles, it was

observed that overweight animals had a lower active metabolic rate compared to the control group. Obese mice also had lower resting energy expenditure during the light and dark cycles, indicating a diminished aptitude for muscle oxidative phosphorylation (Olsen *et al*., 2017). Another study found that diets rich in dietary fat (HFD) spend less energy than diets low in dietary fat (Blaak & Saris, 1996). This could explain the observed increases in body weight, weight gain and fat mass in the HFD intervention groups, despite calorie intake comparable to the control group.

The use of tocotrienols led to a significant decrease in the body weights of animals in the protective experiment, but still the weight gain resembles animal fed HFD. These may be due to a decrease in the accumulation of visceral fat in the tocotrienol-treated rats, which was proven by a decrease in the adiposity index in the current study (table 4- 7) and was consistent with what was demonstrated by Kato and colleagues in their study, which they conducted on mice, that indicated the reason for the lower weight in the tocotrienol group was due to a lower accumulation of perirenal fat compared to the group treated with a high-fat diet (Kato *et al*., 2022).While Shen and his group also recorded no differences in body weight and weight gain between the HFD group and the HFD+TT800 group over the course of the 14-week study in C57BL/6J mice fed either a high-fat diet (HFD control) or HFD supplemented with 800 mg annatto extracted TT/kg (HFD+TT800) (Shen *et al*., 2021).Otherwise, the weight gain therapeutic experiment showed non-significant differences among all the studied groups. These belong to exchange on diet introduced into rats of the study by removal of HFD after inducement of obesity.

Although reduce feed intake for the HFD and HFD supplemented with tocotrienol compared with control group, but the results appeared non-significant difference among them (table 4-5). This reduction continued during the last weeks of the experiment to record significant decrease for HFD and HFD supplemented with tocotrienol. The Significantly similar

values may be an indication of the animals' palatability of high-fat food and the decrease in the last two weeks due to the high energy provided by high-fat feed compared to low-fat feed despite the decrease in the amount of feed consumed referred to in Table (4-5), which subsequently leads to weight gain and its subsequent effects. This was in agreement with Díaz-Urbina *et al*.,(2018) study which showed that a high fat diet can easily lead to excessive food consumption, which can finally result in the development of obesity, due to the affectation of the homeostatic system. Because fat is the most energy-dense component of the diet, at 9 kcal/g, animals require less of an HFD meal to be satisfied on a gram basis than the control group. Obesity models using HFD therapy often show that animals in the intervention and control groups consume the same amount of energy on a daily basis (de Oliveira *et al*., 2014; Masi *et al*., 2017). If weight intake is not increased at least proportionally, this points to high fat diets' high energy density (Hariri and Thibault, 2010). In the therapeutic experiment after obesity was induced, the results showed a significant increase in quantity of the feed consumed in the obese group that was fed a diet low in fat and tocotrienols. This increase may be a result of the occurrence of obesity and thus weight gain and an increase in the energy required to be obtained from diet, consider the use of low-fat diet.

5.2- Effect of tocotrienols supplement on BMI , Lee index and AI in protective and therapeutic experiments.

Obesity in rats may be measured in the same way as it is in humans, using criteria based on weight gain and increasing body fat composition. According to Bastías-Pérez et al. (2020), the human body mass index (BMI) has never been established for rats. Most rodent studies quantify DIO through the comparison of the body weight of the experimental group to that of the untreated group, which is normally fed a low-fat diet (Bastías-Pérez *et al*., 2020). In the current study, all indicators considered to evaluate the

development of obesity agreed to a significant increase in the high-fat diet group when compared with the control group, and the use of tocotrienols improved these indicators compared to the high-fat diet group. Perhaps this result indicates the role of tocotrienols as an anti-inflammatory and antioxidant agent (Shen *et al*., 2021), which works to mitigate the damage resulting from consumption of high fat content in diet, and this finding agrees with Pervez *et al*.,(2022) study which showed a significant BMI reduction after administration of delta -tocotrienol in patients with non-alcoholic fatty liver disease. The anthropometric -reducing effects of δT3 may be due to a variety of processes, such as modulating lipid metabolism enzymes to decrease adipogenesis, inducing apoptosis in pre-adipocytes, and modifying energy sensing (Pang & Chin, 2019). Also, this result disagrees with Li *et al*. who showed in their result that tocotrienol consumption was not associated with BMI(Li *et al*., 2022 a). The Lee index was developed some decades ago to identify obesity in rats in a manner comparable to the BMI used to diagnose human obesity. The Lee index is calculated as the cube root of the body's weight (g) divided by naso-anal length (mm). Lee index values more than 310 g were regarded as a sign of obesity in rats (Bastías-Pérez *et al*., 2020). The current study showed that tocotrienols reduced the Lee index, and this indicates a preventive and improving role in reducing weight and body fat. The current results showed a decrease in the obesity index in the groups fed a diet supplemented with tocotrienols in the two trials: protection and therapeutic. This use of high-fat feeds may generate obesity which leads to increase feed consumption, therefore raising body weight and BMI, and resulting in a rise in body fat index fat mass. This is in agreement with Thamar (2014), Who concluded in their research that the use of high-fat feeds leads to increased body mass index and adiposity index.

5.3-Effect of tocotrienols supplement on relative weight of liver, kidneys and testes of obese induced in male rats as protective and therapeutic experiments:

The ratio of liver, kidney and testes weight to body weight affected by HFD and HFDT (protective groups) compared to the control group (table 4-8). An increase in liver weight in relation to body weight (hypertrophy) were observed in HFD and HFDT groups compared with the control group. Otherwise, testes weight recorded less significant values for the HFD and HFDT group, while kidney showed non-significant weight among the study groups. It could be linked to elevated triglyceride buildup leading to an enlarged liver, which could be due to an increased influx of fatty acids into the liver. Tocotrienol supplement group reduce the relative weight of the liver in the group but statistically showed non-significant difference with HFD group. The reduced relative weight of testes in rats fed HFD belong to the effect of high fat in feeding on hormones concentration and the studies investigated that high fat diet led to modulation in reproductive hormones (table 4- 15). In the therapeutic experiment, the relative weight of the liver showed a significant decrease in the tocotrienol group compared to the obese group, which indicates the therapeutic role of tocotrienol in reducing triglyceride levels, and enhancing the hepatic metabolism for the accumulated fat thus reducing the relative weight. The positive effects of tocotrienols may be due to the antioxidant role generated by free radicals (Jayusman *et al.,* 2017) and were consistent with what Zhao and his colleagues found (Zhao *et al.*, 2015a). Also, the current study showed a decrease in the relative weights of the testes in the experimental groups compared to the control, but tocotrienol supplementation cannot repair the testes weight when compared with HFD. This was consistent with the study of Zhao *et al*. who found γT3 treatment considerably reduced the weight of the liver but it had little effect on the weights of other tissues (Zhao *et al*.,

2015a). It may be due to the difference in the accumulation of tocotrienols in different tissues (Shibata *et al.*, 2012), thus exercising its role as an antioxidant. in the therapeutic experiment; Low kidney weight may be a result of the atrophy that cells have suffered as histological change in present study as a result of the harmful effects of obesity and tocotrienol enhance the tissue of kidney may be by antioxidant role in kidney (Dallner *et al.*, 2021). But this result is non-significant when compared with obese group.

5.4- Effect of tocotrienols supplementation on hematological indices in obese induced male rats as protective and therapeutic experiments.

Obesity is described as an imbalance between energy consumption and expenditure. (Tseng *et al*., 2010). Although an increase in adipocyte mass and number is a physical marker of obesity, the aspects of obesity that should be considered are adipose tissue malfunction and persistent low-grade inflammation (Aigner *et al*., 2014). These situations have unfavorable consequences, such as changes in iron metabolism, the immune system, and platelet activity, and they can also influence hematological parameters (Purdy & Shatzel, 2021). Obese individuals are more likely than thin individuals to develop iron deficiency anemia (Cepeda-lopez *et al*., 2010). Anemia is characterized by a drop in either the total amount of hemoglobin or the RBC count(Alia *et al*., 2019). Iron deficiency is associated with inflammatory markers because obesity is characterised by a systemic low-grade inflammation (Alia *et al*., 2019). Hepcidin expression in adipose tissue is powerfully induced by adipose-derived cytokines like IL-6 and IL-1.2 This elevation may reduce the amount of iron absorbed and reduce the efficiency of iron fortification (Ausk & Ioannou, 2008; Cepeda-lopez *et al*., 2010). The obtained result revealed that the examination of RBC-related parameters, such as RBC count, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin

concentration (MCHC) showed non-significant difference in comparison to the control group. This could be attributed to changes in hematological parameters caused by a high fat diet, which may necessitate more prolonged exposure. These results were identical to the results of Shawky, who used high fat diet for 6 weeks (Shawky, 2015), despite the difference in the components of the diet, the percentage of fat used and, type of fat and duration of exposure. These results were inconsistent with the results of Alia *et al*., (2019) who found that a subchronic high fat diet in mice may reduce the amount of RBC in the high fat diet group compared with the normal control group obesity in individuals is linked to an increase in blood cell count, which could increase blood viscosity (Dixon & O'Brien, 2006; Guiraudou *et al*., 2013). Also, obese mice (since, after a six-month high-fat diet) showed greater red blood cell and white blood cell levels, as well as an increase in packed cell volume PCV (Maysami *et al*., 2015).

In this study the slight significant decrease in the red blood cell with high fat diet in addition to tocotrienols., did not indicate the presence of anemia especially with the significant increase in the MCH value. This was consistent with the results of Tasaki *et al*., 2008). On the other hand, using tocotrienols with high-fat food reduces the number of red blood cells count and MCH, which in turn means improving the health condition resulting from reducing the viscosity that may arise from excessive formation of red blood cells. According to the RBC analysis, the concentrations of HB and PCV in the obese group were lower than in the control group in therapeutic experiment these situations may represent the nature of obesity as a low-grade inflammation that leads to iron deficiency anaemia(Ausk & Ioannou, 2008;Cepeda-lopez *et al*., 2010)

When compared to the control group, the number of WBCs increased in the HFD obese inducement group. This rise in WBC in the treatment groups could be due to an inflammatory response in the bone marrow (Suzana *et al*.,

2017). The amount of neutrophil was tended to increase in HFD group as compared with other groups. Tocotrienol tended to improve this condition in our experiment, and the role played by tocotrienols may be due to its properties as an antioxidant agent (Uzunhisarcikli & Kalender, 2011). Previous research has shown that providing high-fat diets to specific animals can greatly increase the amount of WBC, lymphocyte subsets as well as increasing blood viscosity (Herńandez *et al*., 2002; Hernández *et al*., 2004; Busnelli *et al*., 2013). Due to insufficient iron intake, chronic blood loss, or a combination of the two, iron deficiency anemia—which cannot produce enough iron to form normal RBC—is a condition that is well-known (Johnson Wimbley & Graham, 2011). Dilutional hypoferremia, insufficient dietary iron intake, elevated iron requirements, and/or impaired iron absorption can all contribute to iron deficiency anemia in overweight/obesity patients (Cepeda-lopez *et al*., 2010).

Mature adipocytes and stromal vascular fraction, which includes macrophages, make up adipose tissue. Adipose tissue macrophages (ATMs) are the name for the macrophages that live in adipose tissue (Heilbronn & Campbell, 2008; Patel *et al*., 2013). ATMs exhibit the anti-inflammatory macrophage (M2) in lean phenotype. The phenotype of macrophages changes in obesity from M2 to M1, a traditional pro-inflammatory macrophage (Patel *et al*., 2013; Exley *et al*., 2014). Numerous studies show a link between increased ATM accumulation and obesity-related illnesses like type 2 diabetes, cardiovascular disease, and fatty liver (Patel *et al*., 2013). Obesity affects the lymphoid tissues, the population and distribution of pro-inflammatory leucocytes, as well as the quantity of WBC, lymphocytes, neutrophils, and monocytes (De Heredia *et al*., 2012; Andersen *et al*., 2016). When inflammation and leucocyte activation occurred, the WBC count rose (Andersen *et al*., 2016).

We further performed hematology analysis on a lymphocyte, monocyte, and granulocyte percentage table (4-9). The amount of neutrophil tended to increase in obese group as compared with all treatment groups. These results were consistent with the results of Alia *et al*. who found sub-chronic high fat diet might alter the hematological parameters in mice (Alia *et al*., 2019). The slight increase of monocyte and granulocyte in the obese group showed that there was an acute inflammation occurred and tocotrienol tended to improve this condition in protective and therapeutic experiments. The improvement shown by tocotrienols may be due to the properties of tocotrienols as antioxidants and resistance to free radical formation(Jayusman *et al*., 2017).

5.5-Effect of tocotrienols supplement on liver enzymes and kidney functions in protective and therapeutic experiments

Liver enzymes such as ALT, AST, and ALP are primarily measured to assess liver damage (Ozer *et al*., 2008). The levels of AST and ALP in serum of the obese group were considerably raised during the trial, while there was no significant rise in ALT compared to the control group. Elevation of enzymes activities indicated necrosis of kidneys, skeletal muscles and liver can all contribute to an increase in ALT and AST levels (Lording & Friend, 1991). These liver enzyme readings may have increased, which might be a sign of liver injury or impairment (Welch *et al*., 2013). Obesity and liver disease brought on by underlying cellular death are frequently linked (Fernandez *et al*., 2012). These results are in agreement with the results reported by Marques *et al*., (2015) and Daffalla *et al*.,(2021) in rats fed with high fat diet . Liver of obese rats' were characterized by hepatic steatosis, which included fat accumulation in hepatocytes (Sinha-Hikim *et al*., 2017). Earlier research found that atherogenic diet-induced hepatic TG accumulation caused transaminase leakage such as ALT, ALP, and AST (Kameshwaran, 2011). Endoplasmic reticular, stress-mediated apoptosis, a

significant feature of liver damage, is one of the probable pathways for liver damage caused by a high-fat diet (Feng *et al*., 2003).

A diet high in calories causes lipid buildup, excessive production of inflammatory cytokines, and macrophage infiltration, all of which promote the progression of liver disease (Wei *et al*., 2007). Additionally, eating an HFD resulted in a marked increase in the liver enzymes AST, ALT, and ALP, as well as a significant decrease in the level of total protein (Uthandi and Ramasamy, 2011).

Tocotrienol supplementation may prevent liver damage caused by a high-fat diet by improving plasma AST and ALP activity in protective experiments. This was confirmed by the noticeable improvement in the tissues of rats treated with tocotrienol in the current study. In the therapeutic experiment, Tocotrienol supplementation was found to be effective in treating liver damage induced by a high fat diet, as evidenced by a significant drop in plasma ALP levels. Tocotrienol reduced ALT and AST levels in the treatment group, but statistically insignificant.

The alteration in the equilibrium between ROS and antioxidant levels has been linked to oxidative damage. As a result, tocotrienol's antioxidant activity may play an important role in preventing damage to cells (Jayusman *et al*., 2014).

It is believed that the hepatoprotective effect results from their antioxidant activity. As part of total antioxidant systems, non-enzymatic antioxidants such as vitamin E and C might relieve damage caused by oxidation (Uzunhisarcikli and Kalender, 2011). vitamin E has the ability to intercalate into cell membranes and scavenge chain-propagating peroxyl radicals due to strong lipid solubility. TRF from palm oil has been found as a good antioxidant capable of suppressing ROS production more effectively (Mutalib *et al*., 2003). Their uniform distribution in the lipid bilayer,

effective interaction of the chromanol ring with lipid radicals, and higher recycling efficiency from chromanoxyl radicals, all contribute to their greater antioxidant properties (Watson and Preedy, 2009). As a result, the administration of tocotrienol improved the majority of the biochemical indices in the current investigation.

The results of this study were consistent with some animal studies, like the study by Wong *et al*. (2012 b), when they found that T3 could enhance liver function by lowering AST and ALT levels in plasma brought on by a high-fat diet. Similar findings were made after it was discovered that supplementing with 400 mg of T3 daily could stop the advancement of end-stage liver disorders (Patel *et al*., 2012). While studying Ima-Nirwana *et al*. (2011), they concluded that T3 treatment at dosages of 200 mg/kg, 500 mg/kg, and 1000 mg/kg caused no hepatotoxicity in mice after fourteen and forty-two days of tocotrienol administration, respectively. Nesaretnam *et al*. (2010) conducted a human experiment that also demonstrated that tocotrienol treatment at 200 mg/day did not significantly alter the liver function profile. Lin and his colleagues found no significant changes in the enzymes ALP, AST, and ALT, as well as GGT concentrations, following the tocotrienol supplement (Lin *et al*., 2016). This indicates that tocotrienols either improve liver enzymes or no change occurs, indicating that tocotrienols are not toxic when consumed within the recommended doses.

On the other hand, elevated blood urea levels may be related to kidney illnesses such as glomerulonephritis, urinary tract blockage, and excessive protein catabolism, which can lead to severe toxic and febrile situations (Anosa, 1988). An appropriate explanation for the raised urea levels is increased protein catabolism and quicker amino acid the deamination during gluconeogenesis (Bishop, 2020). This suggests that tocotrienols were not protective against the potential harm caused by a high-fat diet.

5.6- Effect of tocotrienols supplement on total proteins, albumin, globulin, and C-reactive protein levels in protective and therapeutic experiments

Metabolic the variables such as total protein, albumin, globulin, and albumin to globulin ratio are frequently associated with liver functionality, including organic anion transport, endogenous and exogenous substance clearance from blood circulation, and hepatic synthetic function (Khalili *et al.*, 2011). Reduced blood albumin levels might suggest liver impairment (Fabry and Narasimhan, 2006). The current study found an increase in total protein levels in the high fat diet group compared to the control group. This rise was caused by an increase in globulin levels, which resulted in an increase in total protein. But the reduction in total protein level in protective group accompanied by reduced in globulin level when compared to the control group, which indicates a mitigation of the effects of high-fat diet. These findings were in agreement with the findings of Ghasi and colleagues, who discovered that eating high-fat foods increased total protein while also decreasing albumin levels (Ghasi *et al*., 2000). Gabuza *et al.*, (2020) expected that the production of proteins will increase with the increase in the consumption of fats and the increase in their levels in the body, as a result of the different biological functions of proteins that are included as a signal of satiety and control food intake and body weight, in addition to their participation in the transport of fats the current study's high level of total protein for the high-fat diet group to develop obesity contradicted Shawky's study, which found blood protein levels were lowered in rats given HFD (Shawky, 2015). The explanation for the drop was linked to protein depletion caused by localized injury in the endoplasmic reticulum or the hazard impact of energy released during HFD metabolism (Uthandi and Ramasamy, 2011).

According to a study, the incidence of hypertension is positively correlated with parameters like body weight, heart rate, alcohol intake,

carbohydrate dysmetabolism, hematocrit, and hemoglobin as well as serum total proteins and total triglycerides ,therefore in overweight and obese individuals, a rise in serum proteins would be a factor in the development of prehypertension and hypertension (Madhuvanthi & Lathadevi, 2016) . Marques *et al.* (2016) reported that serum albumin levels decreased after HF meals, indicating that albumin, a negative acute phase protein, may be lowered in inflammatory situations such as obesity. It was inconsistent with the current study. Perhaps because our study was a long-term study that lasted 8 and 12 weeks in protective and therapeutic studies, respectively. The use of tocotrienols in our current study resulted in a decrease in total protein levels, which contradicted the findings of Lin *et al.* (2016), who found no significant difference between TRF and placebo interventions on total protein, albumin, globulin, total bilirubin levels, and the albumin-to-globulin ratios.

In the therapeutic experiment, the increase was significant only in albumin, and tocotrienols led to an improvement in its levels compared with the control. This was compatible with the Jayusman *et al.* study whom noted that tocotrienol rich fraction supplementation significantly decreased the level of total protein compared as fenitrothion-treated rats group (Jayusman *et al*., 2014).

CRP is a sensitive acute-phase measure for tissue injury, infection, and systemic inflammation (Pepys & Hirschfield,2003; Musialik *et al*., 2017). Individuals with dual symptoms of insulin resistance, overt T2D (Greenfield *et al.*,2004) overweight, obesity (Pepys & Hirschfield,2003) and metabolic syndrome (Musialik *et al.*, 2017) frequently have elevated CRP levels. Although the physiological processes underlying the relationship between increased CRP and various illnesses are still unclear, it is thought that adipose tissue plays a role in mediating it (Zhao *et al.*, 2008). Unfortunately, CRP cannot be used to identify illness alone since it is part of the nonspecific

acute-phase response to many disorders (Pradhan *et al.* 2001). The current study found that CRP levels were higher in the obese group than in the control group, and that tocotrienols reduced CRP levels, however this did not appear statistically significant compared to the obesity group.

5.7-Effect of tocotrienols supplement on glucose and insulin hormone in protective and therapeutic experiments

The supplementation of tocotrienol in the protective group of the present study reduced the harmful effect of a high-fat diet on glucose levels, although it was still non-significant in the high-fat diet group table (4-12). On the other hand, the use of tocotrienol enhance insulin concentration compared to the HFD group table (4-14). This result is in agreement with study of Patel *et al.* (2011) who showed that tocotrienols enhanced metabolic functions such as insulin sensitivity and glucose utilization.

As for the therapeutic experiment, the levels of the hormone insulin were not significant among the groups, which indicates that the low-fat diet has a lesser role in provoking insulin resistance, which was evident through the rise in glucose and insulin hormone levels in the protection experiment under the influence of high-fat food Triacylglycerol (TAG) content is the most obvious indicator of a lipid content rise, and it's possible that TAGs simply serve as a sign of abnormal muscle glucose metabolism. Also, hyperlipidemia causes visceral fat to lipolyze, resulting in an accumulation of free fatty acids that inhibit insulin release from pancreatic -cells (El-Atat *et al*., 2004).

The observed decline in insulin sensitivity is more likely to be caused by the buildup of bioactive lipids or lipid intermediates, such as diacylglycerol (DAGs), ceramides, acylcarnitines, and/or acyl-CoAs (Adams *et al*., 2004; Itani *et al*., 2005; Holland *et al*., 2007; Bosma *et al*., 2012). It has been demonstrated that increased intracellular DAG levels brought on by fat

consumption trigger protein kinase C (PKC) signaling, which then causes insulin receptor substrate 1 (IRS-1) to be serine phosphorylated. This inhibits the phosphorylation of tyrosine residues on IRS-1, impairing canonical phosphatidylinositol-3 kinase/Akt signaling. This also reduces the translocation of the glucose transporter GLUT-4, which in turn reduces insulin-stimulated glucose uptake (Itani *et al*., 2005;Erion & Shulman, 2010). Leptin resistance and hyperleptinemia are important contributors to insulin resistance (El-Atat *et al*., 2004).

The results of the current study imply that consumption of tocotrienol significantly suppressed the hyperglycemic and hyperinsulinemia responses to HFD in obese rats. These observations are in line with previous studies that have reported tocotrienol rich fraction could improve glycemic control in poor glycemic conditions (Fang *et al.,* 2010; Qureshi *et al.,* 2011; Zhao *et al*., 2015 ;Allen *et al*., 2017), although the hypoglycemic effect of tocotrienol rich fraction was not observed in some investigation (Stonehouse et al., 2016). The stimulatory effect of tocotrienol on PPARs activation (Chung *et al*., 2019), GLUT2 expression in pancreatic β cells, and consequently, glucose interring into β cells (Chia *et al*., 2016) are some of the suggested mechanisms for insulin resistance improvement by tocotrienol. Also, this result may be due to the role of tocotrienol in protecting pancreatic beta cells from inflammation in obesity and hyperglycemia (Kim *et al*., 2018). as well as the overexpression of some insulin gene transcription factors, the regulation of the insulin signaling pathway, and the enhancement of glucose uptake are reported in some other studies (Chia *et al*., 2016; Lee & Lim, 2018).

Hypertriglyceridemia is another manifestation of insulin resistance(Eckel *et al*., 2010). A glucose tolerance defect caused by insulin's failure to enhance glucose uptake and metabolism in insulin-sensitive tissues (Davidson *et al*., 2010; Davidson *et al*., 2011). This indicates that glucose

metabolism and insulin resistance are linked to fat metabolism, which was one of the most prominent effects of high-fat diet in the current study.

5.8-Effect of tocotrienols supplement on lipid profile in protective and therapeutic experiments

The triglyceride, LDL and VLDL levels increased significantly ($P<0.05$) as prognosis of feeding the animals with formula of high fat diet, whereas tocotrienol addition reduced the LDL, triglyceride and elevate HDL levels (table 4-13). Previous research has shown that a high fat consumption increases the quantity of lipids in the enterocytes (Uchida *et al*., 2011). They relate the reasons of increased fat to changes in the expression of food transporters, which might limit intracellular lipid production. While Allen *et al*. (2017) found that mice given HF had more lipid droplets in the liver and greater blood triglycerides than other groups. The increased triglyceride content in rats given a high-fat diet might be related to reduced triglyceride clearance as a result of impaired lipoprotein lipase activity (Nofer *et al*., 2002). Furthermore, our results are in agreement with Garcia *et al*. (2018), who observed that rats fed a high-fat diet had no alteration in their levels of cholesterol. However, this is in finding disagreement with other studies that discovered the content of cholesterol in rats fed a high-fat diet was higher than in normal healthy control rats (Kayhan *et al*., 2014; Amin *et al*., 2015). The difference in impact might have been due to variations in the proportion of fat added to the diet and the length of the intervention.

One of the diagnostic criteria for the metabolic syndrome is hypertriglyceridemia, which arises in rats fed a high-fat diet. Elevated circulating triglyceride levels might be produced by the overproduction of triglyceride-rich very low-density lipoproteins (VLDL) as a result of increased liver-derived free fatty acid flow (induced by an increase in adipose tissue mass) (Eckel *et al*., 2010). They also identified

hypertriglyceridemia as a sign of insulin resistance owing to decrease in glucose tolerance, which is connected to insulin's failure to increase glucose uptake and utilization by insulin-sensitive tissues. There is evidence relating a diet high in fat to a greater amount of droplets of lipid in the liver cells, which occur by integrating lipids into bile and creating VLDL, which is controlled by the liver (Onal *et al*., 2017). The dyslipidemia was characterized as elevated plasma triglycerides, low HDL levels, LDL-C, and increased VLDL production together with a blockage in their release, increasing the risk of hepatic fat buildup, eventually culminating in non-alcoholic fatty liver disease (NAFLD) (Adams *et al*., 2017).

The present study showed that using of tocotrienols improved triglyceride levels when compared to the HFD group. Tocotrienols, which are important dietary components, have been proven to change numerous metabolic syndrome features, including blood pressure, blood glucose levels, and lipid profiles. (Weng-Yew & Brown, 2011). Instead, then treating each risk factor separately, which increases the danger of polypharmacy, treating the metabolic disorder with tocotrienols may ameliorate its different symptoms, such as obesity, insulin resistance, and cardiovascular disease (Grundy, 2006)Tocotrienols influence the expression of genes and proteins encoding fatty acid synthase (FASN), carnitine palmitoyltransferase 1 (CPT1A), and cytochrome P450 3A4 (CYP3A4), lowering TG accumulation (Burdeos *et al*. 2012). A hypothesis that proposes that dietary T3's ability to reduce TG synthesis in human hepatoma cells (HepG2) may be the main benefit (Zaiden *et al*., 2010). Allen *et al*. (2017). found that tocotrienols decreased hepatic steatosis and blood triglycerides in δT3-supplemented groups compared to the HF group, which is consistent with our findings.

Tocotrienols enhance metabolic disorders such as insulin hormone and glucose level in the present study; this may result in decreased plasma triglycerides and non-esterified fatty acids. Since insulin is a powerful

suppressor of circulating non-esterified fatty acid (NEFA) concentrations through suppression of hormone-sensitive lipase and up-regulation of lipoprotein lipase (Coppack *et al*., 1992), and the preservation of a consistent rate of fatty acid free re-esterification, in addition to improving glucose uptake and glycolysis, switching energy production from predominate fat oxidation to predominate carbohydrate utilization (Ferrannini *et al*., 1997).

Burdeos *et al*., (2012a) verified the TG-lowering impact of tocotrienol in both in vivo and in vitro tests, and the effect of tocotrienol might be partially explained by its capacity to down-regulate the hepatic fatty acid synthase gene and up-regulate b-oxidation genes.

Wong *et al*., (2012b) indicated that TRF reduced triglyceride and non-esterified fatty acids NEFA but not total cholesterol concentrations. In the current study, HDL-C levels increased in the obese group, but this increase was not significant compared to the control group. This result was consistent with those published by Daffalla *et al*., (2021) and Amin *et al*., (2015), who found that HDL-C concentrations rose in rats fed a high fat diet. However, this discovery contradicted the results published by Garcia *et al*. (2018) and Ali *et al*. (2019), who found that HDL levels were lower in rats on a high fat diet.

The most obvious sign of dyslipidemia is an increase in TAG in very low-density lipoproteins (VLDL-Ch), which are the primary transporters of endogenous fat synthesized in the liver (Birulina Julia *et al*., 2020).The role of tocotrienols in regulating fat metabolism may be due to increase gene expression of carnitine palmitoyltransferase 1 (CPT1A) involved in fatty acid metabolism and our current study confirmed that and this result agree with Burdeos *et al*., (2012), who found that tocotrienol reduces TG accumulation via modulating fatty acid synthase (FASN), carnitine palmitoyltransferase 1 (CPT1A), and cytochrome P450 3A4 (CYP3A4) gene and protein expression.

5.9-Effect of tocotrienols supplement on Irisin, Leptin, Adiponectin in protective and therapeutic experiments.

Adipose tissue secretes polypeptide hormones such as adiponectin and leptin, which contribute to the evolution of obesity-related disorders such as high blood pressure, cardiovascular disease, and type 2 diabetes (Zahorska, 2006). Circulating levels of leptin, which is primarily secreted by adipocytes, rise in obesity, but its effect on reducing food intake and increasing energy consumption at the hypothalamic level (Koch *et al*., 2014). The current investigation found that leptin levels rose in the diet-induced obesity group compared to the control group in a protective experiment. This might be regarded as an attempt to overcome leptin resistance, which can worsen hyperphagia and obesity (Marques *et al*., 2016). Furthermore, plasma leptin concentration increases proportionally with body fat mass (Klein *et al*., 1996). despite increased leptin concentration, the efficacy of the anorexic effect of leptin is decreased (Farr *et al*., 2015; Morioka *et al*., 2016), with leptin resistance developing due to a defect in intracellular signaling associated with the leptin receptor or decreases in leptin transport across the blood–brain barrier (BBB)(Banks, 2012).

Leptin resistance can also be developed at the BBB, thereby allowing unregulated transport of leptin from the blood to the brain. Brain blood vessels express short forms of *OBR*, which bind leptin and transport it from blood to the interstitial tissue of the brain and into the cerebrospinal fluid (Tartaglia, 1995; Chumakova *et al*.,2015). At serum leptin levels above the range of 25–30 ng/mL, the concentration of leptin in brain tissues and cerebrospinal fluid does not increase (Holtkamp *et al*., 2004). This phenomenon likely plays a role in the development of leptin resistance and obesity, where excessive levels of leptin in the blood result in decreased BBB permeability (Mantzoros, 1999). In the spinal fluid of obese individuals,

decreased leptin concentrations were observed under these conditions (Philbrick *et al*.,2017).

However, obese patients have a high amount of circulating leptin due to leptin resistance (Guerre-Millo, 2003). Leptin influences brain development, immunological function, reproduction, bone density, hemodynamics, respiratory function, sympathetic nerve activity, and insulin levels in the liver (Hekimoğlu, 2006). It contains receptors in both peripheral tissues and the brain.

These findings contradicted the Rocha-Rodrigues *et al*. (2018), which found that HFD 71 Kcal% fat for 17 weeks raised epididymal adipose tissue eWAT leptin levels but had no effect on plasma leptin.

At the end of the trial, animals fed a high-fat diet exhibited greater adiponectin plasma levels. This is in agreement with other studies that show an increase in Adiponectin plasma levels in rats after twenty-four and thirty-two weeks of HF diet supplement (Davidson *et al*., 2010, 2011). Adiponectin is known for its insulin sensitizing action; however, obesity has been linked to a dysfunction in adiponectin signaling (adiponectin resistance) (Scheid & Sweeney, 2014). Adiponectin concentrations are low in obese patients and markedly increase after prolonged caloric restriction, such as in dieting and anorexia nervosa (Delporte *et al*., 2003), and are significantly lower ($P < 0.001$) in obese subjects when compared with nonobese males and females (Arita *et al*., 1999). Adiponectin is more closely associated with visceral fat than with subcutaneous fat (Cnop *et al*.,2003) and is lower in type 2 diabetes, cardiovascular disease, hypertension, and metabolic syndrome (Arita *et al*., 1999; Bluher *et al*., 2004; Li *et al*., 2009), conditions that are often associated with insulin resistance. A recent meta-analysis of prospective studies with a total of 14,598 subjects and 2623 cases of type 2 diabetes indicated that higher adiponectin concentrations were associated with a lower risk of type 2 diabetes. The estimated absolute risk difference (cases/1000 person-

years)/1-log μg/mL increment in adiponectin concentrations was 3.9 for elderly Americans and 30.8 for Americans with impaired glucose tolerance (Li *et al*., 2009). Furthermore, adiponectin is positively correlated with HDL cholesterol and negatively associated with serum triglycerides and apolipoprotein B-100 (Schulze *et al*., 2005). Adiponectin shows anti-inflammatory effects by enhancing nitric oxide production and activating endothelial nitric oxide synthase (Chen *et al*., 2003) and may act as a modulator of vascular remodeling by suppressing smooth muscle cell migration (Arita *et al*., 2002), which possibly plays a role in the regulation of atherosclerosis.

The current study also showed that the use of tocotrienols decreased the blood levels of leptin and adiponectin, but the reduction was not significant in compared to the diet-induced obese group, and this was consistent with what Kok-Yong found, which showed that oral administration of annatto tocotrienol AnTT at 60 or 100 mg/kg (n = 8/group) after treatment for 12 weeks, with buserelin, AnTT does not affect adiponectin and leptin levels in male rats (Kok-Yong, 2019). While a previous study showed that Leptin protein content was significantly reduced by delta tocotrienol in the T400 group but not the T1600 group When compared to mice fed a high fat diet, there were no variations in blood levels of the anti-inflammatory adipokine adiponectin across the groups (Allen *et al.*, 2017).Furthermore, T3 supplementation (six hundred mg/day) for six months significantly reduced liver enzymes, inflammatory markers, oxidative stress, leptin, FLI, and hepatic steatosis compared to placebo (Pervez *et al.*, 2020). The variation in impact of tocotrienols might be attributed to the varying length of the intervention.

The results of our current study showed that the level of the hormone irisin increased in the obesity-inducing group by high-fat diet, compared with the control group. Irisin was initially identified as a protective factor

against diet-induced weight gain; however, several studies have examined the relationship of circulating irisin with Obesity in humans, implying a role mediated by Irisin is involved in the browning of white adipose tissue (WAT) and increasing energy consumption(Rasheed *et al*., 2022). Adipocyte browning increases energy expenditure in subcutaneous adipose tissue by increasing uncoupling protein 1 gene expression (Boström *et al*., 2012). The high level of irisin may be due to the high body mass index, serum triglycerides, and liver enzymes. The results of the current study agreed with what was demonstrated by Rizk *et al*. when showed that serum irisin level increased in patients with metabolic syndrome especially those with elevated liver enzymes and had a positive correlation with parameters of lipid metabolism and glucose homeostasis with the possibility of hepatic clearance to irisin(Rizk *et al*., 2016). A previous study indicated that Weight loss lowers irisin levels, which return after regaining the lost weight (Polyzos *et al*., 2018).

Irisin levels have been shown to correlate positively with leptin and negatively with adiponectin (Palacios-Gonzalez *et al*.,2015; Nigro *et al*., 2017). There is a direct interaction between leptin and irisin, thought to be unlikely, because leptin administration in humans have no effect on circulating irisin (Gavrieli *et al*., 2016).

Obesity raises irisin levels the higher fat mass and, presumably, the higher concentration of adipose tissue derived irisin may explain this increase. It could be a counterbalancing mechanism to increase energy expenditure and improve insulin sensitivity, but more research is needed. It may also indicate irisin resistance, similar to insulin and leptin resistance seen in obesity and T2DM (Polyzos *et al*., 2013). The current study showed that the use of a tocotrienol supplement with a high-fat diet led to a decrease in the level of the hormone irisin, but did not reach a significant difference compared with the HFD group. But it became non-significant different When

compared with the control group. This result consists of Irandoost *et al. (2020).* When combining RJ and a tocotrienol-rich fraction with HFD, irisin concentration was found to be markedly elevated in the RJ and RJ plus TRF groups compared to HFD, but not in the TRF group. Which may indicate that the improving role of tocotrienols follows mechanisms that are independent of irisin.

5.10 Effect of tocotrienols supplement on some sex hormones in protective and therapeutic experiments.

Obesity is thought to be emerging as a significant contributor to adverse health outcomes, including male infertility, as sedentary lifestyles and dietary changes become more common (Fernandez *et al*., 2011).

Obesity can affect seminal parameters as well as other aspects of male fertility (Kahn & Brannigan, 2017). Additionally, it has been shown that having a higher BMI is linked to poorer sperm quality, such as decreased sperm concentration, abnormal sperm morphology, and decreased sperm motility (Yardimci *et al.*, 2022). The luteinizing hormone (LH), which is produced by the anterior pituitary gland, plays a crucial role in the regulation of the gonads. In males, LH stimulates the Leydig cells in the testes to produce and release testosterone, which is a key hormone involved in the development and maintenance of male reproductive tissues and the regulation of secondary sexual characteristics (Hall & Hall, 2020) . This process is known as the hypothalamic-pituitary-gonadal axis, which is essential for the regulation of reproductive function.

In this study, an obesity model induced by a high-fat diet was used. Regarding the effects of HFD-induced obesity on levels of reproductive hormones in male rats, serum LH significantly decreased in HFD and HFDT groups compare with control group and no significant changes in the serum testosterone and FSH levels were observed between different groups,

although reduce they value in in HFD and HFDT groups but these change non-significant. the impact of obesity on the LH hormone may be resulted from several mechanisms, including increased estrogen production in excess fat (Bhardwaj *et al.,* 2019) , which can affect the balance of sex hormones in the body. In addition, obesity-related inflammation can affect the hormonal system (Goldsammler *et al*., 2018) and reduce the secretion of LH hormone. tocotrienols are an antioxidant that may benefit the reproductive system, but its effects on FSH and LH levels are not well-documented. A study on female mice found that δ-tocotrienol supplementation increased FSH and LH levels, suggesting a potential for improved hormonal regulation in females (Mohamad Na'im *et al.,* 2023). The use of tocotrienol led to an increase in LH hormone levels in this study, but it did not reach statistical significance compared to the high-fat diet group. In this study there were not statistically significant difference in levels of testosterone hormone between the HFD and control groups. The results of the current study were consistent with the results (Shawky, 2015), which found that male rats fed a high-fat diet for 6 weeks showed no significant difference in serum testosterone levels between HFD animals and control animals, while there was a significant increase in MDA levels and a significant decrease in SOD in the HFD group in comparison with the control. The current study disagrees with Vigueras-Villaseor *et al.* (2011), who found that Sprague-Dawley rats fed HFD from weaning to 90 days had lower testosterone levels. Also disagreed Bakos *et al*. (2010), who found male mice fed HFD (for 9 weeks) had lower testosterone levels were higher than those in the control group.

5.11 Effect of tocotrienols supplement on seminal analysis and reproductive efficiency in protective and therapeutic experiments.

Obesity is a public health issue due to its rising global prevalence. Obesity has a negative impact on reproduction, which is one of the health

consequences. Some studies have found a link between obesity and infertility, but it is still debatable (Barbagallo *et al*., 2021).

According to Du Plessis *et al*. (2010), obesity is an important risk factor for male infertility. Tortoriello *et al*. (2004) discovered no reduction in the male mice's fertility after feeding them HFD. The result of the current study revealed that sperm account and motility recorded a severe and significant reduction in their values for rats induced obesity. This was consistent with studies by Ghanayem *et al*. (2010) and Bakos *et al*. (2010), which found that mice given HFD had worse sperm motility, fertilization rate, and pregnancy rate. This effect may result from increased sperm DNA damage and intracellular reactive oxygen species (Ghanayem *et al*., 2010). Obesity can cause oxidative stress and increase testicular oxidative stress (Shawky, 2015). And this result may be due to a high-fat diet and elevated testicular malondialdehyde (MDA) levels (Galaly *et al*. 2014). Tocotrienol supplement introduced its ability to improve motility and account for sperm values, whether in protective or therapeutic experiments. Although there was a significant difference between protective and therapeutic experiments, it still appeared to have significantly lower values of sperm motility and account than the control group. The ameliorative effect of the administration of vitamin E protects against testicular oxidative stress and injuries (Al-Attar, 2011).In this study dead and abnormal sperms in table (4-16) investigated the deleterious effect of obesity on sperm viability, by recorded high significant percent for dead and abnormal sperms when compared with all studied groups, and this finding agreed with (Alzubaidi & Al Diwan, 2013), who found that the high diet cholesterol rats group have reduced sperm concentration ,sperm motility, dead sperm , and abnormal sperm compared with control values. While tocotrienol supplement ameliorated the deleterious effect of obesity in protective and therapeutic dose and appeared significantly less than control group. This finding agreed with Jegede *et al*.

(2015) , who found that Red Palm Oil RPO has a potential to attenuate the toxic effect of lead on testicular cells preventing possible resultant male infertility. Also, this result was consistent with a previous study examining the effects of vitamin E who showed that vitamin E is able to compensate the toxic effects of p-NP on testis weight, sperm number, sperm motility and estrogen level, and increases sperm viability in developing rat(Moumeni *et al*., 2009). In our study pregnancy rate and number of births reduced in HFD group compared with another group. This result is in agreement with Ghanayem *et al*. (2010) and Bakos *et al*. (2010), which found that mice given HFD had low fertilization rate, and pregnancy rate. The improving role of tocotrienols was reflected in the results of female reproductive capabilities and led to a significant increase in the pregnancy rate and number of births.

5.12 Effect of tocotrienols on histopathological effects of obesity

The liver plays important roles in detoxification, metabolism, and storage (Hsu *et al*., 2024). The histopathological examination of liver in rats fed high fat diet- induced obese for 12 weeks suffered from pathological lesions such as severe peri central vein vacuolation of hepatocytes (figure 4-2) and this result agreements with Thamer (2014), who indicated that feeding rats with a high-fat diet leads to histological liver changes characterized by the accumulation of fat droplets within the cytoplasm of parenchyma cells, along with inflammation and an increase in the number of Kupffer cells. These results may to increased saturated fatty acids in the liver Also, Stimuli can cause inflammation and collagen deposition in the liver, impairing liver function and raising the risk of damage and disease(Hsu *et al*., 2024). Also, it reflect liver excessive release of lipid from hepatocytes that had chemotactic activity for interleukin-8 which promoting tissue inflammation (Thamer, 2014). This was confirmed by the results of the current study, which included increased gene expression of the IL-1β gene that associated with inflammation. In present study the histopathological examination of

rats' liver that fed high fat diet and supplemented with tocotrienol for 12 weeks showed hepatic changes less than rats fed high fat diet alone such as early regenerated hepatocytes and normal sinusoids. This may indicate that tocotrienol has potent anti-inflammatory and antioxidant properties and may reduce liver injury. This result agreement with study of Allen *et al.* (2017), who found that tocotrienol supplementation improved liver histology by reducing liver damage and inflammation resulted from increased markers of fatty acid oxidation and reduced markers of fatty acid synthesis in adipose tissue and liver. This result may be because the unsaturated side chain of tocotrienol allows for more efficient penetration into the tissues with saturated fat layers, such as liver (Ahsan *et al*., 2014). In a study, δ-tocotrienol is the most potent isoform of vitamin E (Vasanthi *et al*., 2012). Tocotrienol supplementation could prevent liver damage caused by a high-fat diet, according to the current research. It is probable that their antioxidant activity is responsible for their hepatoprotective impact. Non-enzymatic antioxidants such as vitamins C and E, as part of total antioxidant systems, may help to reduce oxidative damage (Uzunhisarcikli & Kalender, 2011). This was confirmed by the noticeable improvement in the tissues of rats treated with tocotrienol in the current study Which indicates that tocotrienols did reduce damage but not prevent it, that may arise from intake of high diet in fat. Our study agreed with Nakamura *et al*. 's study that reported slight hepatocyte hypertrophy in male rats treated with tocotrienols at doses above 0.75% for 13 weeks it is part of a study of tocotrienol on rats at high doses(Nakamura *et al*., 2001). Obesity is a complex health problem that can lead to a number of health problems, including kidney disease (Wickman & Kramer, 2013). According to the National Kidney Foundation, being overweight or obese increases the risk of developing type 2 diabetes and high blood pressure, the two leading causes of kidney disease (Wickman & Kramer, 2013). Excess fatty tissue releases inflammatory molecules that can damage sensitive kidney structures and accelerate the progression of kidney

disease (Khor *et al*., 2021). Obesity has a hemodynamic influence on the kidneys, causing glomerular hyperfiltration and microalbuminuria. It was demonstrated that dogs with obesity generated by a high fat diet had a 30% increase in glomerular filtration rate five weeks after the induction process, as well as amplification of Bowman's capsule and a rise in mesangial matrix after nine weeks (Henegar *et al*., 2001). Alteration in lipoprotein synthesis and the level of cholesterol in the blood have also been associated to poor renal function (Garcia *et al*., 2018). Furthermore, excessive cholesterol and obesity have been associated to glomerular shape alterations in the late stages of kidney disease (Sasatomi *et al*., 2001).Furthermore, obesity often accompanies conditions such as diabetes and high blood pressure, which increases risks to kidney health (Yim & Yoo, 2021). Our current study showed some effects on the kidneys caused by high-fat diet, including: moderate atrophy of glomeruli and dilation of renal tubules as in the figure (4-7) and used of tocotrienol led to normal glomeruli and mild dilation of renal tubules. Our findings are consistent with the findings of the Gupta and Chopra studies, which found that pretreatment with tocotrienol (50 mg/kg/day) for 7 days before Fe-NTA administration in rats protects the kidneys from ferric nitrilotriacetate (Fe-NTA) toxicity, a well-established nephrotoxic agent, by significantly lowering serum creatinine and BUN levels, reducing lipid peroxidation, and restoring kidney tissue histology (Gupta & Chopra, 2009). Also, this result agreements with Siddiqui *et al.* (2010), who showed that administration of tocotrienol rich fraction (TRF) from palm oil and rice bran oil for 8 weeks improved structural abnormalities were seen in the glomerulus and tubules of diabetic rats, indicating an effective protection offered by TRF in diabetic rats and they suggest that tocotrienols rols by the virtue of their hypoglycemic activity, thus preventing hyperglycemia induced glomerular matrix accumulation. Therefore, tocotrienol has a positive effect on kidney tissue histology and can be used to prevent kidney damage caused by nephrotoxic agents. It may be due to the

accumulation of tocotrienols within the kidney tissue and role as antioxidant, as indicated by previous report (Shibata *et al*., 2012), who indicated that the tissue distribution of T3 depends on the affinity of T3 to vitamin E-binding proteins as well as the cytochrome P450 expression level in organ. Histological micrograph of kidney of obese group moderate atrophy of glomeruli and dilation of renal tubules as shown in Figure (4-9) and used tocotrienol in therapeutic group led to atrophy of glomeruli and dilation of renal tubules . Current ideas on the mechanism of action of tocotrienol on the kidneys focus mostly on oxidative and inflammatory stress, however this may not be the complete picture. This was corroborated by a pilot research, which indicated that while renal function improved significantly after three months of supplementation, there was no association between renal function and oxidative or inflammatory stress indicators (Tan *et al*., 2018; Tan *et al*., 2019). In the current study, the high fat diet and obese groups suffered from suppression of spermatogenesis and vacuolation of seminiferous tubules as a result of the harmful effects of obesity resulting from increased the amount of oxidative stress in the reproductive system, which is stress brought on by an increase in the number of molecules containing free oxygen as well as Malondialdehyde (MDA) concentrations in testes were elevated by a high-fat diet (Galaly *et al*., 2014). In our study, the histological micrograph of the testis of the HFDT (protective) group showed normal spermatogenesis, and the use of tocotrienols as therapeutic agents has led to mild suppression of spermatogenesis and vacuolation of seminiferous tubules, which indicates the protective and therapeutic roles that tocotrienols play against the damage caused by a high-fat diet. This was consistent with Taib and his colleagues, which showed that palm oil tocotrienol-rich fraction (TRF) has potential to reduce oxidative stress under various pathological conditions, such as exposure to organophosphates (fenitrothion), which it have been reported to cause testicular oxidative damage (Taib *et al*., 2015). This result may by potent antioxidant ability of tocotrienol, thus protecting against

histopathological alterations. Also, TRF can potentially reduce the expression of Heat shock protein 70 HSP70, thereby reducing apoptotic damage to germ cells, increasing testicular function, and attenuating morphological changes in FNT-treated rats (Taib *et al*., 2015).

5.13 Effect of tocotrienol supplement on gene expression

In the present study we examined the effect of annatto tocotrienol on Expression of genes for enzymes that regulate lipid metabolism carnitine palmitoyl transferase 1CPT1 and factor related to ES stress and inflammation Interleukin-1beta IL-1β. our result showed that tocotrienol increased gene expression of the cpt1b gene, it was 10.17-fold greater in the HFDT protective group and 2.54-fold higher in the therapeutic group compared to the control group and decreased The gene expression of the IL-1β gene as follow it was 8.85-fold greater in the HFD group, 2.67-fold higher in the HFDT (protective group), and 5.09-fold higher in the therapeutic group compared to the control group. These results indicate that tocotrienols increased the expression of the cpt1b gene while decreasing the expression of the IL-1βgene. Carnitine palmitoyl transferase 1 (CPT1), found in mitochondria, is the rate-limiting enzyme for fatty acid oxidation. It contains three isoforms: CPT1A, CPT1B, and CPT1C (Muto *et al*. 2013). The CPT1B gene is a gene that encodes a protein called mitochondrial carnitine 1B. This protein plays an important role in the process of transporting fatty acids into the mitochondria, where they are converted into energy and Disruption of this gene may lead to metabolic disorders (Muto *et al*., 2013). Burdeos *et al*. found that γ-T3 reduced TG accumulation via modulating gene and protein expressions of FAS, CPT1, and CYP3A4 in the HepG2 human liver cell line. (Burdeos *et al*., 2012b). Muto *et al*. (2013) found that γ-T3 did not impact the expression of mRNA for SREBP-1c or CPT1A, indicating that it was ineffective to prevent excessive lipid buildup in hepatocytes. γ-T3 inhibits hepatic fatty acid synthase (FAS) while increasing β-oxidation genes

(CPT1A and CYP3A4). may contribute to its TG-lowering impact in HepG2 cells (Yachi *et al*., 2010;Burdeos *et al*., 2012b). Our study is in agreement with Muto *et al*. who observed that γ-T3 lowered hepatic TG content in normal rat primary hepatocytes by up-regulation of CPT1A and down-regulation of SREBP-1c (Muto *et al*., 2013).

Metabolic Syndrome -related adipocyte dysfunction causes macrophage buildup in adipose tissue, leading to increased pro-inflammatory cytokine production and systemic inflammation (Srikanthan *et al*., 2016). Diabetic individuals with poor glucose metabolism generally have elevated levels of inflammatory markers such IL-1β, IL-6, and TNF-α (Li *et al.*, 2014). Adipose tissue, often known as fat, serves as a store of energy and is a source of hormones, inflammatory mediators, resistin, leptin, adiponectin, and insulin, among other physiologically active molecules (TNF-α, IL-6, IL-1α, IL-1β). Wong *et al*., (2018) showed The beneficial effects of annatto tocotrienol may be attributed to its ability to normalize hormonal changes and inflammatory response but supplement of annatto tocotrienol as administered at sixty or one hundred milligrams per kilogram, annatto had no effect on the level of IL-1β in mice as compared to healthy animals. According to Endo *et al*. (2006), the endoplasmic reticulum ER stress-induced production of the C/EBP homologous protein CHOP triggered caspase-11 and 1 signaling, which in turn enhanced the activation of pro-IL-1B to mature IL-1B in lung cells. As a result, ER stress would stimulate the production of inflammatory cytokines, including IL-1B, and Muto *et al*. (2013) demonstrated that y-T3 decreased IL-1B gene expression. Nevertheless, it is unknown why gamma tocotrienol decreased the expression of the IL-1B gene under normal conditions.

Chapter Six

Conclusions and Recommendations

6.1-Conclusions

Depending on the results of the present study we can conclude the following points:

1. Tocotrienols may have potential as a weight management agent by exhibiting its anti-obesity effect. This occurs clearly through its effect on body weight reduction, body mass index, lee index and AI index in both experiments (protective and therapeutic).
2. The ameliorative effect of tocotrienol supplement in reducing the harmful HFD and obesity risks obviously appeared through the regulation of serum lipid profile and enhance immunity status via total and differential WBC values among the groups. Therefore, tocotrienol can be suggested as a potential natural treatment for dyslipidemia and cardiovascular disease risk factors in obese individuals.
3. The biochemical parameters indicated that tocotrienols supplement reduced the obesity risks through reducing levels of inflammatory markers such as C reactive protein. And balance the metabolic indicator such as total protein, globulin and glucose level. This indicates to that tocotrienols may promote overall health and well-being.
4. Although tocotrienol supplementation was able to reduced obesity risks in many indicators, it had limited impact on the concentrations of metabolic hormones in rats fed high-fat diet except its roles on insulin and leptin levels reduction compared to the obese group. But it did not significantly improve the concentrations of other metabolic hormones.
5. The results also referred to the role of tocotrienol in improving semen profile compared to the obese group and thus improved the reproductive performance of male laboratory rats although there was no significant difference in sexual hormones.

6. Finally, the molecular detection of genetic changes due to Tocotrienol supplements revealed a clear effect on the genes responsible for fat metabolism and inflammation, thus explaining the mechanism by which tocotrienols may exert an effect on reducing high levels of fat.

6.2- Recommendations

This study has some limitations, and the following recommendations are proposed for future research:

1. Studying the effect of tocotrienols as an antioxidant agent in various organs of the body.
2. Studying of other inflammatory cytokines such as TNF-α, IL-6, and IL-18 to support the importance of tocotrienols as an anti-inflammatory.
3. Studying of the effect of tocotrienols on the reproductive capabilities of female rats after the development of metabolic disorders such as obesity or polycystic ovary syndrome.
4. Studying the effect of tocotrienols on the thyroid gland, hormonally and histologically, because of its relationship to weight management and metabolic disorders.
5. Studying the effect of tocotrienols on the gene expression of genes related to sex hormones to provide more understanding of the mechanisms by which tocotrienols influence vital activities in the body.
6. Extracting tocotrienols from plant residues in which tocotrienols are indicated, as well as working to use them as important feed for animals to reduce the risks of harmful obesity and improve production.

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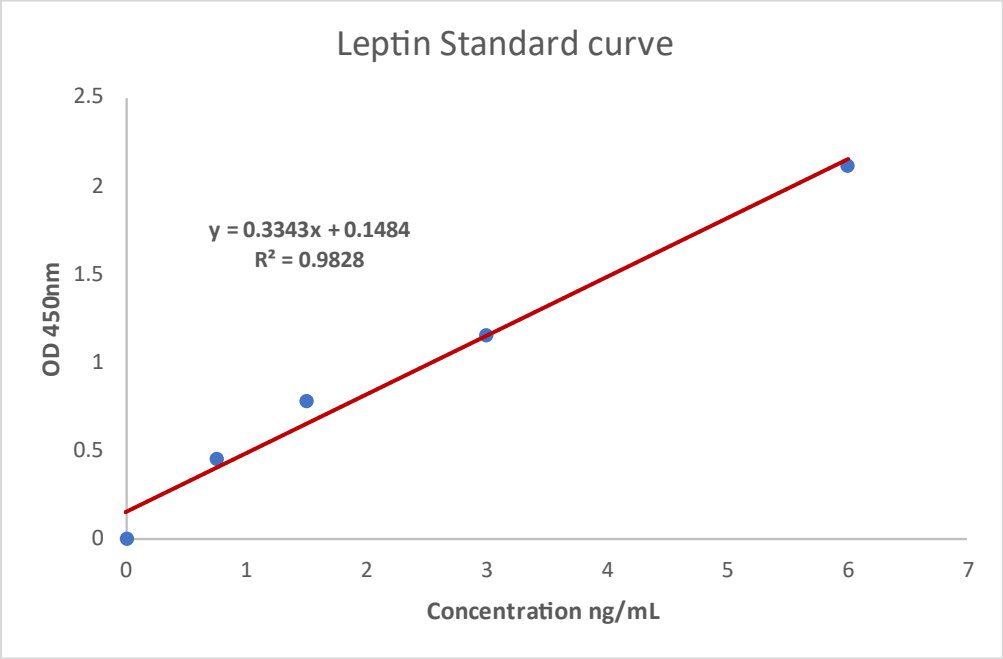
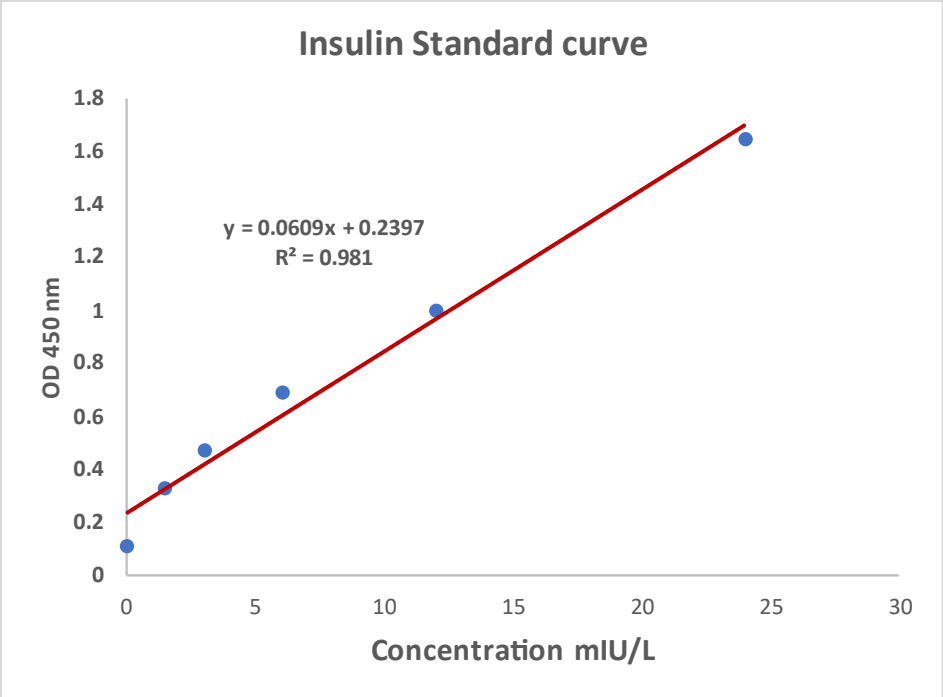
Appendixes

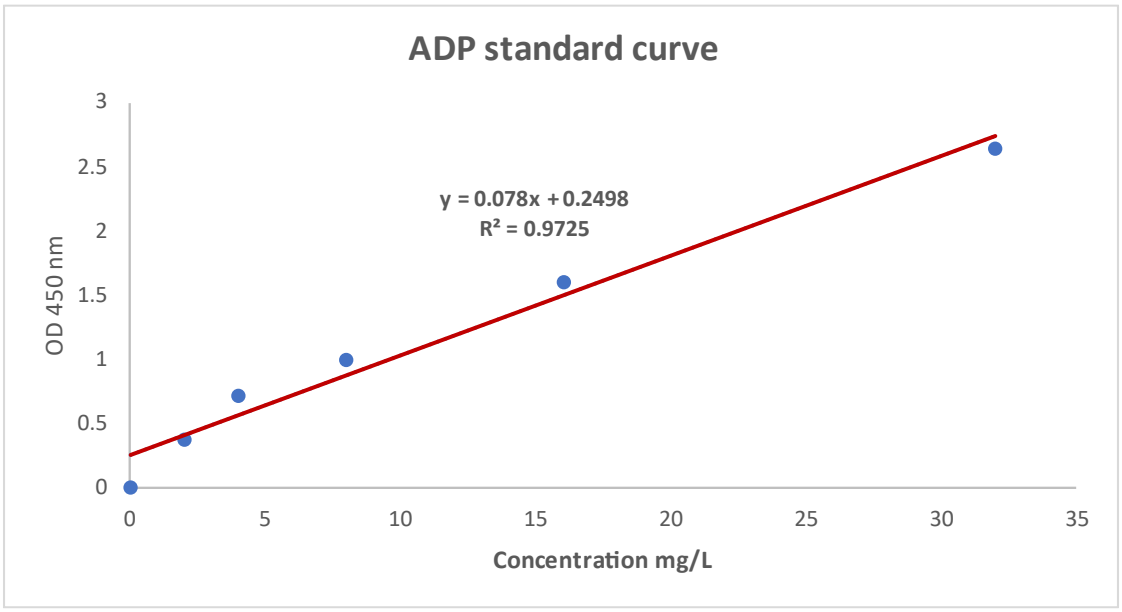
Appendix 1 Leptin standard curve



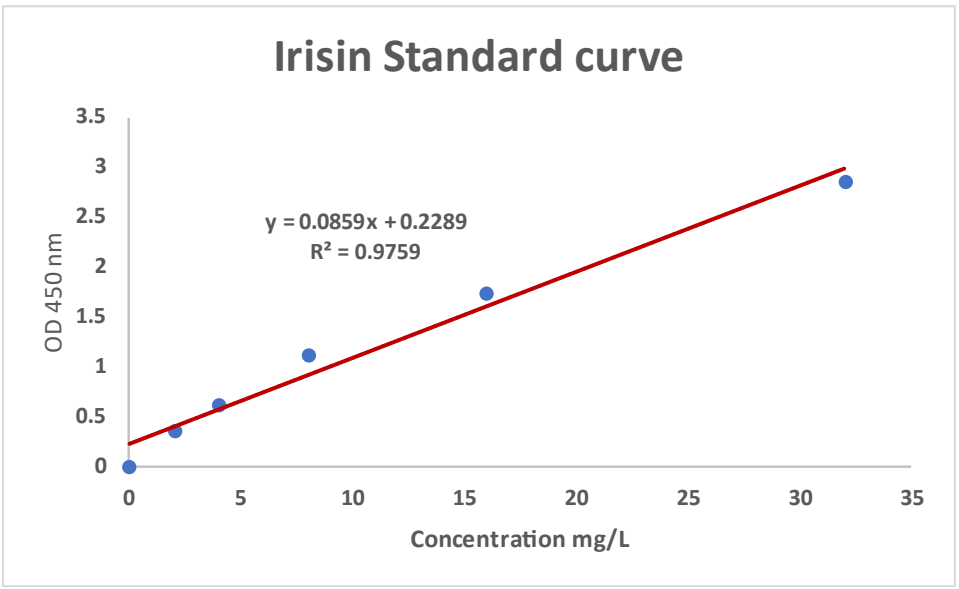
Appendix 2 Elisa insulin standard curve



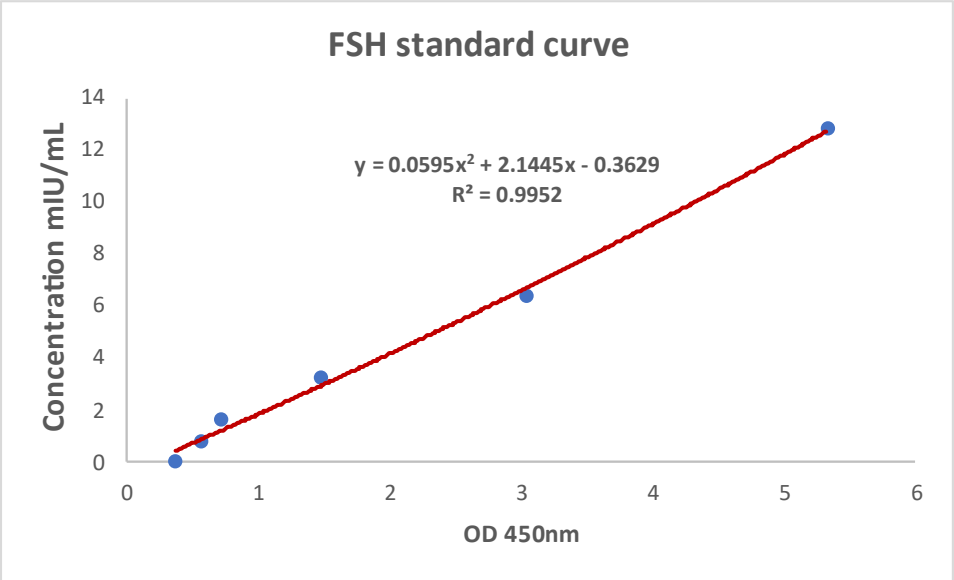
Appendix 3 Elisa adiponectin standard curve Elisa



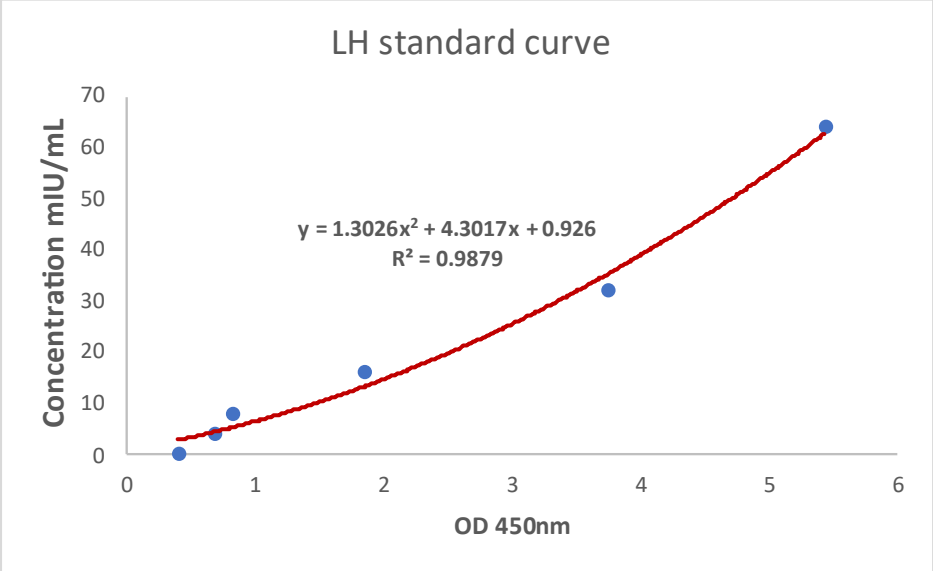
Appendix 4 Elisa Irisin standard curve



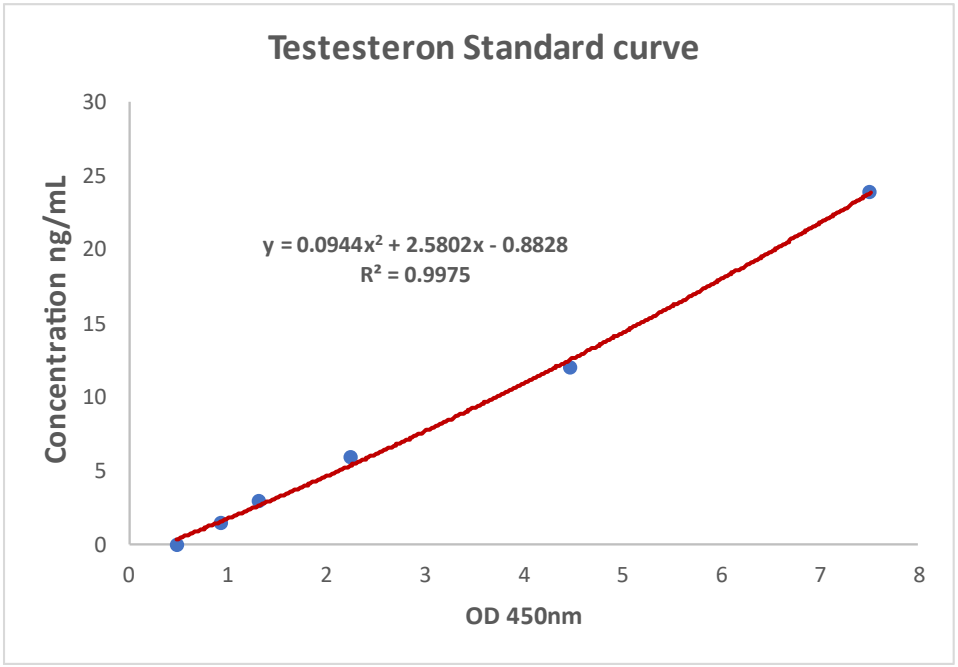
Appendix 5 Elisa FSH standard curve



Appendix 6 Elisa LH standard curve



Appendix 7 Elisa Testesteron standard curve


Testesteron Standard curve
Concentration ng/mL
30
25
20
15
10
5
0
y = 0.0944x² + 2.5802x - 0.8828
R² = 0.9975
0
1
2
3
4
5
6
7
8
OD 450nm

ملخص

أُجريتْ هذه الدراسة في كلية الطب البيطري – جامعة البصرة لتقييم الدور الوقائي والعلاجي لمكملات توكوترينول ضد الآثار الضارة الناتجة عن تناول نظام غذائي عالي الدهون، ومخاطر تحفيز السمنة لدى ذكور جرذان المختبر.

استُخدام أربعة وأربعون جرذاً ذكراً بالغاً تتراوح أعمارهم بين 8 أسابيع، ووزن حوالي 80 ± 25 جرام. وزّعتْ الحيوانات على ثلاث تجارب من الدراسة الحالية. التجربة الأولى (الوقائية) شملت 18 جرذاً ذكراً (6 لكل مجموعة) مقسمة إلى مجموعة ضابطة تغذت على غذاء قليل الدهون ومذاب بزيت الزيتون (1 مل/كجم من وزن الجسم)، ومجموعة HFD تمثل الجرذان التي تغذت على غذاء عالي الدهون ومذاب بزيت الزيتون، زيت (1 مل / كجم من وزن الجسم)، بينما غُذيت المجموعة الوقائية بـ HFD وتوكوترينول (60 ملجم / كجم من وزن الجسم) المذاب في زيت الزيتون لمدة 12 أسبوعًا. التجربة الثانية (العلاجية) شملت 18 جرذاً ذكراً (6 لكل مجموعة) قُسّمتْ عشوائياً إلى مجموعة السيطرة ومجموعة السمنة (السمنة المُستحثة بتغذية الجرذان غذاء عالي الدهون HFD لمدة 12 أسبوعاً)، والمجموعة العلاجية التي حُثّتْ السمنة فيها وعُلجتْ بتجريع توكوترينول (60 ملغم/كغم من وزن الجسم) مذاب في زيت الزيتون (1مل/كغم من وزن الجسم) لمدة 8 أسابيع، وقد تغذتْ جميع المجموعات على غذاء قليل الدهون. وفي التجربة الثالثة التي تعدّ تجربة تكاثرية أستخدم 8 ذكور مُستخرجين من التجارب الوقائية والعلاجية و16 أنثى، قُسمت عشوائياً إلى 4 مجموعات (2 ذكور و4 إناث). المجموعة الأولى كانت عبارة عن جرذان ذكور طبيعية تزاوجت مع إناث طبيعية. وكانت المجموعة الثانية عبارة عن جرذان ذكور تغذت غذاءً عالي الدهون لمدة 12 أسبوعًا وتزاوجت مع إناث طبيعية. وكانت المجموعة الثالثة عبارة عن جرذان ذكور تغذت غذاءً عالي الدهون فضلاً عن توكوترينول لمدة 12 أسبوعًا. اما المجموعة الرابعة عبارة عن جرذان ذكور تغذت غذاءً عالي الدهون لمدة 12 أسبوعًا ثم اعطيت توكوترينول وغذاء منخفض الدهون لمدة 8 أسابيع اخرى ، وقد أدى استخدام توكوترينول إلى تحسين القدرات الإنجابية.

سُجّل وزن الحيوان واستهلاك العلف طيلة مدة التجربة. ثم جّمعتْ عينات الدم لفحص دمها، وقيس مستوى البروتين الكلي والجزئي والبروتين التفاعلي C ومستوى الكلوكوز في المصل. وقيستْ بعض الهرمونات الأيضية مثل هرمون اللبتين والأديبونيكتين والأنسولين والإيريسين، فضلاً عن الهرمونات الجنسية، وتسجيل تقييم حيوية الحيوانات المنوية، وقد لوحظت تغيّرات نسجية في أعضاء الجسم (الكبد والكلى والخصيتين)، وفُحص التعبير الجيني (PCR الكمي في الوقت الحقيقي) في الكبد والأنسجة الدهنية.

أظهر تأثير تجريع توكوترينول على جرذان المختبر انخفاضًا ملحوظًا في وزن الجسم بعد الأسبوع الخامس مقارنة بالجرذان التي غُذيتْ بـ HFD في التجربة الوقائية، على الرغم من عدم وجود اختلاف معنوي في التجربة العلاجية لمدة ثمانية أسابيع، وهذا بدوره يُضعف زيادة وزن الجسم المكتسب لمجموعات HFD وHFDT على الرغم من أن توكوترينول قلل من قيم مؤشر كتلة الجسم ومؤشر LEE وAI ولكنه لا يزال أعلى من قيمهم في المجموعة الضابطة.

سجّل استهلاك الغذاء لحيوانات الدراسة فروقاً غير معنوية في التجربة الوقائية، على الرغم من أنّ التجربة العلاجية أظهرتْ انخفاضاً معنوياً في استهلاك الغذاء لجرذان المجموعة السمينة.

كشفت نتائج الدراسة الحالية أنّه لا يوجد مؤشر محدد لاختلاف معنوي في مؤشرات الدم. في حين سجّل إجمالي كريات الدم البيض والعدلات انخفاضًا ملحوظًا في قيمهما بالنسبة للجرذان التي تغذتْ على HFD لمدة 12 أسبوعاً، لغرض حثّ السمنة، ثم جُرّعتْ توكوترينول مقارنة بمجاميع الدراسة الأخرى.

إنّ تجريع توكوترينول حسّن وظائف الكبد وخفّض مؤشرات الدهون الضارة (الدهون الثلاثية، LDL-c وVLDL-c) وحسّن قيم HDL-c في الجرذان المُعالجة بتوكوترينول للتجربة الوقائية.

أدى تأثير مكمل توكوترينول على HFD إلى انخفاض معنوي في مستويات البروتين الكلي والجلوبيولين وفشل في تعزيز تركيز البروتين التفاعلي C، كما ظهر الكلوكوز على عكس قيمهم في مجموعة السيطرة، في المقابل أدت إضافة توكوترينول العلاجية إلى جرذان المختبر السمينة إلى تعزيز قيم الألبومين والكلوكوز عند مقارنتها بجرذان المختبر التي تعاني من مخاطر السمنة. أظهر تأثير إعطاء توكوترينول على بعض الهرمونات الأيضية انخفاضاً معنوياً في تركيز اللبتين وتحسّن تركيز الإيريسين والأنسولين في الجرعة الوقائية مقارنة مع HFD والجرذان السمينة في كلا التجربتين، وفي المقابل يعمل توكوترينول على تعزيز تحسين LH وتقليل التأثير الضار لـ HFD على حيوية الحيوانات المنوية في التجارب الوقائية والعلاجية. تم تأكيد النتائج المذكورة في أعلاه من خلال الفحص النسيجي المرضي للكبد والكلى والخصيتين، بينما كان التعبير الجيني لجين cpt1b أكبر بمقدار 10,17 ضعفًا في مجموعة الحماية HFDT وأعلى بمقدار 2,54 ضعفًا في المجموعة العلاجية مقارنة بمجموعة التحكم. كان التعبير الجيني لجين IL-1β أكبر بمقدار 8,85 ضعفًا في مجموعة HFD، وأعلى بمقدار 2,67 ضعفًا في مجموعة HFDT (المجموعة الوقائية)، وأعلى بمقدار 5,09 ضعفاً في المجموعة العلاجية مقارنة بالمجموعة الضابطة.

في ضوء الدراسة الحالية، نستنتج أن التوكوترينول قد تكون له أدوار حيوية في تقليل خطر التمثيل الغذائي لـ HFD والسمنة وتعزيز أداء الخصوبة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة البصرة كلية الطب البيطري
فرع الفسلجة والادوية والكيمياء

التأثيرات المحسنة لتوكوترينول المضاف على بعض المعايير الفسيولوجية للسمنة المستحثة في ذكور الجرذان المختبرية

اطروحة

مقدمة إلى مجلس كلية الطب البيطري- جامعة البصرة كجزء من متطلبات نيل درجة دكتوراه فلسفة في علوم الطب البيطري (الفسلجة)

من قبل

ايمان حنش راهي

ماجستير علوم الحياة (2018)

باشراف

الأستاذ الدكتور نمير عبد الكريم خضير

1445 هجري

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