AMELIORATIVE EFFECT OF PHENETHYL ISOTHIOCYANATE ON HIGH FAT DIET (*SAUM*) INDUCED SPERMATOGENESIS DYSFUNCTION IN WISTAR ALBINO RATS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY, MIZORAM UNIVERSITY, AIZAWL

CERTIFICATE

This is to certify that **Lalrinzuali Sailo**, Department of Zoology, Mizoram University, has completed her thesis work entitled **"Ameliorative effect of phenethyl isothiocyanate on high fat diet (*saum*) induced spermatogenesis dysfunction in Wistar albino rats"** in partial fulfillment for the Degree of Doctor of Philosophy in Zoology from March 2017-2023 under my guidance and supervision. No part of this dissertation has been submitted for any other degree, diploma, associateship, fellowship or other similar titles in this University or any other University or institution of higher learning.

Dated:

Place: Aizawl, Mizoram

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DECLARATION

MIZORAM UNIVERSITY

JULY, 2023

I Lalrinzuali Sailo, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institude.

This is being submitted to the Mizoram University for the Degree of Doctor of Philosophy in Zoology

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Aizawl, Mizoram (**LALRINZUALI SAILO**)

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ABBREVIATIONS	
%	Percentage
µg	Microgram
µl	Microliter
3β-HSD	3β-hydroxysteroid dehydrogenase
AI	Apoptotic index
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AO	Acridine orange
AOCS	Association of Official Analytical Chemists
Apaf-1	Apoptotic protease activating factor-1
ARRIVE	Animal research: reporting of in vivo experiments
AST	Aspartate aminotransferase
AtI	Atherogenic index
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BMI	Basal metabolic index
BUN	Blood urea nitrogen
B:C ratio	BUN:Creatinine ratio
CAI	Coronary artery index
cAMP	Adenosine 3',5'-cyclic monophosphate
CAT	Catalase
CI	Cardiac index
DCA	Detrended correspondenceanalysis
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DSP	Daily sperm production
EI	Electron bomb ionization mode,

FAME	Fatty acid methyl esters
FID	Flame ionization detector
FPF	Fermented pork fat
FPF-H	Fermented pork high fat diet (high calorie, high fat and high FAME levels)
FPF-L	Fermented pork low fat diet (low calorie,low fat and low FAME levels)
FPF-M	Fermented pork medium fat diet (moderate calorie, moderate moderate FAME levels)
FSH	Follicle-stimulating hormone
FSANZ	Food Standard Australia New Zealand
G	Gram
GC-MS	Gas chromatography mass spectrometry
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GST	Glutathione S transferase
H&E	Hematoxylin and eosin
HDL	High density lipoprotein
HOMA-IR	Homeostasis model assessmentof insulin resistance index
HOMA-β	Pancreatic β-cell function
HPG	Hypothalamic–pituitary–gonadal axis
HRP	Horse radish peroxidase
H2O2	Hydrogen peroxide
IL-1	Interleukin 1
IL-10	Interleukin-10
IL-6	Interleukin 6
IS	Interstitial space
IFIC	International Food Information Council Foundation
JTBS	Johnsen's mean testicular biopsyscore
JECFA	The joint FAO/WHO Expert Commmittee on Food Additives
K,fe (CN)	Potassium ferricynide

K2HPO4	Potassium hydrogen phosphate
kg	Kilogram
KH2PO4	Potassium dihydrogen phosphate
LDH	Lactic dehydrogenase
LDL	Low density lipoprotein
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LLL	Trilinolein
LPO	1-linolein-2-palmitin-3-olein
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
l	Litre
MDA	Malondialdehyde
MANOVA	Multivariate analysis of variance
MPO	Myeloperoxidase
MSTD	Mean seminiferoustubule diameter
MUFA	Monounsaturated fatty acid
MZU-IAEC	Mizoram university: Institutional animal ethics committee
mg	Milligram
MZU	Mizoram university
Na2CO3	Sodium carbonate
NAFLD	Non-alcoholicfatty liver disease
NIH	National institute of health
NNN	Trinonadecanoyl-glycerol
NO	Nitric oxide
Na2HPO4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NaoH	Sodium hydroxide
NBT	Nitrobluetetrzolium
OLL	1,2-linoleoyl-3-oleoyl-sn-glycerol

OOL	1,2-dioleyl-3-linolenoylglycerol
OOO	1,2,3-trioleylglycerol
OOP	1,2-oleoyl-3-sn-palmitoylglycerol
OOSt	1-Stearoyl-2-oleoyl-3-oleoyl-glycerol
OPO	1,3-oleoyl-2-palmitoyl-sn-glycerol
C	Degree Celsius
PBS	Phosphate buffer
PARP	Poly (ADP-ribose) polymerase
PAST	PAleontological Statistics
PCA	Principalcomponent analysis
PCNA	Proliferating cell nuclear antigen
PLL	Palmitodilinolein
PLP	1,3-palmitoyl-2-linoleoyl-sn-glycerol
POP	1,3-palmitoyl-2-oleoyl-sn-glycerol
PPL	1,2-palmitoyl-3-linolein-sn-glycerol
PPO	Dipalmitoyl-oleoyl-glycerol
PPP	Tripalmitoyl-glycerol
PPS	1,2-palmitoyl-3-stearoyl-sn-glycerol
PEITC	Phenethylisothiocyanide
PMS	Phenazenemethosulfate
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SFA	Saturated fatty acid
SOD	Superoxide dismutase
ssDNA	Single stranded DNA
StAR	Steroidogenic acute regulatory protein
STEH	Seminiferous tubuleepithelial height
SEM	Standard error of mean
SGOT(AST)	Serum glutamic oxaloacetic transaminase(aspartate transaminase)

SGPT(ALT)	Serum glutamic pyruvate transaminase (alanine transaminase)
SH	Sulphur, hydrogen atom
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TAG	Triacylglycerols
TAT	Tunica albugineathickness
TC	Total cholesterol
TDI	Tubule differentiation index
TL	Tubular lumen
TNF-α	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TBARS	Thiobarbituric acid
TCA	Trichloroacetic acid
U	Units
VLDL	Very low-density lipoprotein
WHO	World health Organisation

CHAPTER 1

INTRODUCTION

1.1. Introduction

High fat diets are diets which consists of high density lipoproteins (HDL) and low density lipoproteins (LDL) in the diet. Diets can have a significant impact on normal physiology, with high calorific foods and sedentary lifestyles which contributes to the development of obesity, an epidemic in adults and children of all ages all over the world (NCD-RisC., 2017). The type, source and composition of an FPF-diet strongly impact bioenergetics, modifying metabolic function through changes in gene and protein expression, metabolism, fatty acid composition and cholesterol content of cell membranes (Augustin *et al.*, 2018), resulting in mitochondrial dysfunction and the development of metabolic diseases. SFAs, particularly palmitate and stearate, are harmful and toxic to normal cellular processes and can induce abnormal plasma lipid profles, insulin resistance, infammation, activation of stress-associated protein kinases, protein oxidative stress, disruption of spermatogenesis and steroidogenesis, apoptosis, male reproductive disorders and infertility (Ly *et al.*, 2017; Dallak 2019; Li *et al.*, 2020).

1.2. High fat diet- a mulifactorial disease:

Dietary pork-fat comprises an expansive amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, high in calorie and fat content, which is a potential risk factor for hypertension, hyperlipidemia, hypercholesterolemia, imbalance in glucose and lipid metabolism, inflammation, gut dysbiosis and promotes the production of body obesity in rats, gastric cancer and ulcer, and type-2 diabetes (Emelyanova *et al*., 2019; Chakraborty *et al*., 2021; Lalrohlui *et al*., 2021; Zhu *et al*., 2021).Obesity is considered a serious disease affecting a large population worldwide characterized by excess adipose tissue that contributes to numerous chronic diseases and early mortality. (Bessesen, 2008; Kushner, 1993; Simopoulous, 1984). It is a complex metabolic disease that is a serious detriment to children and adult health, which induces a variety of diseases, such as cardiovascular disease, type II diabetes, hypertension and cancer. The integrity of the blood-testis barrier can be severely interrupted consistent with declines in the tight junction related proteins, occludin, ZO-1 and androgen receptor,

but instead endocytic vesicle-associated protein, clathrin rose. Obesity can impair male fertility through declines in the sperm function parameters, sex hormone level, whereas during spermatogenesis damage to the blood-testis barrier (BTB) integrity may be one of the crucial underlying factors accounting for this change. Thus, efforts are continuously underway to prevent obesity in the population. (Nordmann *et al*., 2006; Yancy *et al*., 2004; Volek *et al*., 2008).

Chronic ingestion of a low-carbohydrate diet is that it usually contains a high percentage of fat to compensate for carbohydrate-calorie reduction, and most in the form of saturated fat which is one potential problem. In rats a high-saturated fat diet is used as a diabetogenic factor increasing insulin and lipid levels and it has been shown to induce severe insulin resistance in skeletal muscles (Surwit *et al*., 1988; Chun *et al*., 2010). It is certain that with the increasing prevalence of unhealthy dietary behaviours and sedentary life styles, obesity is emerging as an important risk factor for non-insulin-dependent diabetes, hypertension, cardiovascular disease, cancer, and relevant metabolic and reproductive disorders (Mayes *et al*., 2004).

1.3. Food and water consumption in relation to high fat diet

Food and water consumption are the basic parameters while consuming high fat diets which will show the effects of high fat diets. Many factors including technological developments in the food industry and the prevalence of high fat food plays role in increasing the prevalence of obesity (Pherson, 2014). Dietary fat is a significant contributor to hyperphagia, weight gain, and fat deposition, with body fat increasing in direct proportion to the fat content of the diet.4 Together with elevated body fat, long term high-fat diet (HFD) consumption causes a rise in circulating levels of leptin, insulin, triglycerides (TG), and glucose. (Buettner *et al*., 2000 and Leibowitz *et al*., 2004). Its metabolic effects include an increase in lipoprotein lipase (LPL) activity in adipose tissue, which enhances the uptake of excess lipids, and a reduction in energy expenditure, sympathetic nervous system activity, and carbohydrate oxidation in muscle. In addition to fat consumption, there is evidence that the overeating of carbohydrate may also contribute to obesity. A carbohydrate-rich diet or meal compared to a balanced diet stimulates the secretion of the anabolic hormone, insulin,

which promotes body fat synthesis. (Wang *et al*., 1998; Leibowitz *et al*., 1998 and Kersten, 2001).

1.4. Effects of high fat diet on body weight and lipid profiles

A worldwide survey was conducted in 2013, which shows that the body mass index (BMI) of adults had increased by 36.9% in men and by 38.0% in women (Ng *et al*., 2014). Many factors including technological developments in the food industry and the prevalence of fast food plays role in increasing the prevalence of obesity (Mc Pherson., 2014). Men with higher body mass index (BMI) are most likely to have unfavourable conditions like semen parameters with a higher chance of azoospermia (Chavarro *et al.*, 2010; Paasch *et al*.,2010; Luque *et al.*, 2015). It is also increasingly recognised that being overweight or obese may have a deleterious effects on female potential fertility. (Gesink *et al*., 2007; Zain *et al*., 2008).

Although weight gain is ultimately the result of an overall positive energy balance, the environmental and genetic connections that accounts for the enormous rise in obesity is not fully understood. Although the traditional weight loss approach advises a high carbohydrate low fat diet, a very low carbohydrate high fat diet has been suggested to have greater effectiveness in weight loss and metabolic improvement (Nordmann *et al*., 2006; Yancy *et al*., 2004; Volek *et al*., 2008). The type, source and composition of an FPF-diet strongly impact bioenergetics, which modifies metabolic function through changes in gene and protein expression, metabolism, fatty acid composition and cholesterol content of cell membranes (Augustin *et al*., 2018), resulting in mitochondrial dysfunction and the development of metabolic diseases. SFAs, particularly palmitate and stearate, are harmful and toxic to normal cellular processes and induce abnormal plasma lipid profiles, insulin resistance, inflammation, activation of stress-associated protein kinases, protein oxidative stress, disruption of spermatogenesis and steroidogenesis, apoptosis, male reproductive disorders and infertility (Ly *et al*., 2017; Dallak 2019; Li *et al*., 2020). Increases in the ratio of n-6:n-3 PUFA, distinct of the FPF-diet, could potentiate oxidative and inflammatory processes and increase in SFA and PUFA-ω6consumption alter the production of inflammatory mediators and regulators and immune responses on the way to a pro-

inflammatory process (Schulze *et al*., 2020). An increase in the ratio of n-6:n-3 PUFAs, distinct from the FPF-diet, could potentiate oxidative and infammatory processes, and an increase in SFA and PUFA-ω6 consumption could alter the production of infammatory mediators and regulators, as well as immune responses, leading to a pro-infammatory process (Schulze *et al.*, 2020).

1.5. Effects of high fat diet on blood glucose levels and rectal temperature

Insulin resistance (IR) is considered the key mechanism inducing obesity, diabetes and heart disease (Bray, 2004). In the course of months or years, IR is followed by the increase in β-cell insulin secretion and by several complications known as the insulin resistance syndrome, which is associated with dyslipidemia, hypertension, hyperglycemia and cardiovascular disease. Diabetes mellitus (DM) is recognized as the world most common endocrine disorder (WHO, 1999; 2003; Ingrid and Mathias., 2006) and a major degenerative multi-factorial disorder (Barham and Trinder., 1972; Ogbonnia *et al*., 2008) which is characterized by hyperglycemia-the primary clinical manifestation (Nodestgarrd *et al*., 1998), and raised metabolic rate (Owu *et al*., 2006). Associated factors include imbalance and/or abnormalities in carbohydrate, fat and lipoprotein metabolism (Ugochukwu *et al*., 2003; Scoppola *et al*., 2001), reactive oxygen species and oxidative stress (Kesavulu *et al*., 2002; Nayeemunnisa., 2009). A study was performed on mice which determined that the consumption of a high fat diet (HFD) resulted in elevated levels of insulin, specifically fasting insulin and pro-insulin (Eisinger., 2014). Obesity is associated with type II diabetes, hypertension and coronary heart disease (Kyeong-Mi Choia *et al*., 2013). It is associated with metabolic syndrome, a group of risk factors for cardiovascular disease, type 2 diabetes mellitus (T2DM) and other pathologies, including hypertension, hyperlipidemia and cancers (Esser *et al*., 2014 and Lei *et al* ., 2007) It is associated with excessive growth and expansion of adipose tissue due to an imbalance between energy intake and expenditure (Spiegelman *et al*., 2001).Adipose tissue is a central regulator of energy metabolism and secretes numerous adipokines such as leptin and adiponectin (Ouchi *et al*., 2001 ; Rajala *et al*., 2003). Adipose tissue mass can be expanded by adipocyte hyperplasia and hypertrophy. (Spalding *et al*., 2008 and Cowherd *et al*., 2008). Adipocytes can be differentiated from preadipocytes by adipogenic signals. Adipocyte

differentiation is regulated by a complex network of transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα). (Cowherd *et al.*, 1999; Gregoire *et al.*,1998). AMP-activated protein kinase (AMPK), a αβγ heterotrimer, is a regulatory enzyme involved in lipid and energy metabolism (Hardie, 2003). (Hardie *et al.*, 2003; Kahn *et al.*, 2005). Activation of AMPK regulates lipogenesis, fatty acid oxidation and glucose transport (Daval *et al.*, 2008). Phosphorylation of AMPK inhibits lipid biosynthesis by the inactivation of key metabolic enzymes involved in fatty acid and cholesterol synthesis, such as acetyl-CoA carboxylase (ACC) and hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (Kyeong *et al.*, 2013).

1.6. Effects of high fat diet on oxidative stress, antioxidant enzymes and spermatogenesis:

It is well demonstrated that obesity and also high fat diets are strong inducers of oxidative stress, and also, among the many biological targets of oxidative stress, lipids peroxidation is the most prominent. Lipid peroxidation generates a number of by products and the unsaturated aldehydes such as 4-HNE. 4-HNE is the most toxic molecule among them and also an important biomarker of lipid peroxidation and oxidative stress. (Barrerra, 2012; Bełtowski, 2000). Some reactive oxygen species have the potential of damaging cells and tissues (Diplock A, 1998). Catalase (CAT) activity is significantly upregulated after the consumption of a HFD; an increased CAT protein content translates to increased enzyme activity in the heart of the animal (Rindler *et al.*,2013). Also, HFD-fed mice have been recognised to have lower superoxide dismutase (SOD) levels compared with normal mice (Wang and Ryu., 2015), and treated rats have been shown to have increased triglyceride (TG) and glucose levels, and hypertension after 8 weeks on a HFD (Vargas *et al.*, 2015).

Oxidative damage primarily occurs via production of reactive oxygen species such as superoxide anion, peroxides, and it can cause damage to lipids, proteins and DNA. Therefore, it may cause to loss of enzymatic activity and structural integrity of enzymes and activate inflammatory processes (Özyurt *et al.*, 2004). Oxidative stress plays an important role in the etiology of defective sperm formation, function, sperm

count profile and male infertility (Acharya, 2008; Shen and Ong, 2000).SOD is an enzyme that catalyses dismutation of two superoxide anion into hydrogen peroxide and molecular oxygen. SOD is one of the most important enzyme in the front line of defence against oxidative stress.These enzymes are also considered as an important indicator of the balance status between the first and second step of the enzymatic antioxidant pathway (Jihen *et al*., 2009. The testis,epididymis, sperm and seminal plasma contain high activities of antioxidant enzymes (Aruldhas *et al*., 2005). Whereas SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, CAT converts hydrogen peroxide into water (Mansour and Mossa, 2009). Therefore, SOD–CAT system provides the first defense system against oxidative stress and these enzymes work together to eliminate active oxygen species (El-Demerdash, 2011 and Wafa *et al*., 2011). Glutathione peroxidases are antioxidant seleno enzymes that are present in the cytosol of cells. The major function of these enzymes, which use glutathione (GSH) as a substrate, is to reduce soluble hydrogen peroxide and alkyl peroxidases (Demir *et al*., 2011). Catalase catalyses the dismutation of H_2O_2 and thus competes with GSHPs for the common substrate. It is considered to be the primary scavenger of intracellular H_2O_2. In the UV range H_2O_2 absorbs maximally at 240nm.Catalase rapidly breaks down H_2O_2 leading to a decrease in absorbance. A difference in the absorbance at 240 nm per unit time is measured of Catalase activity

1.7. Effects of high fat diet on hormones and male fertility

The regulation and maintenance of spermatogenesis requires hormone testosterone. It is the major androgenic hormone in male reproduction and is responsible for the development of secondary sexual characteristics, sexual desires and erectile function (Bain, 2007)and a normal testosterone level is required to maintain bone density, muscle growth, the brain nervous system and cognitive health (Tyagi *et al*., 2017).Testosterone production is regulated by the hypothalamic–pituitary–gonadal axis with appropriate levels of gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion. In testosterone biosynthesis, cholesterol is transported into Leydig cells by the steroidogenic acute regulatory protein (StAR). Cholesterol then transforms into pregnenolone,

progesterone, androstenedione and testosterone through cytochrome P450 families and hydroxysteroid dehydrogenases (HSD) (Hanukoglu *et al*., 1992).Despite cholesterol being the starting precursor for biosynthesis of steroid hormones including testosterone, studies have shown that a high plasma cholesterol level leads to a notable decrease in semen quality (Ramírez-Torres *et al*., 2000; Maqdasy *et al*., 2013). High fat diets affect androgen receptor (AR) expression essential for male fertility (Fan *et al*., 2015) also causing testicular inflammation, increased oxidative stress and causing sperm DNA damage (Fullston *et al*., 2013; Bisht *et al*., 2017).

Male C57BL/6 mice fed with a high-fat diet (HFD) for 10 weeks served as a model of diet-induced obesity clearly showed that the percentage of sperm motility and progressive motility significantly decreased, whereas the proportion of teratozoospermia dramatically increased in HFD mice compared to those in normal diet fed controls. Besides, the sperm acrosome reaction fell accompanied by a decline in testosterone level and an increase in estradiol level in the HFD group. (Nordmann *et al*., 2006; Yancy *et al*., 2004; Volek *et al*., 2008). Most of studies revealed that spermatogenesis is affected by altered levels of sex hormones in obese men, such as decreased free or total testosterone and increased estradiol levels in serum (Shayeb *et al*., 2011). Meanwhile, there is increasing evidence that obesity may also impair male fertility (Hammoud *et al*., 2007; Hofny *et al*., 2010; Hammiche *et al*., 2012), although several reports failed to document this association (Jensen *et al*., 2004; Rybar *et al*., 2011). Besides, diet-induced obesity is highly susceptible to increases in the DNA fragmentation index in spermatozoa due to oxidative stress, resulting in an obvious decline in male fertility (Liu *et al*., 2014). However, the mechanism is poorly characterized describing how obesity can cause male sub-fertility and warrants elucidation. The complete process of spermatogenesis is the key component in normal sperm quality and male fecundity. Briefly, spermatogonia located at the basement membrane of the seminiferous tubules enter mitosis and generate primary spermatocytes, which then undergo two meiotic divisions to form secondary spermatocytes and spermatids. The spermatids differentiate into mature spermatozoa through spermiogenesis (De Kretser *et al*., 1998).

The association between obesity and impaired male reproductive function is multifactorial, involving alterations at the level of the hypothalamic-pituitary-gonadal (HPG) axis, as well as direct testicular effects on spermatogenesis and somatic cell function (Sermondade *et al*., 2013). Rodent studies which use high fat diet (HFD) to induce obesity reveal reductions in sperm volume and motility with a higher percentage of morphologically abnormal sperm (Saez Lancellotti *et al*., 2010). Obesity may also increase testicular vulnerability to environmental insults and subfertility from pathophysiological states (e.g., cholestasis) (Ghanayem *et al* ., 2010; Gutzkow *et al*., 2016;Vega *et al*., 2015). Effects continue after conception affecting embryo quality and implantation rates (Aitken *et al*., 2004; Fullston *et al*., 2015). Furthermore, HFD may also affect the sperm epigenome with trans-generational effects to the offspring of obese males (Fullston *et al*., 2015). This alteration of sperm function parametersstrongly indicated that the fertility of HFD mice was indeed impaired, which was also validated by a low pregnancy rate in their mated normal female. Moreover, testicular morphological analyses revealed that seminiferous epithelia were severely atrophic, and cell adhesions between spermatogenic cells and Sertoli cells were loosely arranged in HFD mice. (Nordmann *et al*., 2006; Yancy *et al*., 2004; Volek *et al*., 2008). HFDs are linked with both leptin and insulin resistance (Koch *et al*.,2014), and importantly, are associated with hepatic steatosis. Moreover, mice fed with HFD had increased levels of serum glutamic oxaloacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT) compared with mice fed a normal diet (ND) and those fed HFD supplemented with α-lipoic acid for 24 weeks (Yang *et al*., 2014). In addition, it has been shown that HFD-fed mice have greater adipocyte hypertrophy and increased levels of inflammatory cytokines monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α, as well as hepatic steatosis (Gao *et al*., 2015).

1.8. Importance of Biochemical parameters and lipid profiles (ALT, AST, cholesterol, AP, Creatinine and Urea)

SGPT (ALT) is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, obstructive jaundice and other hepatic diseases. Slight

elevation of the enzymes is also seen in myocardial infarction. SGPT converts L-Alanine and α-Ketoglutarate to Pyruvate and Glutamate. The Pyruvate formed reacts with 2,4Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured (Reitman and Frankel.,1957). SGOT (AST) is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood stream. Elevated levels are found in myocardial infarction, Cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases, and primary muscle diseases. Decreased levels may be found in pregnancy, beri-beri and diabetic ketoacidosis. SGOT converts L-Aspartate and α Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. (Reitman and Frankel.,1957).

Cholesterol is the main lipid found in blood, bile and brain tissues. It is the main lipid associated with arteriosclerotic vascular diseases. It is required for the formation of steroids and cellular membranes. The liver metabolizes the cholesterol and it is transported in the blood stream by lipoproteins. Increased levels are found in hypercholesterolemia, hyperlipidaemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome and cirrhosis. Decreased levels are found in mal absorption, malnutrition, hyperthyroidism, anaemia and liver diseases. Cholesterol esterase hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-animoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. In tensity of the colour formed is directly proportional to the amount of cholesterol present in the sample. (Allain *et al*., 1974)

Cholesterol Esterase

Cholesterol esters + H_20 cholesterol + Fatty acids

Cholesterol Oxidase

Cholesterol + 0_2 cholesterol + H_20_2

Peroxidase

H_20_2 + 4 Aminoantipyrine + Phenol Red Quinoneimine dye + H_20

Alkaline Phosphatase (ALP) is an enzyme of the hydrolase class of enzymes and acts in an alkaline medium. It is found in high concentrations in the liver, biliary tract epithelium and in the bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone disease. Moderate increases are seen in Hodgkins disease and congestive heart failure.

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy

Creatinine + Alkaline Picrate →Orange Coloured Complex

Urea is the end production of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from when it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy. (Mod. Berdthelodt, 1859). Urease hydrolyses urea to ammonia and CO.

Urease

Urea+H_2O → Ammonia + CO_2

Triglycerides (Trinder ,1969) Lipoprotein lipase hydrolyse triglycerides to glycerol and free fatty acids. The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxide further reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex. Triglycerides are used for transporting and storing fatty

acids in the body. These fatty acids are important because they can be burned as fuel for the body's needs. When food is plentiful, the fatty acids are stored in the body's fat cells, and body fat accumulates.

Triglycerides Glycerol+Free fatty acids

Glycerol+ATP Glycerol3 Phosphate + ADP

Glycerol3 Phosphate +02------- Dihydroxyacetone phosphate +H2O2

H2O2 + 4 Aminoantipyrine -------Red Quinoneimine dye+ H2O+Phenol

Very Low-density lipoproteins

1.9. High fat diet in relation with serum hormones

1.9.1. Testosterone

The strongest naturally released androgen is testosterone (17-hydroxyandrost-4-ene-3-one), a C19 steroid. It is the most significant hormone secreted into the circulation and is mostly produced by the Leydig cells of the testes, androgen, and ovaries. In males, the Leydig cells of the testes are principally responsible for the release of testosterone; in females, about 50% of the circulating testosterone is produced peripherally through androstenedione conversion, with the remaining 20% coming directly from the adrenal and ovarian glands. Due to stimulation from the placenta and foetal pituitary gonadotropins, testosterone levels in male foetuses rise during the last trimester of pregnancy, then fall and rise again 30–60 days after birth. Following this, testosterone levels drop to low levels in childhood. Gonadotrophin secretion causes an increase in testosterone production in the testicles at the beginning of male puberty. Serum testosterone levels in adult males vary throughout the day, peaking in the morning. Testosterone is essential for the growth of secondary male sex traits, and measuring it is useful for assessing hypogonadal conditions.. In prepubertal males, elevated testosterone levels are found in both gonadotrophin-dependent and independent precocious puberty (e.g. testotoxicosis, adrenal hyperplasia or adrenal tumor), as well as in androgen receptor defects. In adult males, high levels of testosterone are associated with various pathologic conditions, including primary hypogonadism (e.g. testicular

dysgenesis, Klinefelter syndrome) and gonadotrophin deficiencies (e.g. hypogonadism, Kallman syndrome). In woman, there is a much smaller increase in serum testosterone levels during the third trimester, followed by low levels in childhood, and a small increase during puberty. In females of all ages, elevated testosterone levels can be associated with variety of virilizing conditions, including congenital adrenal hyperplasia, arrhenoblastoma, mix-gonadal dysgenesis, polycystic ovarian disease, and ovarian and adrenal tumors. Testosterone concentration in serum may be raised by certain drugs, such as 19-nortestosterone, epitestosterone, ethisterone and Danazol. Similar to typical oral contraceptives, CPA-containing medications, and analogues of gonadotropin-releasing hormone (GnRH) are highly effective at reducing testosterone levels. While measures taken before and after exogenous gonadotropin injection can help to detect cryptorchidism and other structural anomalies, measurements taken in the immediate postnatal period can help in the differential diagnosis of ambiguous genitalia. (Diane, 1992)

1.9.2. Leutenising Hormone

Luteinizing hormone (LH) is produced in both men and women from the anterior pituitary gland in response to luteinizing hormone-releasing hormone (LH-RH or Gn-RH), which is released by the hypothalamus. LH, also called interstitial cell-stimulating hormone (ICSH) in men, is a glycoprotein with a molecular weight of approximately 30,000 daltons. It is composed of two noncovalently associated dissimilar amino acid chains, alpha and beta. The alpha chain is similar to that found in human thyroid stimulating hormone (TSH), follicle-stimulating hormone (FSH), and human chorionic gonadotropin (hCG). The differences between these hormones lie in the amino acid composition of their beta subunits, which account for their immunological differentiation. The basal secretion of LH in men is episodic and has the primary function of stimulating the interstitial cells (Leydig cells) to produce testosterone. The variation in LH concentrations in women is subject to the complex ovulatory cycle of healthy menstruating women, and depends on a sequence of hormonal events along the gonado-hypothalamic-pituitary axis. (Diane, 1992). The decrease in progesterone and estradiol levels from the preceding ovulation initiates each menstrual cycle. As a result of the decrease in hormone levels, the hypothalamus

increases the secretion of gonadotropinreleasingfactors(GnRF), which in turn stimulates the pituitary to increase FSH production and secretion. The rising FSH levels stimulate several follicles during the follicular phase, one of these will mature to contain the egg. As the follicle develops, estradiol is secreted, slowly at first, but by day 12 or 13 of a normal cycle increasing rapidly. LH is released as a result of this rapid estradiol rise because of direct stimulation of the pituitary and increasing GnRF and FSH levels. These events constitute the pro-ovulatory phase. Ovulation occurs approximately 12 to 18 hours after the LH reaches a maximum level. After the egg is released, the corpus luteum is formed which secretes progesterone and estrogen feedback regulators of LH.

The luteal phase rapidly follows this ovulatary phase, and is characterized by high progesterone levels, a second estradiol increase, and low LH and FSH levels. Low LH and FSH levels are the result of negative feedback effects of estradiol and progesterone on the hypothalamic-pituitary axis. After conception, the developing embryo produces hCG, which causes the corpus luteum to continue producing progesterone and estradiol. The corpus luteum regresses if pregnancy does not occur, and the corresponding drop in progesterone and estradiol levels results in menstruation. The hypothalamus initiates the menstrual cycle again as a result of these low hormone levels. Patients suffering from hypogonadism show increased concentrations of serum LH. A decrease in steroid hormone production in females is a result of immature ovaries, primary ovarian failure, polycystic ovary disease, or menopause; in these cases, LH secretion is not regulated. A similar loss of regulatory hormones occurs in males when the tests develop abnormally or anorchia exists. High concentrations of LH may also be found in primary testicular failure and Klinefelter syndrome, although LH levels will not necessarily be elevated if the secretion of androgens continues. Increased concentrations of LH are also present during renal failure, cirrhosis, hyperthyroidism, and severe starvation. A lack of secretion by the anterior pituitary may cause lower LH levels. As may be expected, low levels may result in infertility in both males and females. Low levels of LH may also be due to the decreased secretion of GnRH by the hypothalamus, although the same effect may be seen by a failure of the anterior pituitary to respond to GnRH stimulation. Low LH values may therefore indicate some dysfunction of the pituitary or hypothalamus, but the actual source of the problem must

be confirmed by other tests. In the differential diagnosis of hypothalamic, pituitary, or gonadal dysfunction, assays of LH concentration are routinely performed in conjugation with FSH assays since their roles are closely interrelated. Furthermore, the hormone levels are used to determine menopause, pinpoint ovulation, and monitor endocrine therapy**.**

1.9.3. Follicle Stimulating Hormone

FSH is a glycoprotein secreted by the basophile cells of the anterior pituitary. Gonadotropin releasing hormone, produced in the hypothalamus controls the release of FSH from the anterior pituitary. Follicle-Stimulation Hormone (FSH) and Luteinizing Hormone (LH) are intimately involved in the control of the growth and reproductive activities of the gonadal tissues, which synthesize and secrete male and female sex hormones. The levels of circulating FSH and LH are controlled by these sex hormones through a negative feedback relationship. Tumors of the testis generally depress serum FSH concentrations, but levels of LH are elevated as determined by radioimmmunoassay. It has been postulated that the apparent LH increase maybe caused by cross reactivity with hCG like substances secreted by tumors of the testis. High levels of FSH in men may be found in primary testicular failure and Klinefelter syndrome. Elevated concentrations are also present in cases of starvation, renal failure, hyperthyroidism and cirrhosis. The growth of the seminiferous tubules and maintenance of spermatogenesis in men are regulated by FSH. However, androgens, unlike estrogen, do not lower FSH levels, therefore demonstrating a feedback relationship only with serum LH. For some reasons, azospermic and oligospermic males usually have elevated FSH levels. (Diane, 1992)

1.10. Sperm parameter and motility

The sperm parameters include the sperm concentration in testis and epididymis. The complete process of spermatogenesis is the key component in normal sperm quality and male fecundity. Briefly, spermatogonia located at the basement membrane of the seminiferous tubules enter mitosis and generate primary spermatocytes, which then undergo two meiotic divisions to form secondary spermatocytes and spermatids. The spermatids differentiate into mature spermatozoa through spermiogenesis (Kretser *et*

al., 1998). The association between obesity and impaired male reproductive function is multifactorial, involving alterations at the level of the hypothalamic-pituitary-gonadal (HPG) axis, as well as direct testicular effects on spermatogenesis and somatic cell function (Sermondade *et al*., 2013). Rodent studies which use high fat (HF) diet to induce obesity reveal reductions in sperm volume and motility with a higher percentage of morphologically abnormal sperm (Saez Lancellotti *et al*., 2010). Obesity may also increase testicular vulnerability to environmental insults and subfertility from pathophysiological states (e.g., cholestasis) (Ghanayem *et al*., 2010; Gutzkow *et al*., 2016; Vega *et al*., 2015). Effects continue after conception affecting embryo quality and implantation rates (Aitken *et al*., 2004; Fullston *et al*., 2015). Furthermore, HF diet may also affect the sperm epigenome with trans-generational effects to the offspring of obese males (Fullston *et al*., 2015). This alteration of sperm function parameters strongly indicated that the fertility of HFD mice was indeed impaired, which was also validated by a low pregnancy rate in their mated normal female. Moreover, testicular morphological analyses revealed that seminiferous epithelia were severely atrophic, and cell adhesions between spermatogenic cells and Sertoli cells were loosely arranged in HFD mice. (Nordmann *et al*., 2006; Yancy *et al*., 2004; Volek *et al*., 2008).

1.11. Testis histopathology

‘The word "histology" is a Greek word: Histos, which refers to the study of tissues for living organisms, is different from logia, which is the study of or knowledge. All categories of microscopic anatomy are included in histology. The relationship between structure and function serves as proof that histology is an engaging and easily comprehendable subject. The processing of tissue in order to observe it under a microscope is a key factor in the study of histology. Unless they are properly prepared for microscopic study, cells and tissues cannot be investigated effectively. In terms of the overall technique for tissue preparation, there are two divisions: the technique that involves direct observation of a living cell and the technique that uses a dead cell (fixed and stained). Though some tissues that connect to one another are difficult. This study provided with a full processes which allow the observation of tissue become easier. (Boodnard *et al.*, 2001) mentioned that administration of high fat diets

to rats led to atrophic changes in enhancers widely used in many countries and is found in the testis and destruction of Sertolli cells and Leydig cell. (Das and Ghosh., 2010) observed loss of spermatogenic cells injected with high fat diet. Treating rats with high fat diet at short-term showed slight to moderate seminiferous tubules including cytoplasmic vacuolization of spermatogonia and loss of spermatids. Long–term treatment of high fat diet caused severe damage of germ cells and vacoulisation of seminiferous tubules (Mohamed, 2012).

1.12. Acridine orange staining and TUNEL assay on high fat diet

Acridine orange is an organic compound that serves as a nucleic acid selective fluorescent dye with cationic properties useful for cell cycle determination. It is cell permeable, and it allows the dye to interact with DNA by interaction, or RNA via electrostatic attractions. The staining of spermatozoa were used to upgrade the information observed by a semen analysis. As a test of DNA integrity, this cytochemical method allowed the differentiate between double (green fluorescence) and single stranded (red fluorescence) DNA because of the metachromatic properties of acridine orange. According to (Kosower *et al.*,1992), the color of acridine orange (AO) fluorescence of acid-treated sperm heads is determined by the thiol-disulfide status of DNA-associated protamines in the nucleus. The nucleoprotein of somatic cells is histone, whereas that of mature spermatozoa is predominantly exposed. Thiols in this DNA-associated protamine change gradually to disulfides during epididymal spermatozoa maturation (Calvin and Badford., 1971). In the mature sperm nucleus, DNA associated with disulfide-rich protamines is resistant to disruption by acid or heat and remains intact and double stranded. DNA associated with thiol-rich protamines in the immature sperm nucleus become single stranded after denaturation. The fluorochrome AO insinuated into double-stranded DNA as a monomer and binds to single stranded DNA as a heap. The monomeric AO bound to native DNA fluorescence green, whereas the aggregated AO on denatured DNA fluorescence red (Ichimura *et al*., 1971; Peacocke., 1973). The maturity of mammalian sperm nuclei can be seen and assessed by the AO nuclear fluorescence of sperm.One distinctive sign of apoptosis is DNA fragmentation. TUNEL is a well-known technique for

examining and identifying DNA fragments. It uses terminal deoxynucleotidyl transferase and dUTP nick end labelling. Apoptotic cells are prominently arising in high fat diet induced rats testicular activity.

1.13. Immunohistochemical and Western blotting analysis on rats fed with high fat diet

Proliferating cell nuclear antigen (PCNA) is a nuclear non histone protein, originally identified as an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle. (Leonardi *et al.*, 1992) with a molecular weight of 36kD which plays a role in the initiation of cell proliferati on by amplifying DNA polymerase; stains for proliferating cell nuclear antigen in tumors correlated with grade and mitotic activity. The initial member of the Bcl-2 family of proteins was B-cell lymphocyte/leukemia-2 (Bcl-2) oncoprotein. Bcl-2 was identified at the breakpoint of the chromosomal translocation that occurs in a large majority of patients with non-Hodgkin's B-cell lymphoma (Tsujimoto *et al.*, 1984). As a result of the translocation, the expression of bcl-2 comes under the control of the immunoglobulin heavy chain enhancer and is therefore constitutively expressed in B cells (Graninger *et al.*, 1987; Reed *et al.*, 1987; Seto *et al.*, 1988). Some studies had demonstrated that deregulation of Bcl-2 markedly prolongs the survival of mature B cells, resulting in an immortalized cell population (Vaux *et al* 1988; McDonnell *et al* 1989). The biochemical function of Bcl-2 was eventually elucidated in 1990, when it was discovered that deregulation of Bcl-2 prevents cell death by blocking apoptosis (Hockenbery *et al.,* 1990), although how Bcl-2 achieves this block continues to be a matter of controversy (Vander and Thompson., 1999; Belka and Budach., 2002; Annis *et al.*, 2004). BCL2 family of proteins is the hallmark of apoptosis regulation. In the past years, new members of BCL2 gene family were discovered and cloned and were found to be differentially expressed in many types of cancer. The protein family, through its role in regulation of apoptotic pathways, is possibly related to cancer pathophysiology and resistance to conventional chemotherapy, is well known that leukemias are haematogenesis malignancies characterized by biological diversity, varied cytogenetics, different immunophenotype profiles, and diverse results. The

protein (and its antiapoptotic orthologues) seems to stop apoptosis by the preservation of mitochondrial membrane probity as its hydrophobic carboxyl-terminal domain is linked to the outer membrane. It prevents BAX/BAK oligomerization, which would otherwise lead to the release of several apoptogenic molecules from the mitochondrion. BCL2 also binds to and inactivates BAX and other pro-apoptotic proteins, thereby inhibiting apoptosis. The protein might also synchronize the activation of several initiator caspases like caspase-2 that act independently of cytochrome c release from mitochondria. Therefore, BCL2 directly blocks cytochrome c release and somehow prevents APAF-1 and caspase-9 activation. (Khemtemourian *et al*., 2008). Steroidogenic enzymes that have a high basal level of expression, such as 3/3HSD, include cholesterol sidechain cleavage cytochrome P450. The effects are mostly seen in Leydig cells. (Diane *et al*., 1992). Steroidogenesis is complex process in which cholesterol is converted into potent androgens estrogens and progesterone through several catalytic and metabolic activities. There are number of steroidogenic enzymes involved in production of several kinds of steroid hormone. The initial step of steroidogenesis is start with cholesterol transport by steroidogenic acute regulatory (StAR) protein to mitochondrial membrane in steroidogenic organs or tissue. Further, in adrenal and testis, both cytochrome P450 (CYP) and 3 - hydroxysteroid dehydrogenase (3βHSD) enzyme involved in steroid biosynthesis and produces more potent steroid progesterone, 17 α hydroxyprogesteron and androstenedione (Payne and Hales., 2004). All enzymes involved in steroidogenesis are regulated by several endocrine and paracrine factors. 3β-HSD is regulated by LH receptor hence activation of LH receptor is necessary for initiation of actual steroidogenesis (Rahman and Rao., 2008). In another hands, LH secretion from anterior pituitary is necessary for stimulation of enzymes involved in steroid production therefore LH receptor plays a regulatory control over 3β-HSD expressions. In testicular tissue, LH receptor controls the proliferation and maturation of Leydig cells that is essential for testosterone production (Dufau, 1998). LHR interact with luteinizing hormone (LH) to further precede proliferation and maturation of germ cells and Leydig cells (Roess *et al* ., 2000) in adults as well neonatal.

The process of western blotting involves electrotransferring proteins from a gel to a

membrane. The specificity of immunological identification is combined with the tenacity of electrophoretic protein separation in a quick and extremely sensitive manner. High fat diets are effecting the specific proteins showing traces of the specific bands.

1.14. Polymerase chain reaction in analysing the high fat diet

A laboratory technique known as reverse transcription polymerase chain reaction (RT-PCR) combines RNA reverse transcription into complementary DNA (cDNA) with PCR amplification of specified DNA targets. (Deepak et al., 2007). The main function of it is to quantify the quantity of a certain RNA. Real-time PCR or quantitative PCR (qPCR) is a method for accomplishing this that uses fluorescence to monitor the amplification reaction. In both academic and medical settings, combined RT-PCR and qPCR are frequently employed to analyse gene expression and quantify viral RNA.The close association between RT-PCR and qPCR has led to metonymic use of the term qPCR to mean RT-PCR. Such use may be confusing, (Bustin , 2002) as RT-PCR can be used without qPCR, for example to enable molecular cloning, sequencing or simple detection of RNA. Conversely, qPCR may be used without RT-PCR, for example to quantify the copy number of a specific piece of DNA.

In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase (RT). The cDNA is then used as a template for exponential amplification using PCR. The use of RT-PCR for the detection of RNA transcript has revolutionized the study of gene expression in the following important ways:

- Made it theoretically possible to detect the transcripts of practically any gene.
- Enabled sample amplification and eliminated the need for abundant starting material required when using northern blot analysis.
- Provided tolerance for RNA degradation as long as the RNA spanning the primer is intact (Freeman *et al.,* 1999)

1.15. PHENETHYL ISOTHIOCYANATE

Phenethyl isothiocyanate (PEITC) is a naturally occurring isothiocyanate whose precursor, gluconasturtiin is found in some cruciferous vegetables especially in watercress and cabbages (Tusskorn *et al.*, 2013) . Watercress is a rich source of gluconasturtiin, the precursor of phenethyl isothiocyanate (PEITC), while garden cress is rich in glucotropaeolin, the precursor of benzyl isothiocyanate (BITC). PEITC has been studied for its potential for chemoprevention of cancers (Stan *et al.*, 2013) such as prostate cancer.(Thomson *et al.*, 2013).In terms of biosynthesis, PEITC is produced from gluconasturtiin by the action of the enzyme myrosinase. PEITC is found in plants of the Brassicaceae family, including broccoli, cabbage, and radish.

1.16. Ameliorative role of Phenethyl isothiocyanate

Phenethyl isothiocyanate has been demonstrated to have a series of pharmacological actions, including antioxidant, antimutative, anti-inflammatory and anti-tumorigenic actions. PEITC which is a naturally occurring cruciferous vegetable-derived compound that inhibits cell growth and induces apoptosis in oral cancer cells (Yeh *et al*., 2014). It induces DNA damage-associated G2/M arrest and subsequent apoptosis in oral cancer cells with varying p53 mutations (Yeh *et al*., 2014). PEITC is one of the most studied members of the ITC family of compounds because of its ability to chemically inhibit induced cancers, including oral cancer, in animal models (Solt *et al*., 2003; Shabany *et al*., 2002). In addition, PEITC displays minimal or no toxicity toward normal cells, thus making this class of compounds ideal chemopreventive agents against various malignancies (Trachootham *et al*., 2008; Musk *et al*., 1995). The chemopreventive and therapeutic effects of PEITC on lung cancer in smokers are currently being explored in clinical studies. Isothiocyanates are best known for their antioxidative, anticancer chemotherapeutic, chemopreventive, anti-angiogenic, and antibiotic properties. In vitro, PEITC increases caspase 3 activity and cleavage of poly (ADP)-ribose polymerase (PARP), inducing caspase-mediated apoptosis in Jurkat T cells and other cellular models. PEITC increases activation of JNK1, one potential mechanism behind its regulation of phase II detoxifying enzyme gene expression.

The LD50 of PEITC for female rats was 1.47 g/kg. The maternal body weight gain and the number of implanted and live fetuses were decreased with the increase of PEITC dosage given during pre-implantation period. There was also a dose-dependent effect of PEITC given during post-implantation period on fetal weight/growth and placental weight. No toxicity on the organ weight of pregnant rats was observed. Given at different duration of gestation PEITC exhibited some embryo-toxicity on pregnant rats, and the no observable adverse effect level was 15 mg/kg (Liu *et al*., 2011).Over the last decade, the therapeutic effects of Phenethyl isothiocyanate on various cancer types and Alzheimer's disease have been confirmed by clinical trials (Yang *et al*., 2016). Additionally, PEITC decreases levels of Bcl-2 and increases levels of Bax, also decreasing the mitochondrial membrane potential and inducing intracellular influx of free Ca2+, resulting in cell death. This compound decreases oxidation of carcinogen NNK and increases activity of NADPH: quinone oxidoreductase and glutathione S-transferase in vitro and in vivo. In glioma cells, PEITC alters PI3K/MAPK signaling to inhibit accumulation of HIF-1α and secretion of VEGF during hypoxia. Initially, phenethyl isothiocyanate was shown to be cytotoxic to the Jurkat T lymphoma cell line with an LD50 of 7.4 μM. Bcl-2 expression had little protective effect, and even greater than 50-fold overexpression only increased the LD50 to 15.1 μM. Morphological and biochemical assays indicated that death still occurred by apoptosis despite overexpression of Bcl-2. (Annis *et al*., 2004).Sulforaphane is associated with the inhibition of adipocyte differentiation, the induction of adipocyte lipolysis and a reduced risk of cardiovascular disease (Choi *et al*., 2012; Kwon *et al*., 2012; Jayakumar *et al*., 2013; Lee *et al*., 2012). Sulforaphane inhibits adipocyte differentiation by blocking clonal expansion via cell cycle arrest in 3T3-L1 preadipocytes, and stimulates lipolysis via hormone sensitive lipase activation in 3T3-L1 adipocytes (Choi *et al*., 2012; Lee *et al*., 2012). This compound also inhibits neointima formation through suppression of NF-κB pathways and regulates migration and proliferation in vascular smooth muscle cells Furthermore, it has been shown to activate adenylate cyclase and inhibit the PI3-kinase/Akt, p38MAPK and PLCγ2-PKC-p47 cascades, thus ultimately inhibiting platelet aggregation and thrombotic formation (Kwon *et al*., 2012; Jayakumar *et al*., 2013).

PEITC inhibited the migration of tumor cells to the brain after injection into the heart of mice, limiting the growth of metastatic brain tumors (Gupta *et al.*, 2013). To fuel their rapid growth, invasive tumors must also develop new capillaries from pre existing blood vessels by a process known as angiogenesis. Isothiocyanates have been shown to prevent the formation of capillary-like structures from human umbilical endothelial cells. Isothiocyanates likely inhibit the expression and function of hypoxia inducible factors (HIFs) that control angiogenesis, as reported in endothelial cells and malignant cell lines (Cavell *et al*., 2011)

PEITC inhibited androgen receptor (AR) transcriptional activity in prostate cancer cells by repressing miR-141 expression and miR-141-mediated downregulation of small heterodimer partner (shp), a repressor of AR (Xiao *et al*., 2012).

In this study, we initially established a high fat diet (HFD) induced obese rats in order to determine whether obesity affects declines in male fertility as well as serum reproductive hormone levels and disrupts testicular morphology. Furthermore, during spermatogenesis in obese rats testicular changes in relevant biomarkers of blood-testis barrier (BTB) function was studied.We hypothesized that exposure to a FPF-diet with excessive calories, a high fat content, and high FAME levels alters testicular physiology and metabolism, leading to permanent damage to the testicular system and its function. Therefore, the purpose of this study was to compare the effects of long-term consumption of FPF-based diets (FPFH, FPF-M, and FPF-L), FPF-M for 30 days, 60 days and 90 days and PEITC on anthropometrical indices (body and organ weights, body fat mass distribution, food and calorie intake nutritional parameters), metabolic syndrome (liver and kidney function profles, hyperinsulinemia, hyperglycemia, insulin resistance, dyslipidaemia, leptinemia), infammatory (IL-1, IL-6, LTB4, prostaglandin, NO, MPO, LDH, and TNF-α) and pro-oxidant activities (conjugated dienes, lipid hydroperoxides, malondialdehyde, protein carbonyl and fragmented DNA), compromised testicular antioxidant defenses (CAT, SOD, GST and GSH), variations in reproductive sex hormones (testicular and serum testosterone, estradiol, FSH and LH), sperm quality and functional indices, sperm DNA fragmentation and damage, progressive deterioration of spermatogenesis (histology

and histomorphometrics), germ cell proliferation (PCNA expression) and testicular function, steroidogenic impairment (StAR, 3β-HSD and LHR expression), and abnormally enhanced testicular germ cell apoptosis (TUNEL assay, BAX and BCL-2 expression) in Wistar albino rats compared to the control diet.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Many studies revealed that tobacco, alcohol, different food habit, infection etc. promote the occurrence of most of the cancers including stomach cancer worldwide. Diet has been associated as a co-factor in the progression from gastritis to gastric cancer; accordingly the incidence of stomach cancer varies around the world depending on dietary patterns (Ward *et al.,*, 1999). The lifestyle and dietary habit of the people of Mizoram are different from other parts of the country, as they consume many uncommon foods which includes smoke and sun dried salted meat and fish, soda (alkali), traditional fermented food, Sa-um (fermented pork fat) and bekang (fermented soya bean) etc. (Phukan *et al.,*, 2006). Obesity comes with a plethora of adverse health consequences, including cardiovascular diseases, dyslipidaemias, non-alcoholic fatty liver disease and higher incidence of type 2 diabetes and various cancers including female cancers (Johnson *et al.,*, 2015; Abdullah *et al.,*, 2 010; Park *et al.,*, 2014 ; Foong *et al.,*, 2017. Diets high in saturated fat are negatively correlated with sperm concentration and this has been described to occur in a dose dependent manner (Jensen *et al.,*, 2013). Diet has important effects on normal physiology and the potential deleterious effects of high fat diets and obesity on male reproductive health are being increasingly described (Jarvis *et al.,*, 2020). Obesity is often defined simply as a status of excessive or abnormal fat accumulation arising from an imbalance between caloric intake and metabolic expenditure (Nammi *et al.,*, 2004). Currently, epidemiological studies show that the proportion of adults with a body-mass index (BMI) of 25 kg/m2 or greater significantly increased between 1980 and 2013 worldwide and over 31% of the male adult population in USA is obese in 2013 (defined as a BMI_30 kg/m2.In the past decades, the adverse effects of obesity on female fertility have been well recognized. They include menstrual disorder, anovulation, polycystic ovarian syndrome, an increased risk of miscarriage and a reduced conception rate (Zain *et al.,*, 2008; Gesink *et al.,*, 2007). Moreover, obesity, especially abdominal obesity, causes serum adipokine imbalance, insulin resistance and endothelial dysfunction (Ritchie and Connell., 2007). It is also associated with dyslipidemia and an increase in the ratio of total cholesterol (TC) to high-density lipoprotein cholesterol (HDL-C) (Paccand *et al.,*, 2000). A study was performed on

mice which determined that the consumption of a (HFD) has resulted in elevated levels of serum cholesterol (Eisinger ., 2014).

However, key aberrations in gene targets or pathways in the testis from HF diets are still relatively unknown with modest changes in RNA transcripts in the mouse testis making it challenging to understand pathways by which HF diets exerts such deleterious consequences (Grandjean *et al.,*, 2015; An *et al.,*, 2017).Gene expression studies are unlikely to be the most relevant investigation to comprehensively understand the effects of HF on the testis, particularly as male germ cells become transcriptionally silent in late spermatogenesis. At the protein level, one recent study used proteomic analysis in conjunction with long non-coding RNA arrays to study testes from rats fed a HF diet; cytoskeleton changes and oxidative stress were found to be important (Yang *et al.,*, 2018).

2.2. Phenethyl isothiocyanate (PEITC), extracted from cruciferous vegetables, showed anticancer activity in many human cancer cells. PEITC significantly reduced the levels of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β genes, in transcriptional levels and modulated ERK- and Akt-dependent and NF-κB signaling pathways in GBM 8401 cells. PEITC may have anti inflammatory effects on GBM, which can be a basis for further experiments to explore the immune regulation of PEITC on glioblastoma in vivo. (Sheng-Yao Hsu *et al.,*, 2022). PEITC inhibited the binding of NF-κB on promoter site of DNA in GBM 8401 cells. PEITC also altered the protein expressions of protein kinase B (Akt), extracellular signal-regulated kinase (ERK), and NF-κB signaling pathways. The inflammatory responses in human glioblastoma cells may be suppressed by PEITC through the phosphoinositide 3-kinase (PI3K)/Akt/NF-κB signaling pathway. Thus, PEITC may have the potential to be an anti-inflammatory agent for human glioblastoma in the future. (Sheng-Yao Hsu *et al.,* 2022).

PEITC prevents the initiation of carcinogenesis and suppresses the progression of tumorigenesis (P.Gupta., 2014). The anticancer effects of PEITC on cell proliferation, apoptosis, angiogenesis, metastasis, autophagy, inflammation, and immunomodulation in different cancer models have been reported. PEITC reduced

the cell viability of GBM 8401 cells in our previous experiments, including the studies of apoptosis, migration, and invasion (Chou *et al.*,, 2015)

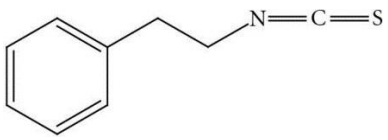
PEITC administration including the high ingestion of cruciferous vegetables can be expected to be beneficial for preventing and improving obesity without adverse effects. However, long-term in vivo studies are required to establish the anti-obesity efficacy, efficient dosage, and safety profile of PEITC. In the future, we must adequately assess the long term potency of PEITC ingestion for suppression of food intake and body weight gain, and improvement of leptin responsiveness in obese mice fed a high-fat diet or in obese human subjects. (Yagi *et al.*,, 2018). PEITC is a relatively nontoxic compound that occurs abundantly in water cress and broccoli as gluconasturtiin, and has reached the level of phase 2 clinical trials for lung and oral cancer prevention in the US (Gupta *et al.*,, 2014).

PEITC exhibited an inhibitory effect on the adhesion and invasion of HeLa cells by induction of G2/M phase arrest, it reduced the expression of CDK1, MMP-2/9, CD44, ICAM-1, increased the production of TGF-β, IL-6 and IL-8, and increased the phosphorylation of Smad2. PEITC may be a potential antitumor compound, acting through the TGF-β/Smad2 pathway; and it has the potential for future use as a therapy for cervical carcinoma subsequent to further studies. PEITC serves an import function in anti-metastatic processes in various tumor cells, including human hepatoma, human colon cancer and breast carcinoma cells (Hunakova *et al.*,, 2009; Hwang and Lee HJ., 2008 and Lai., 2010). This effect is associated with the regulation of the expression of metastasis-related genes, cytokines and signal transduction molecules. PEITC has been demonstrated to suppress growth and metastasis in lung, prostate and ovarian cancer. (Zhang *et al.*,, 2014). PEITC is known to selectively kill cancer cells, but not normal cells, by generating reactive oxygen species (ROS) to trigger signal transduction, resulting in cell cycle arrest and/or apoptosis (X.-J. Wu and X. Hua., 2007). PEITC is also an effective inhibitor of hypoxia inducible factor (HIF), a transcription factor that plays an important role in the expression of proangiogenic factors (X.-H. Wang *et al.*,, 2009). PEITC may slow down the development of colon carcinogenesis in an inflammatory intestinal

setting which is potentially associated with epigenetic modulation of NFκB1 signaling. At non-cytotoxic concentrations, a key pro-inflammatory transcription factor NFκB1 and associated expression of interacting genes were down regulated after PEITC exposure. The PEITC associated reduced expression of the NFκB1, correlated with changes in a gene-suppressing histone modification mark. This indicates at least partially that PEITC activity leads to a potential epigenetic regulation of NFκB1 that relates to its anti-inflammatory effects. (Liu and Moul Dey., 2017).

The aim of this study was to determine whether diet-induced obesity impairs male fertility and furthermore to uncover its underlying mechanisms. HFD impairs the reproductive system by decreasing IL1β and enhancing testosterone levels in rats which provides new ideas for the treatment of obesity-induced infertility (Zhang *et al.*,, 2017). The purpose of this study was, therefore, to compare the effects of long-term consumption of FPF-based diets with the amelioration of PEITC on anthropometrical indices (body and organ weights, body fat mass distribution, food and calorie intake nutritional parameters),metabolic syndrome (liver and kidney function profiles, hyperinsulinemia, hyperglycemia, insulin resistance, dyslipidaemia, leptinemia), augmentation of inflammatory (IL-1, IL-6, LTB4, prostaglandin, NO, MPO, LDH, and TNF-α) and pro-oxidant (conjugated dienes, lipid hydroperoxides, malondialdehyde, protein carbonyl and fragmented DNA) activities and compromised testicular antioxidant defences (CAT, SOD, GST and GSH),variations in reproductive sex hormones (testicular and serum testosterone, estradiol, FSH and LH), sperm quality and functional indices, sperm DNA fragmentation and damage, progressive deterioration of spermatogenesis (histology and histo-morphometrics), germ cell proliferation (PCNA expression)and testicular function, steroidogenic impairment (StAR, 3β-HSD and LHR expression), and abnormally enhanced testicular germ cell apoptosis (TUNEL assay, BAX and BCL-2 expression)in the Wistar albino rats compared to the standard control-diet and the amelioration of PEITC.

A. Chemical structure of phenethyl isothiocyanate



B. Food sources of PEITC

FIG 1: A. Chemical structure of phenethyl isothiocyanate

B. Food sources of PEITC

CHAPTER 3

OBJECTIVES

Based on the literature survey the following queries have been raised to validate the scientific finding:

Is fermented pork fat really a toxic substance or may be used as a flavour enhancer?

Is Fermented pork fat causing organ toxicity?

If it causes organ toxicity what is the dose at which it impairs the organ function?

Is there any ameliorative measure for fermented pork fat toxicity?

Hence, the objectives of the present work is to study the effect of phenethyl isothiocyanate on reproductive parameters of Fermented pork fat fed with Wistar albino rats and their role in amelioration of the testicular toxicity induced by Fermented pork fat in relation to:

- **Assessment of fermented pork high fat diet (saum) and its implications on biochemical parameters, sperm quality, spermatogenesis and steroidogenesis, oxidative stress and apoptosis.**

- **Ameliorative effects of phenethyl isothiocyanate on fermented pork high fat diet (saum) induced metabolic alterations and reproductive toxicity.**

Chapter 4

Materials and Methods

4.1. Animal ethics

Male Wistar albino rats (150–200 g, 13 weeks old) were inbred in the Animal Care Facility at the Department of Zoology, Mizoram University, Aizawl, Mizoram, India. Pathogen-free polypropylene cages (421×290×190 mm) and bedding materials were used to house experimental rats in an environmentally controlled chamber (23±1 °C; relative humidity, 50±10%; photoperiod, 12L:12D). The study was approved by the Institutional Animal Care Committee of Mizoram University (Approval Number: MZUIAEC/2018/15 dt. 26/03/2018) and conducted in accordance with the ARRIVE and NIH guidelines for the care and use of laboratory animals. All the experiments were conducted with minimal suffering and distress.

4.2. Experimental design

After seven days of acclimation, rats were randomly divided into 4 groups (n=20): Group 1: Control rats fed with standard pellet diet (Carbohydrate-65%, Protein-24%, Fat-11% and Total energy-12.56 kJ/g), Group 2: FPF-M, a low-calorie and low-fat diet (Carbohydrate-65%, Protein 20%, Fat 15%, and Total energy 14.21 kJ/g).), Group 3: FPF-M, moderate calorie and moderate-fat diet (Carbohydrates 50%, Protein 20%, Fat 30%, and Total energy 17.5 kJ/g), and Group 4: FPF-M, high-calorie and high-fat diet (Carbohydrate-20%, Protein-20%, Fat-60% and Total energy-23 kJ/g) for 30, 60 and 90 days respectively. Based on the energy content another experiment was done in which rats were randomly divided into 4 groups (n=20): Group 1: Control rats fed with standard pellet diet (Carbohydrate-65%, Protein-24%, Fat-11% and Total energy-12.56 kJ/g), Group 2: FPF-L, a low-calorie and low-fat diet (Carbohydrate-65%, Protein 20%, Fat 15%, and Total energy 14.21 kJ/g).), Group 3: FPF-M, moderatecalorie and moderate-fat diet (Carbohydrates 50%, Protein 20%, Fat 30%, and Total energy 17.5 kJ/g), and Group 4: FPF-H, high-calorie and high-fat diet (Carbohydrate-20%, Protein-20%, Fat-60% and Total energy-23 kJ/g). Rats were fed the diets and water ad libitum twice daily at 9:00 AM and 4:00 PM for 90 days. Another experiment was performed in which rats were divided into 6 groups fed with FPF diet and PEITC. Group 1: Control rats fed with standard pellet diet (Carbohydrate-65%, Protein-24%, Fat-11% and Total

energy-12.56 kJ/g), Group 2: FPF-M diet (fermented pork fat), Group 3: PEITC 100mg/kg, Group 4: PEITC 200mg/kg, Group5: Fermented pork fat +PEITC 100mg/kg, Group 6: Fermented pork fat +PEITC 200mg/kg. Routine analyses (body weight, food and water consumption, and rectal temperature) were performed at regular intervals. After the experiment, the rats were fasted overnight, anesthetized (ketamine, 60 mg/ kg), euthanatized, and serum was collected and stored at -20 °C for hormonal and biochemical analyses. Livers, kidneys, testes, and accessory sex organs, as well as fat contents (epididymal, retroperitoneal, peri-renal, mesenteric, and dorsal subcutaneous) were excised, weighed, and stored at -80 °C for further use. The left testis from each group was fixed in Bouin's fixative for 24 h for histological and immunohistochemical evaluation, while the right testis was preserved for biochemical assays

Table 1: High fat diet doses and experimental design

	EXPERIMENTAL GROUPS		
DOSE	High Fat Diet	No of animals	No of days
control	-	5	30
FPF-M	Fermented pork fat	5	30
control		5	60
FPF-M	Fermented pork fat	5	60
control		5	90
FPF-M	Fermented pork fat	5	90

Table 2: High fat diet doses and experimental design

	EXPERIMENTAL GROUPS		
DOSE	High Fat Diet	No of animals	No of days
control	-	5	90
FPF-L	Fermented pork fat	5	90
FPF-M	Fermented pork	5	90

	fat		
FPF-H	Fermented pork fat	5	90

Table 3 : High fat diet doses and PEITC doses experimental design

	EXPERIMENTAL GROUPS		
	Chemicals	No of animals	No of days
control	-	5	60
fermented pork fat	-	5	60
100mg/kg	PEITC	5	60
200mg/kg	PEITC	5	60
FPF+100mg/kg	PEITC	5	60
FPF+100mg/kg	PEITC	5	60

4.3. FPF, diet composition, FAME and TAG analysis

The production and consumption of fermented pork fat (FPF) has expanded throughout the world as well as Northeast India as a source of daily food, which leads to the progress of multifactorial diseases, chronic non-communicable diseases, a global socio-economic and health problem, which affects human health (Emelyanova *et al*., 2019; Lebret and Candek-Potokar., 2022). Sa-um, a high fat diet which is obtained from pork fat, is used as flavour enhancer in Mizo cuisine. The high fat diet, an indigenous fermented pork fat is a product in Northeast India, especially in Mizoram, is prepared using methods based on cultural traditions under cottage-industrial scale and as a result of variation in the organoleptic, nutritional and product quality (De Mandal *et al*., 2018; Deka *et al*., 2021). Fats of pigs are mainly collected from the inner abdominal portion and sometimes fats from other parts of the body, it is then torn/chopped into pieces and cooked and are placed in a special container called sa-um bur, which is prepared from the dried fruit of the plant ûm bottle gourd (*Lagenaria siceraria)*. The container is then placed over the fireplace and approximately after three days or even longer which is called Sa-um, (fermented) and

is ready for use in the preparation of other foods such as bawl, bai, etc. (Lalthanpuii *et al*.,2015). FPF is rich in fat content (90 – 95%) with high calorific value (830 Kcals/100 g) and comprises of pathogenic organisms such as *Clostridium* (7.61%), *Bacteroides*(4.57%), *Oscillospira* (4.15%), *Corynebacterium* (1.80%), *Megamonas* (1.52%), *Faecalibacterium* (1.38%),*Proteus* (1.38%), *Ruminococcus*(1.24%), and *Prevotella*, which can have adverse health effects in human (De Mandal *et al*.2018).

The nutrients and mineral composition of FPF were determined according to AOAC methods (Association of Official Analytical Chemists, 2019). FPF-diet composition and analysis of FAME and TAG composition Three FPF diets were developed, and their respective caloric composition, percentage contribution of calories from proteins, carbohydrates, and fat are presented. Fatty acid methyl esters (FAME) composition of the diets was determined gas chromatography-mass spectrometry (a TRACE GC–MS with a Polaris Q mass spectrometer, Thermo Finnigan, USA) (Aldai et al., 2006; Liu et al. 2010; Li *et al.*, 2021). A capillary column (J&W DB-WAX, 30 m×0.25 mm I.D., 0.25 mm flm thickness), helium carrier gas (fow rate 1.0 mL/min), oven temperature (60— 250 °C at a rate of 4 °C/min) and 1 µL sample (1:9 with hexane) were used for GC analysis (1:10 split ratio, 24.79 psi, inlet temperature 230 °C, solvent delay time 5 min, and scanning at 30–450 m/z with electron energy at 70 eV and 0.58 s/scan velocity). FAMEs were separated at a constant flow with an oven program of initially 50 °C for 2 min, followed by an increase in temperature of 10 °C per minute up to 200 °C and maintained at 200 °C for 10 min, and finally an increase in temperature of 10 °C per minute up to 220 °C and maintained at 220 °C for 15 min. The transfer line was maintained at 230 °C. The ion trap mass spectrometer was operated under electron ionization (EI) mode (Liu *et al.*, 2010). Solutions of 16 individual triacylglycerol (10 mg TAG:10 mL dichloromethane w/v) standards (Table 1) were prepared and 1 µL was used for GC analysis [Thermo Scientifc Trace 1300 gas chromatograph, capillary column (RTX-65TG, 30 m×0.25 mm i.d. 0.1 µm), split ratio of 1:30, oven temperature (250—360 °C at 4 °C/min), FID detector, carrier gas (hydrogen, 99.9%, fow rate 1.5 mL/min)]. Trinonadecanoyl-glycerol (NNN) was used as an internal reference. The validation parameters (ftness of analytical curves, linearity, recovery, limit of

detection, limit of quantitation, precision, relative standard deviation and repeatability) were computed using the peak area. Peak identifcation was based on the elution profle of known FAME chromatographic standards (fatty acid methyl esters, C4eC24, Nuchek Prep, CDDE-GLC-617-50MG, USA) and previous reports (Aldai *et al.,* 2006; Liu *et al.,* 2010; Li *et al.,* 2021). Relative quantifcation was normalized with the sum of the detected species and is shown as mg/g of total species.

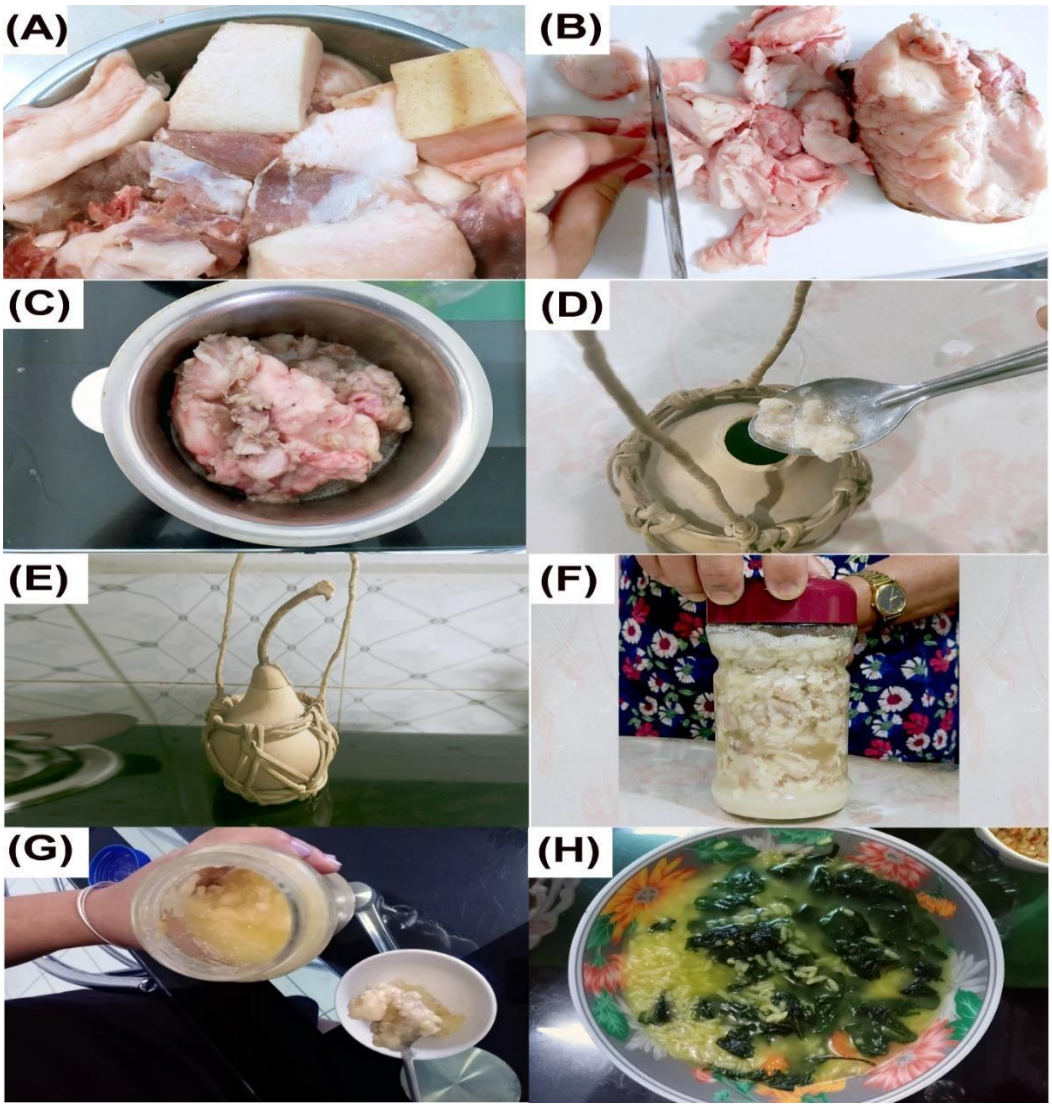


FIG 2: Traditional method of preparation of fermented pork (Sa-um) fat diet. **A** Cleaned and washed pork back fat and lean meat from *longissimus thoracis et*

lumborum. **B** Separation of extracellular fat to obtain lean pork fat. **C** Chopped and trimmed of Caul fat adipose tissue. **D** Process of Lard rendering, i.e., minced fat boiled at 100 ℃ for 15 min. **E** Transfer of boiled caul fat adipose tissue to a "Sa-um bur" dry container made of bottle gourd (*Lagenaria siceraria*) for ripening. **F** Dry bottle guard container, comprising boiled caul fat adipose tissue, is kept for 3-5 days under the sun for fermentation. **G** Light brownish-yellow, creamy, melted butter like textured fermented pork fat. **H** Vegetable soup [Bai] prepared by mixing different types of vegetables with fermented pork fat (Sa-um)

4.4. Anthropometrical and nutritional measures

Body length (nose-to-anus), absolute and relative organ weights, gonadosomatic index, body mass index, Lee index, food and water consumption, body fat mass distribution, specifc rate of body mass gain, energy intake, feed effciency, and rectal temperature were measured. (Bernardis, 1970; Novelli *et al.*, 2007).

4.5. Sperm analysis and acridine orange staining

The daily sperm production (DSP) was calculated as the number of homogenization-resistant spermatids. Sperm motility, viability, morphology, epididymal sperm concentration, sperm DNA damage (acridine orange staining, AO), sperm head and tail abnormalities, and sperm transit time were evaluated (World Health Organization 1999). The dsDNA/ssDNA ratio was calculated as the number of normal dsDNA (green fuorescent) divided by the number of damaged ssDNA (yellowish orange/red fuorescent).

4.6. Insulin resistance, hepato-renal function and lipid profle

Serum insulin, glucose, aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), creatinine, urea, total cholesterol (TC), HDL, TAG, and leptin levels were estimated using the procedure outlined in commercial diagnostic kits (Coral Clinical systems, Goa, India). The Homeostasis Model Assessment of Insulin Resistance Index (HOMA-IR), Pancreatic β-Cell Function (HOMA-β), AST:ALT Ratio, Blood Urea Nitrogen, BUN:Creatinine Ratio, VLDL,

LDL, Cardiac Index (CI), Atherogenic Index (AtI), and Coronary Artery Index (CAI) were computed (Ajiboye *et al.*, 2016)

4.7. Serum and testis reproductive hormone assay

Serum and testicular testosterone (Labor Diagnostika Nord, Germany), serum estradiol (DiaMetra kit, Italy), serum luteinizing hormone (LH, DiaMetra kit, Italy), and serum follicle stimulating hormone (FSH, MyBiosource, USA) hormones were estimated using ELISA kit according to the manufacturer's instructions. Serum and testis infammatory markers Serum interleukins (IL-1, IL-6, and IL-10), leukotriene B4 (LTB4), and prostaglandin levels were assessed using ELISA kits (Bioassay Technology Laboratory, China) and the testicular levels of nitric oxide (NO), myeloperoxidase (MPO), lactate dehydrogenase (LDH), tumour necrosis factor-alpha (TNF-α) were estimated using ELISA kits (R&DSystems, Thermo Fisher Scientifc) based on the manufacturer's instructions. Testis oxidative stress markers The levels of conjugated dienes, lipid hydroperoxides and malondialdehyde (Reilly and Aust., 2001), protein carbonyl (Levine *et al.*, 1990) and the fragmented DNA (Burton, 1956) were determined according to the protocols described earlier. The enzymatic activity of antioxidant enzymes (CAT: catalase, SOD: superoxide dismutase, GST: glutathione S transferase and GSH: reduced glutathione and GSSG: glutathione disulfde) was estimated in the testicular extract using commercial kits (ELBA Science, USA and Cayman Chemical Company, USA) according to the standards and protocols provided by the manufacturer. GSH:GSSG ratio was calculated to measure the mitochondrial or cellular redox state.

4.8. Serum FAME profiles

Serum FAME levels in rats fed diferent FPF-diets, along with control-diet, were analyzed by GC–MS as described previously in the section "FPF-diet composition and analysis of FAME and TAG composition" (Aldai *et al.*, 2006; Liu *et al.*, 2010; Li *et al.*, 2021).

4.9. Testis histology and histo-morphometrics

The left testes were harvested, fxed in Bouin's fxative overnight, followed by parafn embedding, sectioned at 5 µm thicknesses, and counterstained with hematoxylin and eosin (H&E) for histopathological analysis. Testis tissue damage was assessed using the blindfold method and scored in all tissue Sects. (10 random non-overlapping felds/diet group) at 10×and 40×magnifcations, respectively. A lesion scoring system was employed to measure the testis damage (Abdelhamid *et al.,* 2020). Spermatogenesis impairment was evaluated in terms of histomorphological changes in the seminiferous tubules (JTBS: Johnsen's mean testicular biopsy score, MSTD: mean seminiferous tubule diameter, TAT: tunica albuginea thickness, STEH: seminiferous tubule epithelial height, IS: interstitial space and TL: tubular lumen) and the quantifcation of germ cells (spermatogonia, spermatocytes, spermatids, Sertoli cells) and Leydig cells in the testis was performed (10 random non-overlapping felds per diet group), respectively. The Tubule Differentiation Index (TDI, %) was calculated as the percentage of tubules presenting Type A or Type B diferentiation in 600 seminiferous tubules for each treatment group (5 tissue sections per group) (Johnsen 1970; Russell *et al.,* 1990).

Table 4: Score for accessing spermatogenesis in testicular biopsy

Score for assessing spermatogenesis in testicular biopsy

Score	Description
10	complete spermatogenesis and perfect tubules
9	Many late spermatids or many spermatozoa present but disorganized spermatogenesis
8	only a few spermatozoa present
7	no spermatozoa but many spermatids present
6	only a few spermatids present
5	no spermatozoa or spermatids present but many spermatocytes present
4	only a few spermatocytes present
3	only spermatogonia present
2	no germ cells present only sertoli cells
1	No cells

4.10. Immunohistochemistry

Immunohistochemical staining was performed on the testis tissue using PCNA (1:100, Santa Cruz Biotechnology Inc., USA), StAR (1:2000, St. John's laboratory, London, UK), 3β-HSD (1:200, Santa Cruz Biotechnology, Dallas, USA), LHR (1:500, Santa Cruz Biotechnology, Dallas, USA), BAX (1:50, Elabscience, China) and BCL-2 (1:500, Elabscience, China) polyclonal rabbit primary antibodies and a secondary antibody HRP-conjugated goat-anti-rabbit (1:500, Merck Specialties Pvt. Ltd, Mumbai, India), along with 0.05% diaminobenzidine solution, to confrm the efect of FPF-diet on testicular steroidogenesis, cell proliferation and apoptosis. The area immunostained for the antibodies was obtained using ImageJ, and the percentage area of staining at 10×and 40×magnifcations was calculated. The number of germ cells and Leydig cells that had positive reactions to PCNA, BAX, BCL-2, StAR, 3β-HSD, and LHR were manually counted and computed as the ratio of positively reacted cells to the total number of cells multiplied by 100 (Jeremy *et al.,* 2019).

Table 5: Details of antibodies used for immunohistochemical analysis

Sl no	Protein	Dilution of blocking serum and incubation	Dilution of primary antibody and incubation	Dilution of secondary antibody and incubation	Hematoxylin
1	Hsp70	1:100µL(30min)	1:200 (12h)	1:200 (3h)	No
2	PCNA	1:100µL(30min)	1:200 (12h)	1:200 (3h)	No
3	3 beta Hsd	1:100µL(30min)	1:200 (12h)	1:500 (3h)	Yes
4	BCl2	1:100µL(30min)	1:200 (12h)	1:200 (3h)	Yes
5	Bax	1:100µL(30min)	1:200 (12h)	1:200 (3h)	Yes
6	LH receptor	1:100µL(30min)	1:500 (12h)	1:500 (3h)	Yes
7	Caspase 3	1:100µL(30min)	1:200 (12h)	1:200 (3h)	Yes
8	Aromatase	1:100µL(30min)	1:500 (12h)	1:500 (3h)	Yes

4.11. TUNEL assay

Testis germ cell apoptosis was evaluated by TUNEL assay using the Apo-Brdu-IHC in situ DNA fragmentation assay kit (BioVision Inc., USA) following the manufacturer's instructions. The TUNEL-positive apoptotic cells were identifed as dark brown cells. The apoptotic index (AI) was calculated as AI (%)=(number of apoptotic cells/number of total cells)×100)

4.12. Univariate and multivariate analyses

The Kolmogorov and Smirnof test, Levene test, one-way analysis of variance (ANOVA) with Tukey's multiple range post hoc test, and correlation and regression analyses were performed to detect signifcant diferences ($p<0.05$) between the treatment groups. A multivariate analysis of variance (MANOVA) comprising principal component analysis (PCA), hierarchical cluster analysis (using an Euclidean distance measure), and detrended correspondence analysis (DCA) was

conducted using PAST (PAleontological Statistics, version 4.03) software (Hammer *et al.,* 2001).

4.13. Western blotting

Western blot analysis for StAR, **3 beta Hsd, BCl2, HSP 70** protein was undertaken as previously described by (Clark *et al.,,* 1994), except that 250 mg aliquots of mitochondrial protein was analyzed in each gel lane and a Protean II system was used for electrophoresis.5 samples were selected randomly from each groups. β tubulin was used as loading control. Protein estimation was done by the protocol of (Lowry *et al.,,* 1951). 50µ of protein was loaded in each lane followed by separation using SDS-PAGE which is then electroblotted in PVDF membrane (Millipore, Massachusetts, USA). The membrane was blocked for 1hour at 37°C with 5% bovine serum albumin (BSA) solution. The membrane was incubated with anti-rabbit and anti-mouse polyclonal antibody (1:1000) for overnight at 4°C, which is then followed by incubation with alkaline conjugated anti-rabbit and anti-mouse for 4 hours at a ratio of (1:1000). After washing with PBS, the membrane was developed using ECL (BIO-RAD, Cat.# 170-5060,USA). All blottings were done under same condition. The result is the representative of one western blot data. The densiometry analysis was done using Image J software and Graph pad prism.

Table 6: Details of antibodies used for Western blotting

Sl no	**Antibody**	**Type a dilution**	**Host specie**	**Catalogue, source**
Primary antibody				
1	Hsp70	1:1000	mouse	SC32239,SantaCruz Biotechnology Inc , Dallas, USA
2	PCNA	1:1000	rabbit	SC7907,SantaCruz, Dallas USA
3	3 beta Hsd	1:1000	rabbit	Sc30820,SantaCruz Biotechnology Inc, Dallas, USA
4	17 beta Hsd		rabbit	

5	BCl2	1:1000	rabbit	SC7382,SantaCruz Biotechnology Inc, Dallas, USA
6	Bax	1:1000	rabbit	SC6326,SantaCruz Biotechnology Inc, Dallas, USA
7	LHR	1:1000	rabbit	SC26341,SantaCruz Biotechnology Inc, Dallas, USA
8	PARP	1:1000	rabbit	SC74470,SantaCruz Biotechnology Inc, Dallas, USA
9	Caspase 3	1:1000	rabbit	SC56053,SantaCruz Biotechnology Inc, Dallas, USA
10	Caspase9	1:1000	rabbit	SC56076,SantaCruz Biotechnology Inc, Dallas, USA
11	NF-kB	1:1000	rabbit	SC8008,SantaCruz Biotechnology Inc, Dallas, USA
12	TNF alpha	1:1000	rabbit	SC133192,SantaCruz Biotechnology Inc, Dallas, USA

4.14. Analysis of gene expression

Total RNA was extracted from testicular samples using QIAzol reagent. Integrity of the RNA was visualized using 1.5% denatured agarose gel electrophoresis, followed by ethidium bromide staining. Total RNA (2 µg) in a total volume of 11 µl, together with 0.5 ngoligodT primers and sterilized diethylpyrocarbonate (DEPC) water, was used to synthesize cDNA. Briefly, the mixture was incubated in a T100™ Thermal

Cycler at 70˚C for 10 min for denaturation. Subsequently, 2 µl 10X reverse transcription (RT)-buffer, 100 U Moloney Murine Leukemia Virus Reverse Transcriptase (Thermo Fisher Scientific, Inc.), 1 µl 10 mMdNTPs and 5 µl DEPC water was added (total volume, 20 µl). This mixture was then incubated in the thermal cycler at 37˚C for 1 h, and at 70˚C for 10 min in order to induce inactivation of the enzyme. For RT-polymerase chain reaction (PCR) analysis, specific primers were used:

Table 7 : Details of genes used for RT-PCR

Gene	Product size (bp)	Annealing temp (˚C)	Direction Sequence (5'-3')	
			Sense	Antisense
StAR	389	58	TTGGGCATACTC AACAACCA	ATGACACCGCTT TGCTCAG
MPO	108	56	TACCAGGAAGCC CGG AAG AT	TGAGTCATTGTA GGA ACG GT
iNOS	252	65	GTGTTCCACCAG GAGATGTTG	CTCCTGCCCCCTG AGTTCGTC
LT-B4	187	58	ACCAAAGAGCGG ATACGCAG	CCGCCACCTACT GGAAAGTT
IL4	277	63	TGCACCGAGATG TTTGTACC	GGATGCTTTTTA GGCTTTCC
COX1	179	54	GCAGCCTCTGTT CCACATACAC	AATCTGACTTTCT GAGTT- GCC
PG-E2	145	58	ACATGGTGCTTT ATCCGGCA	GGCTTCTTCTGCT CCGACG
TNF alpha	402	50	ACTGAACTTCGG GGTGATTG	GCTTGGTGGTTT GCTACGAC
IL6	260	62	TCCTACCCCAAC TTCCAATGCTC	TTGGATGGTCTT GGTCCTTAGCC
IL-1B	235	61	CACCTCTCAAGC AGAGCACAG	GGGTTCCATGGT GAAGTCAAC
GAPDH	309	52	AGATCCACAACG GATACATT	TCCCTCAAGATT GTCAGCAA

In a total volume of 25 µl [1 µl synthesized cDNA, 1 µl each primer (10 pM), 12.5 µl PCR master mix and 9.5 µl sterilized deionized water], PCR was conducted. The PCR cycling conditions was set as follows: Initial denaturation for 1 cycle at 95˚C for 4 min, followed by 27 cycles (each consisting of denaturation at 94˚C for 1 min, annealing as stated in Table I for each gene, and extension at 72˚C for 1 min) with a final extension step at 72˚C for 7 min. G3PDH were used as an internal control. PCR products were separated by 1.5% agarose gel electrophoresis for 30 min and stained with ethidium bromide in Tris-borate-EDTA buffer. The gels were visualized under ultraviolet light and subsequently photographed using gel documentation system. The band intensities were densitometrically quantified and calculated. (Alkhedaide *et al.*, 2016).

CHAPTER 5

RESULTS

5.1. Time course change of high fat diet

5.1.1. FPF, diet composition, FAME and TAG analysis

FPF composed of high-fat with a high calorific value (160g/kg). The control contained 9.67%, of the total calories from fat with 111.31, 139.2, 138.76 and162.85 g/kg of calories originating from SFA, MUFA, PUFA-ω6 and PUFA-ω3 fatty acids, respectively. The FPF diet comprised 19.67 %of total calories from fat with 419.73, 58.37, 249.01 and 66.33 g/kg of calories arising from SFA, MUFA, PUFA-ω6 and PUFA-ω3 fatty acids, respectively. The total quantity of calories in the control was less than in the FPF- diets **(Table 8).** Analysis of FAME composition in the diets revealed that SFA (lauric, myristic, palmitic, stearic acid: (6.65 – 3.05 fold) and PUFA-ω6 (linoleic, γ-linolenic, arachidonic acid: (1.64 – 1.69 fold) levels were elevated to high quantity in compared with the control. In contrast, MUFA (palmitoleic, oleic, and vaccenic acid: 2.35 – 3.14 fold) and PUFA-ω3 (α-linolenic, eicosatrienoic, and eicosapentaenoic acid: 3.61 -3.66 fold) levels were substantially higher quantity in in the FPF-diets. The n-6: n-3 PUFA ratio (4.44 fold) was higher in FPF-diets than in the control. The levels of twenty triacylglycerol species were also detected in high quantity in the FPF-diets than the control. (**Table 8).**

Table 8: Formula and calculated nutrient composition of fermented pork fat (Sa-um) diet and their fatty acid methyl ester and triacylglycerol profiles

Formula and calculated nutrient composition of fermented pork fat (Sa-um, FPF) diet		
Content	**Control diet (g/kg)**	**FPF- diet (g/kg)**
Casein	180.30	180.30
Corn starch	519.80	350.40
Dextrin	119.00	119.00
Sucrose	90.10	90.01
Fermented Pork fat	0	160.00
Crude fat	40.00	0
Cellulose	45.00	50.00
Minerals	31.50	35.00
Vitamins	9.00	10.00
L-cystine	2.70	3.00
Choline chloride	2.20	2.50
Tert-butyl hydrogen	0.01	0.01
Total (g/kg)	1000	1000
Total energy (kJ/g)	15.48	15.48
Protein (%)	19.40	19.40
Carbohydrate (%)	63.60	63.60
Fat (%)	9.67	30.10
Fatty acid methyl esters composition analysis (mg/g)		
Fatty acid methyl esters (FAME)	**Control diet**	**FPF- diet**
Saturated fatty acids (SFA)		
C12, Lauric acid	13.33 ± 0.04	88.70 ± 1.33
C14, Myristic acid	10.12 ± 0.30	74.45 ± 1.20

C16, Palmitic acid	55.78 ± 1.48	158.51 ± 3.75
C18, Stearic acid	32.09 ± 1.38	98.07 ± 1.22
Monounsaturated fatty acids (MUFA)		
C16:1, Palmitoleic acid, ω7	54.12 ± 1.92	22.99 ± 2.11
C18:1, Oleic acid, ω9	56.00 ± 1.12	26.12 ± 1.16
C18:1, Vaccenic acid, ω7	29.08 ± 1.42	9.26 ± 0.85
Polyunsaturated fatty acids (PUFA)		
C18:2, Linoleic acid, ω6	36.00 ± 1.82	59.06 ± 0.22
C18:3, α-Linolenic acid, ω3	73.60 ± 0.03	20.35 ± 1.22
C18:3, γ-Linolenic acid, ω6	24.22 ± 1.02	56.55 ± 1.80
C20:3, Eicosatrienoic acid, ω3	66.02 ± 1.55	39.65 ± 0.55
C20:4, Arachidonic acid, ω6	78.54 ± 1.26	133.40 ± 1.12
C20:5, Eicosapentaenoic, ω3	23.23 ± 0.12	6.33 ± 0.44
SFA (mg/g)	111.32 ± 0.26	419.73 ± 2.11
MUFA (mg/g)	139.2 ± 1.18	58.37 ± 1.65
PUFA ω6 (mg/g)	138.76 ± 1.05	249.01 ± 3.52
PUFA ω3 (mg/g)	162.85 ± 1.22	66.33 ± 0.52
n-6 : n-3 PUFA ratio	0.85	3.75
Triacylglycerol composition analysis (mg/g)		
Triacylglycerol (TAG)	**Control diet**	**FPF- diet**
1,2-palmitoyl-3-stearoyl-sn-glycerol, PPS	2.45 ± 0.36	20.08 ± 0.45
Trilinolein, LLL	2.66 ± 0.48	12.33 ± 0.85
1,2-linoleoyl-3-oleoyl-sn-glycerol, OLL	23.81 ± 0.18	58.95 ± 0.04
Palmitodilinolein, PLL	35.85 ± 0.26	81.16 ± 1.08
1,2-dioleyl-3-linolenoylglycerol,	34.31 ± 0.74	85.05 ± 0.68

OOL		
1-linolein-2-palmitin-3-olein, LPO	92.65 ± 1.66	248.39 ± 3.65
1,2-palmitoyl-3-linolein-sn-glycerol, PPL	27.88 ± 1.42	60.92 ± 1.72
1,2,3-trioleylglycerol, OOO	25.89 ± 1.28	55.19 ± 1.88
1,3-palmitoyl-2-oleoyl-sn-glycerol, POP	26.56 ± 3.75	212.48 ± 2.11
Dipalmitoyl-oleoyl-glycerol, PPO	32.85 ± 1.21	156.18 ± 1.82
Tripalmitoyl-glycerol, PPP	10.12 ± 0.36	34.66 ± 0.51
1-Stearoyl-2-oleoyl-3-oleoyl-glycerol, OOSt	16.37 ± 0.52	42.48 ± 0.38
1,3-palmitoyl-2-linoleoyl-sn-glycerol, PLP	20.55 ± 0.02	194.25 ± 2.05
Trinonadecanoyl-glycerol, NNN	3.05 ± 0.21	16.42 ± 1.02
1,2-oleoyl-3-sn-palmitoylglycerol, OOP	4.48 ± 0.05	14.45 ± 1.35
1,3-oleoyl-2-palmitoyl-sn-glycerol, OPO	6.06 ± 0.84	29.38 ± 1.28

Data on nutrient composition of the diet, FAME and TAG profiles are presented as mean ± standard error mean.

5.1.2. Long-term FPF feeding afflicted anthropometrical measures

Food and water consumption analysis of 30 days, 60 days and 90 days exposure of FPF diet to Wistar albino rats showed significant differences ($p > 0.05$) in terms of their food and water consumption in comparison with control. Food consumption in FPF diets are (2.48-1.54 fold) as compared to control in 30 days,(1.50-1.63) in 60 days and (2.78-3.33 fold) in 90 days and water consumption in FPF diets are (2.47-2.48 fold) as compared to control in 30 days, (4.17- 4.88 fold) in 60 days and (5.35-6.30 fold) in 90 days(**Table 9**). Rectal temperature and blood glucose level showed significant changes in (30 days, 60 days and 90 days) treatment. Stress behaviour was observed in treatment groups compared to control which may be the cause of significant ($P>0.05$) changes in body temperature which is evidenced from (**Table 10**) **.**The rectal temperature in the FPF diet (1.00-1.02 fold) in 30 days, (1.05-1.07 fold) in 60 days and (1.05-1.09 fold) in 90 days and blood glucose levels are (1.03-1.66 fold) in 30 days, 1.03-2.03fold)in 60 days and (1.11-2.75 fold) in 90 days and the correlation and regression relationship observed between fasting serum blood glucose level and rectal body temperature.

Table 9: Details of food and water consumption for 30, 60 and 90 days treatment with high fat diet.

	30 Days			
Length of time	**Control diet**	**FPF- diet**	**Control diet**	**FPF- diet**
	Food consumed(mg)	Food consumed(mg)	Water consumed(ml)	Water consumed(ml)
1 week	44.78 ± 1.80	46.77 ± 2.11*	13.33 ± 1.50	18.09 ± 0.11***
2 week	56.20 ± 2.01	59.08 ± 1.01**	17.88 ± 1.80	22.99 ± 1.01***
3 week	61.09 ± 3.10	77.89 ± 1.31***	23.33 ± 2.03	31.66 ± 0.11***
4 week	69.03 ± 2.08	83.34 ± 1.28***	33.00 ± 1.02	44.99 ± 0.74***
60 Days				
1 week	57.68 ± 3.80	60.27 ± 4.33	12.28 ± 1.55	19.11 ± 2.17***

2 week	69.20 ± 2.01	73.72 ± 1.71	22.50 ± 1.89	22.33 ± 1.84***
3 week	84.02 ± 1.10	86.23 ± 1.31	25.25 ± 2.63	28.25 ± 3.65***
4 week	81.05 ± 2.48	81.17 ± 1.28	43.00 ± 3.94	49.09 ± 4.74***
5 week	66.71 ± 5.83	68.55 ± 0.81	47.62 ± 5.80	55.09 ± 4.46***
6 week	59.55 ± 2.86	87.33 ± 0.26	48.23 ± 2.55	62.33 ± 4.69***
7 week	83.15 ± 4.54	88.41 ± 0.25	50.22 ± 2.40	67.89 ± 3.33***
8 week	86.70 ± 3.06	98.77 ± 0.33	51.24 ± 2.33	77.89 ± 0.28***
90 Days				
1 week	55.89 ± 3.01	56.89 ± 1.30ns	12.31 ± 1.50	16.78 ± 2.10***
2 week	59.04 ± 2.01	61.23 ± 1.02ns	17.89 ± 1.01	22.89 ± 1.10***
3 week	67.89 ± 1.11	72.33 ± 1.32**	22.33 ± 2.02	25.67 ± 3.01**
4 week	78.08 ± 2.01	85.66 ± 1.21***	32.99 ± 3.11	39.09 ± 2.11**
5 week	80.99 ± 1.09	.88 ± 0.81***	47.62 ± 2.12	43.44 ± 1.22***
6 week	89.01 ± 2.01	97.77 ± 0.20***	52.77 ± 2.12	59.08 ± 1.02***
7 week	99.88 ± 1.54	112.34 ± 0.20***	61.78 ± 2.03	61.97 ± 3.00ns
8 week	105.09 ± 2.06	20.89 ± 0.31***	65.55 ± 2.01	69.89 ± 0.12***
9 week	111.24 ± 1.11	33.89 ± 1.10***	67.89 ± 1.34	70.67 ± 1.22***
10 week	121.34 ± 0.89	40.89 ± 2.12***	71.22 ± 1.23	74.08 ± 1.23***
11 week	130.44 ± 0.34	158.99 ± 0.21***	73.21 ± 2.13	78.89 ± 0.89***
12 week	155.90 ± 2.11	189.98 ± 1.23***	77.67 1.12	89.89 ± 1.23***

Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Datamarked with ***,**,* are statistically significant ($p< 0.0001$,$p<0.005$,$p<0.001$) and ns=non-significant. The results show that the intragastric administration of FPF led to increased food consumption and water consumption , ***Control diet**: standard pellet diet - protein-19.40%, carbohydrate-

63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g

Table 10: Details of rectal temperature and blood glucose levels for 30, 60 and 90 days treatment with high fat diet

30 Days				
Length of time	**Control diet**	**FPF- diet**	**Control diet**	**FPF- diet**
	Rectal temperature		**Blood glucose level**	
1 week	91.90 ± 0.12	94.20 ± 0.12**	112.57 ± 0.10	20.00 ± 1.11***
2 week	92.20 ± 0.13	95.20 ± 0.21***	113.91± 0.20	33.89 ± 1.01***
3 week	92.20 ± 0.11	97.30 ± 0.01***	115.01± 0.11	80.09 ± 2.22***
4 week	94.30 ± 0.01	97.40 ± 0.02***	116.00 ± 0.09	00.01 ± 2.10***
60 days				
1 week	89.07 ± 0.10	92.10 ± 0.12**	112.78 ± 0.01	22.87 ± 0.21***
2 week	91.19 ± 0.02	93.20 ± 0.21***	112.07 ± 0.02	137.89 ± 0.12***
3 week	92.33 ± 0.11	94.30 ± 0.01*	113.04 ± 0.03	45.00 ± 0.01***
4 week	92.47 ± 0.01	95.10 ± 0.02**	114.00 ± 0.11	165.89 ± 0.03***
5 week	93.78 ± 0.02	96.77 ± 0.10**	115.60 ± 0.10	190.00 ± 0.03***
6 week	94.02 ± 0.12	97.07 ± 1.02***	115.09 ± 0.11	220.00 ± 2.02***
7 week	94.00 ± 0.01	98.89 ± 0.12**	116.00 ± 0.01	230.77 ± 2.01***
8 week	94.00 ± 0.02	98.99 ± 0.23***	117.02 ± 0.03	250.01 ± 2.01***
90 days				
1 week	89.80 ± 0.09	91.04 ± 0.09*	111.45 ± 0.01	123.33 ± 0.03**
2 week	90.90 ± 0.02	92.90 ± 0.02*	111.67 ± 0.03	140.00 ± 0.02***
3 week	91.30 ± 0.12	94.33 ± 0.22**	113.45 ± 0.02	150.45 ± 0.01***
4 week	91.70 ± 0.02	95.70 ± 0.03**	114.67 ± 0.03	162.89 ± 0.02***
5 week	92.60 ± 0.02	95.61 ± 0.12**	115.77 ± 0.04	177.05 ± 0.03**

6 week	92.08 ± 0.01	96.07 ± 0.11***	118.62 ± 0.02	189.78 ± 0.10***
7 week	93.67 ± 0.12	96.00 ± 0.02**	119.78 ± 0.01	200.35 ± 0.10***
8 week	93.67 ± 0.01	97.67 ± 0.01***	120.21 ± 0.02	220.34 ± 0.12***
9 week	94.56 ± 0.02	97.56 ± 0.10**	121.34 ± 0.10	250.66 ± 0.06***
10 week	94.50 ± 0.01	98.50 ± 0.01***	122.45 ± 0.03	290.90 ± 0.07***
11 week	94.70 ± 0.04	99.70 ± 0.12***	123.56 ± 0.10	320.89 ± 0.08***
12 week	94.70 ± 0.10	99.99 ± 0.13***	124.00 ± 0.02	340.02 ± 0.09***

Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Datamarked with ***,**,* are statistically significant ($p< 0.0001$,$p<0.005$,$p<0.001$) and ns=non-significant. Rectal temperature and blood glucose levels ***Control diet**: standard pellet diet - protein-19.40%, carbohydrate-63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g

Body weight gain (6.9 % of initial weight), organ weights (especially wet weight of liver and fat) are the indices to assess obesity and food and calorie intake determine the high fat diet induced obesity. FPF diet-fed rats showed a significant increase in body (1.01–1.52 fold) and liver (1.01 – 1.06 fold) weight, body fat mass distribution [epididymal (1.05 – 2.77 fold), retroperitoneal (1.14 – 1.73 fold), perirenal (1.03 – 1.06 fold), mesenteric (1.11 – 1.24 fold), and dorsal sub-cutaneous fat (1.16 – 2.28 fold)], body mass index (BMI, 1.12 – 8.20 fold), specific rate of body mass gain (1.42 – 2 fold), energy intake (1.08 – 1.29 fold), feed efficiency (0 – 1.2 fold) whereas, decrease in the kidney (1.02 – 1.61 fold), testis (1.11 – 1.61 fold,) were observed compared with the control diet, respectively. Increment in the absolute and relative liver weights, body fat mass and decrement in the absolute and relative kidney and reproductive organ weights, in the three FPF diet groups compared with the control diet further evidenced the high fat diet

induced obesity and its pathological processes. **(Table11)** significant increase in body weight was observed in treatment groups in comparison to control ($P<0.05$) and significant differences ($P>0.05$) in the organ weight and their respective relative weight were observed in control and treatment groups.

Table 11: Long-term feeding effects of fermented pork fat diet on body and organ weights, body fat mass distribution and food and calorie intake nutritional parameters in male Wistar albino rats

Parameters	**Experimental groups**					
	30 days		**60 days**		**90days**	
	Control diet	**FPF-Diet**	**Control diet**	**FPF-Diet**	**Control diet**	**FPF-Diet**
Body weight(g)	136.40 ± 0.01	138.60 ± 0.02*	130.60 ± 1.60	155.09 ± 2.36***	143.89 ± 2.13	219.4 ± 5.85***
Testis (g)	0.66 ± 0.01	0.59 ± 0.02**	0.66 ± 0.03	0.44 ± 0.04***	0.68 ± 0.01	0.42 ± 0.07***
Testis (g/100g)	1.17 ± 0.02	1.48 ± 0.01***	1.16 ± 0.19	0.62 ± 0.04***	1.02 ± 0.01	0.20 ± 0.01***
Liver (g)	7.44 ± 0.03	7.94 ± 0.02***	8.24 ± 0.02	8.34 ± 0.01**	7.27 ± 0.02	7.36 ± 0.02*
Liver (g/100g)	7.45 ± 0.30	2.08 ± 1.29***	7.22 ± 0.02	5.84 ± 0.05***	6.78 ± 0.09	3.73 ± 0.29***
Kidney (g)	0.44 ± 0.05	0.42 ± 0.05**	0.43 ± 0.02	0.42 ± 0.02*	0.42 ± 0.01	0.26 ± 0.06***

Kidney (g/100g)	0.65 ± 0.13	1.10 ± 0.13*	0.61 ± 0.08	0.34 ± 0.08*	0.55 ± 0.05	0.19 ± 0.03***
Seminal vesicles (g)	0.62 ± 0.05	0.58 ± 0.01*	0.62 ± 0.04	0.44 ± 0.05*	0.59 ± 0.01	0.41 ± 0.03***
Seminal vesicles (g/100g)	1.24 ± 0.01	1.07 ± 0.02***	1.22 ± 0.02	0.35 ± 0.08***	1.20 ± 0.04	0.27 ± 0.01***
Vas deferens (mg)	0.26 ± 0.01	0.22 ± 0.01 *	0.26 ± 0.01	0.21 ± 0.01**	0.26 ± 0.05	0.21 ± 0.05 ns
Vas deferens (g/100g)	0.48 ± 0.02	0.57 ± 0.02	0.46 ± 0.02	0.23 ± 0.01***	0.39 ± 0.02	0.11 ± 0.02***
Cauda (g)	0.46 ± 0.02	0.43 ± 0.01***	0.44 ± 0.03	0.26 ± 0.05*	0.33 ± 0.04	0.18 ± 0.03*
Cauda (g/100g)	0.74 ± 0.17	1.16 ± 0.20*	0.72 ± 0.02	0.46 ± 0.06**	0.66 ± 0.03	0.21 ± 0.02***
Caput (g/100g)	0.86 ± 0.28	0.67 ± 0.16ns	0.82 ± 0.02	0.25 ± 0.04***	0.55 ± 0.03	0.15 ± 0.03***
Body fat mass distribution						
Epididymal fat (mg/g)	1.78 ± 0.01	1.88 ± 0.02***	1.80 ± 0.02	4.99 ± 0.23***	3.89 ± 0.13	5.10 ± 0.14***
Retroperitoneal fat (mg/g)	1.55 ± 0.01	1.77 ± 0.02***	1.61 ± 0.01	1.81 ± 0.02***	1.67 ± 0.21	2.89 ± 0.22***
Perirenal fat (mg/g)	1.60 ± 0.04	1.67 ± 0.04*	1.63 ± 0.12	1.68 ± 0.11ns	1.77 ± 0.03	1.89 ± 0.04***

Mesenteric fat (mg/g)	0.61 ± 0.01	0.68 ± 0.02*	0.62 ± 0.02	0.77 ± 0.01***	1.68 ± 0.09	1.99 ± 0.06**
Dorsal sub-cutaneous fat (mg/g)	0.25 ± 0.01	0.29 ± 0.01	0.31 ± 0.01	0.44 ± 0.02***	0.38 ± 0.12	0.87 ± 0.11***
Food and calorie intake nutritional parameters						
Body mass index (BMI, g/cm^2)	0.47 ± 0.01	0.53 ± 0.02*	0.43 ± 0.01	3.53 ± 0.01***	4.51 ± 0.01	6.75 ± 0.03***
Specific rate of body mass gain (g/kg)	5.00 ± 0.20	7.00 ± 0.30***	11.00 ± 0.10	22.00 ± 0.10***	17.00 ± 0.01	34.00 ± 0.02***
Energy intake (kJ/day)	1234.00 ± 2.90	1343.00 ± 3.40***	1433.00 ± 3.30	1567.00 ± 3.10***	2679.00 ± 4.30	3456.00 ± 3.90***
Feed efficiency (FE; %)	0.11 ± 0.01	0.10 ± 0.02ns	0.09 ± 0.01	0.09 ± 0.03ns	0.05 ± 0.01	0.06 ± 0.02ns

Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Datamarked with ***,**,* are statistically significant ($p< 0.0001$,$p<0.005$,$p<0.001$) and ns=non-significant. **Relative organ weight**(g/100g) = {[organ weight/body weight] × 100},**BMI** = body weight (g) / length^2 (cm^2), **Specific rate of body mass gain** (g/kg) =dM / M dt, where dM represents the gain of body weight during dt = t_2 - t_1 and M is the rat body weight at t_1,**Energy intake** (kJ/day) = mean food consumption x dietary metabolizable energy, **Feed efficiency** (FE; g gained/kcal) = (mean body weight gain (g) 100) / total energy intake (kcal)***Control diet**: standard pellet diet - protein-19.40%, carbohydrate-63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g

5.1.3. Long- term feeding effects on Sperm morphology and sperm parameters

Sperm morphology analysis revealed a significant increase in sperm abnormalities in rats treated for 30 days, 60 days and 90 days. Sperm were classified as normal and abnormal sperms i.e., tail (amorphous, banana, detached) abnormality and head (coil or broken) abnormality. As shown in table 8, the normal sperms were found maximum in control groups and abnormality increased with FPF treated groups. .Normal phenotypes in FPF diets compared to control (1.05 – 1.42 fold), total head abnormalities (1.99 – 3.59 fold) and total tail abnormalities (2.50 – 3.73) were observed in **(Table 12**). The sperm concentration in treated groups (FPF diet) decreased compared to control groups. **Table 13** shows sperm concentration and daily sperm production in different groups compared to control. FPF treatment resulted in a significant decrease in sperm count in testis (daily sperm production) and epididymis (caput and cauda). Furthermore, there was a reduction in daily sperm production per testis in FPF treated rats. Furthermore, there was a reduction in daily sperm production per testis as shown in **(Table 13).** The sperm number of FPF diet (1.14-1.42 fold) in 30 days, 60 days and 90 days, daily sperm production (1.14-1.50 fold) in 30 days, 60 days and 90 days, sperm number in caput in (1.15-1.50 fold) 30 days, 60 days and 90 days, sperm number in cauda in (1.02-1.61fold) 30 days, 60 days and 90 days.

Table 12: Long-term feeding effects of fermented pork fat diet on sperm morphology in male Wistar albino rats

Parameters	**30 days**		**60 days**		**90 days**	
Sperm morphology	**Control diet**	**FPF-diet**	**Control diet**	**FPF-diet**	**Control diet**	**FPF-diet**
Sperm morphology – Normal (%)						
Normal phenotypes	95.00 ± 0.77	90.02 ± 0.12***	88.57 ± 0.83	69.00 ± 0.89***	77.00 ± 0.99	54.01 ± 1.10***
Sperm morphology - Head abnormality (%)						
Amorphous	1.9 ± 0.04	2.89 ± 0.02***	2.78 ± 0.12	7.49 ± 0.06***	3.24 ± 0.77	9.43 ± 1.21**
Banana head	1.01 ±	1.99 ± 0.02***	2.11 ±	8.89 ±	3.12 ±	12.67 ±

	0.04		0.02	0.04***	0.23	0.21***
Detached head	1.21 ± 0.05	3.34 ± 0.07***	2.89 ± 0.13	7.11 ± 0.25**	2.31 ± 0.02	9.07 ± 0.11**
Total of head abnormalities	4.12 ± 0.03	8.22 ± 0.04***	7.78 ± 0.03	23.49 ± 0.06***	8.67 ± 0.21	31.17 ± 0.27***
Sperm morphology - Tail abnormality (%)						
Coiled tail	1.02 ± 0.01	4.03 ± 0.03	2.99 ± 0.04	9.54 ± 0.04***	3.33 ± 0.12	7.89 ± 0.12***
Broken tail	1.10 ± 0.11	3.88 ± 0.01**	1.87 ± 0.04	7.22 ± 0.01**	4.56 ± 0.88	11.89 ± 0.98**
Total of tail abnormalities	2.12 ± 0.02	7.91 ± 0.03**	4.86 ± 0.19	16.76 ± 0.51***	7.89 ± 0.07	19.78 ± 0.06***

Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. ***,**,* indicate that treatment groups are significantly different at $p < 0.0001$,$p > 0.01$ and $p < 0.005$. The sperm abnormality was weighed as percent abnormality = {[No. of abnormal sperm/Total no. of sperm] × 100} **Control diet**: standard pellet diet - protein-19.40%, carbohydrate-63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g

Table 13: Long-term feeding effects of fermented pork fat diet on sperm parameters in male Wistar albino rat

Parameters	**Experimental Groups**					
	30 days		**60 days**		**90 days**	
Sperm count	**Control diet**	**FPF-Diet**	**Control diet**	**FPF-Diet**	**Control diet**	**FPF-Diet**

Testis						
Sperm number ($\times 10^6$)	170.91 ± 4.18	149.27 ± 2.16 **	163.89 ± 4.12	121.66 ± 4.14***	162.09 ± 2.87	113.94 ± 0.86***
Sperm number ($\times 10^6$/g)	480.99 ± 10.61	358.86 ± 13.54***	477.00 ± 11.22	351.47 ± 10.3***	577.90 ± 9.08	206.78 ± 11.40***
DSP ($\times 10^6$/testis/day)	29.83 ± 1.22	26.02 ± 0.92*	28.43 ± 1.02	18.84 ± 0.43***	22.22 ± 0.78	17.69 ± 0.62***
DSPr($\times 10^6$/testis /day/g)	85.57 ± 6.70	65.89 ± 4.40***	84.62 ± 3.67	62.83 ± 8.55***	82.33 ± 3.78	32.11 ± 2.53***
Epididymis						
Caput						
Sperm number($\times 10^6$)	768.20 ± 13.36	664.80 ± 11.70***	758.90 ± 7.70	555.80 ± 6.87***	729.09 ± 7.45	483.00 ± 6.45***
Sperm number ($\times 10^6$/g)	238.56 ± 12.66	150.85 ± 10.72***	234.90 ± 14.06	130.49 ± 13.38***	230.87 ± 15.67	97.27 ± 18.31***
Sperm transit time (days)	1.20 ± 0.01	1.28 ± 0.01***	1.33 ± 0.02	1.48 ± 0.02***	1.88 ± 0.01	1.40 ± 0.02***
Cauda						
Sperm number ($\times 10^6$)	856.20 ± 7.70	832.20 ± 5.70***	834.09 ± 11.23	629.80 ± 9.07***	777.89 ± 6.78	483.00 ± 6.45***
Sperm number ($\times 10^6$/g)	218.16 ± 12.09	121.65 ± 36.66*	213.98 ± 11.44	116.52 ± 16.91***	190.88 ± 11.23	81.60 ± 14.15***

Sperm transit time(days)	1.55 ± 0.01	1.93 ± 0.03***	1.26 ± 0.01	1.87 ± 0.01***	1.76 ± 0.06	1.13 ± 0.05***

Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. ***,**,* indicate that treatment groups are significantly different at $p< 0.0001$,$p> 0.01$ and $p< 0.005$. The sperm abnormality was weighed as percent abnormality = {[No. of abnormal sperm/Total no. of sperm] × 100} **Control diet**: standard pellet diet - protein-19.40%, carbohydrate-63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g

5.1.4 Long-term FPF feeding altered serum FAME profiles

Serum SFA (lauric, myristic, palmitic, stearic acid: 1.89 – 4.44 fold) and PUFA-ω6 (linoleic, γ-linolenic, arachidonic acid: 1.23 – 3.44 fold) levels were increased significantly while MUFA (myristic, palmitic, palmitoleic, stearic, oleic acid: 1.09 – 1.23 fold) and PUFA-ω3 (α-linolenic, eicosatrienoic, and eicosapentaenoic acid: 0.11 – 0.21fold) levels were decreased significantly in the serum of rats fed with FPF-diets compared to the control-diet group (Table2) implicated with the induction of obesity and onset of chronic inflammation and oxidative stress in rats. Our study demonstrated that the n-6:n-3 PUFA ratio levels were increased significantly in the FPF-diets to the tune of 1.26 – 3.22 fold, respectively **(Table14)**, which is associated with dietary intake-mediated change in the production of inflammatory mediators and regulators toward a pro-inflammatory profile.

Table 14: Long-term feeding effects of fermented pork fat diet (Sa-um) on serum fatty acid methyl esters composition in male Wistar albino rats exposed for 30,60 and 90 days

Parameters	**Experimental groups**					
	30 days		**60 days**		**90 days**	
	Control diet	**FPF- diet**	**Control diet**	**FPF- diet**	**Control diet**	**FPF- diet**
Serum fatty acid methyl esters composition (mg/g)						
Saturated fatty acids (SFA)						
C12, Lauric acid	17.11 ± 0.21	42.26 ± 2.08***	18.19 ± 0.21	61.45 ± 2.02 ***	16.11 ± 0.11	88.17 ± 1.35 ***
C14, Myristic acid	14.46 ± 1.16	48.12 ± 0.16***	13.26 ± 0.11	6 9.05 ± 1.01***	13.26 ± 0.16	83.18 ± 1.12***
C16, Palmitic acid	131.10 ± 3.91	289.4 ± 4.28***	131.20 ± 3.94	3 88.21 ± 3.96***	141.20 ± 3.94	399.75 ± 2.06***
C18, Stearic acid	40.66 ± 4.28	99.51 ± 5.02***	41.26 ± 3.28	1 20.56 ± 3.03***	41.66 ± 1.28	133.82 ± 4.03***
Monounsaturated fatty acids (MUFA)						
C16:1, Palmitoleic acid, ω7	61.19 ± 1.20	55.31 ± 1.10***	59.10 ± 1.10	3 4.74 ± 1.03***	61.29 ± 1.10	25.41 ± 1.20***
C18:1, Oleic acid, ω9	267.18 ± 2.16	139.12 ± 2.08***	260.89 ± 01.89	118.02 ± 4.30***	255.06 ± 0.19	76.05 ± 1.05***
C18:1, Vaccenic acid, ω7	20.45	15.10	19.15	8.10	18.50	5.95

	± 1.08	± 1.02**	± 1.08	± 0.22***	± 1.38	± 0.60***
Polyunsaturated fatty acids (PUFA)						
C18:2, Linoleic acid, ω6	141.14 ± 6.30	177.18 ± 5.12***	134.44 ± 3.28	244.85 ± 3.16***	131.14 ± 4.20	422.84 ± 6.21***
C18:3, α-Linolenic acid, ω3	94.55 ± 0.24	58.45 ± 0.28***	91.51 ± 0.14	39.43 ± 0.31***	91.58 ± 0.94	22.28 ± 0.11***
C18:3, γ-Linolenic acid, ω6	19.28 ± 1.50	44.24 ± 1.05***	18.67 ± 1.30	65.31 ± 1.10***	16.66 ± 2.50	83.15 ± 2.01***
C20:3, Eicosatrienoic acid, ω3	33.22 ± 1.05	28.81 ± 1.01*	30.15 ± 1.05	20.38 ± 1.07**	29.95 ± 0.75	15.60 ± 1.90***
C20:4, Arachidonic acid, ω6	26.66 ± 3.01	50.11 ± 3.64**	26.36 ± 2.01	66.77 ± 3.03***	27.06 ± 4.01	82.66 ± 3.08***
C20:5, Eicosapentaenoic, ω3	22.11 ± 0.22	20.22 ± 0.36*	23.33 ± 0.54	18.99 ± 0.10***	20.12 ± 0.92	8.86 ± 0.26***
SFA (mg/g)	203.53 ± 0.12	479.3 ± 0.13***	203.91 ± 1.12	639.27 ± 1.02***	212.23 ± 1.32	704.92 ± 1.02***
MUFA (mg/g)	348.82 ± 1.2	209.53 ± 1.1***	339.14 ± 0.23	160.86 ± 0.32***	334.85 ± 1.13	107.41 ± 1.12***
PUFA ω6 (mg/g)	187.08 ± 2.02	271.53 ± 2.11***	179.47 ± 1.34	376.93 ± 1.67***	174.86 ± 1.23	588.65 ± 1.45***

PUFA ω3 (mg/g)	149.88 ± 1.32	107.48 ± 1.44***	144.99 ± 0.33	78.8 ± 0.22***	141.65 ± 1.11	46.74 ± 1.21***
n-6 : n-3 PUFA ratio	1.24 ± 0.03	2.52 ± 0.22***	1.23 ± 0.01	4.78 ± 0.04***	1.23 ± 0.89	12.59 ± 0.98***

Datas are presented as mean ± standard error mean (n = 5). Abbreviations: **Control diet**: standard pellet diet - protein-19.40%, carbohydrate-63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g

5.1.5. Long-term FPF feeding triggered serum and testis inflammatory markers

FPF-diets promoted inflammation, which is evidenced through a significant increase in the serum and testis pro-inflammatory markers, including IL-1 (2.40 – 5.06 fold), IL-6 (7.08 – 8.50 fold), LTB4 (1.22 – 1.64 fold), prostaglandin (1.48 – 2.31 fold), NO (1.84 – 3.79 fold), MPO (5.31 – 6.93 fold), LDH (2.97 – 3.29 fold) and TNF-α (2.87 – 3.37 fold) and a significant decline in the anti-inflammatory marker, IL -10(1.49 – 2.92 fold) compared with the control-diet **(Table 15).**

5.1.6. Long-term FPF feeding activated testis oxidative stress and depleted of antioxidant reserve, altered hepato-renal functions markers, elevated serum lipid profiles and instigated insulin resistance

Long-term feeding of FPF diets) and fragmented DNA (6.54 – 9.36 fold)concentration in the testis compared with the control diet **.**The enzymatic (CAT:0.52 – 0.89 fold,; SOD:1.24 – 4.58 fold, GST:0.22 – 0.87 fold,) and non-enzymatic (GSH: 6.99 – 17.33 fold, antioxidant reserves were depleted drastically in the FPF diets compared to the control diet, substantiating antioxidant enzymes are responsible for reducing the redox imbalance and these antioxidants were suppressed due to the intensification of oxidative stress in testis caused by FPF diet supplementation. Further, GSH:GSSG ratio (reduced-to-oxidized glutathione ratio) is a benchmark of the mitochondrial or cellular redox state and observed that the GSH level is significantly lower (1.77 – 4.62 fold), but that the GSSG level is higher(5.23 – 7.67 fold) in FPF diet groups, signifying that oxidative stress in the testis occurs in groups supplemented with dietary FPF than in a control diet. The increased GSH:GSSG in the testis of rats fed a FPF diet persuade the rats to oxidative stress **(Table 15).**

In the testis tissue sections of the control diet group, the spermatogonia and early-stage spermatocytes were strongly positive with PCNA staining , whereas there were significantly fewer PCNA-positive germinal cells (0.15 – 0.42 fold,) in the FPF diet

group. The PCNA ratio decreased significantly up to 59.98-22.33% in FPF diet groups than the control indicated the inhibition of germ cell proliferation in testis tissue and testis atrophy (Table19). Further, FPF diet contributed to defective spermatogonial differentiation that was reflected as a significant decrease in the TDI (66.75 – 22.09%, the status of type B spermatogonial differentiation into spermatocyte and spermatids) compared with the control diet (Table 19). Intense BAX immunostaining (9.22 – 44.55%,), and BAX/BCL-2 (0.13 – 2.60%) immunoreactivity ratios were observed in the testis of FPF-fed rats, whereas mild to faint BCL-2 (69.89 – 17.11%), StAR (77.89 – 26.79 %), 3β-HSD (42.66 – 22.32%), and LHR (33.00 –5.99%) immunostaining.
compared with the control group **(Table15)** defining germ cells, Leydig cells, and Sertoli cells undergoing cell death, inhibition of cholesterol transfer within mitochondria leading to testis apoptosis and disruption of spermatogenesis and steroidogenesis.

Serum ALT (2.14 – 4.55 fold), AST (2.13 – 5.14 fold), ALP (2.16 – 4.80 fold), urea (3.15 – 4.55 fold), creatinine (1.18 – 2.96 fold), AST:ALT ratio (1.14 – 1.18 fold)and BUN:Creatinine ratio (1.11 – 1.02 fold) were significantly increased in the three FPF-diet groups compared with the control-diet group because of metabolic syndrome resulting in hepatic and renal damage **(Table 16).** The rate of germ cell apoptosis (measured by TUNEL assay) was computed in terms of apoptotic index (AI). Increased incidence of apoptotic index (apoptotic germ cells) was observed in the 30 days (27.88%), 60 days (48.99%) and 90 days (57.23%) diet group than the control diet group (4.46 %) indicating activation of apoptosis and induction of DNA damage in the germ cells **(Table 16).**

The serum TC, TAG, VLDL, LDL and leptin levels in the serum of rats fed a FPF-diets increased significantly by (1.88 – 5.24, 2.07 – 6.30, 2.07 – 6.30, 5.62 – 9.69 fold) respectively, and HDL level (2.11 – 7.76 fold) decreased compared with the control-diet group, indicating disturbance in lipid metabolism like hypercholesterolemia and hyperleptinemia which are associated with the pathogenesis of insulin resistance, obesity,

atherosclerosis and cardiovascular disease by elevation in cardiovascular risk factors (CI, AI and CAI: 4.01 – 13.44, 3.22 – 6.33, 12.29 – 22.44 fold, respectively). **(Table 16).** Compared to the control-dietgroup, FPF-diet groups showedsignificant elevation in serum glucose (1.33 – 4.48 fold) and insulin (3.22 – 4.33 fold) levels induced by FPF-diets further redirected toward insulin resistance and pancreatic defect in β-cell function as indicated by a significant increase in the HOMA-IR (12.33 –16.63 fold) and a reduction inHOMA-β (0.43 – 0.72 fold) than the control-diet group **(Table 16).**

Table 15: Long-term feeding effects of fermented pork fat diet (Sa-um) on serum fatty acid methyl esters composition and testicular inflammatory, oxidative stress, apoptotic and steroidogenic markers in male Wistar albino rats exposed for 30,60 and 90 days.

Parameters	**Experimental groups**					
	30 days		**60 days**		**90 days**	
	Standard diet	**FPF- diet**	**Standard diet**	**FPF- diet**	**Standard diet**	**FPF- diet**
Serum inflammatory markers						
Interleukin-1 (pg/mL)	18.18 ± 1.92	43.65 ± 2.08***	17.19 ± 1.92	49.51 ± 3.26***	13.13 ± 2.72	66.48 ± 2.10***
Interleukin-6 (pg/mL)	15.14 ± 1.06	38.25 ± 1.81***	16.54 ± 1.06	47.19 ± 2.10***	16.14 ± 1.06	52.22 ± 1.15***
Interleukin-10 (pg/mL)	42.15 ± 1.05	28.28 ± 2.05***	44.15 ± 1.45	22.00 ± 1.09***	46.05 ± 1.05	15.72 ± 1.10***
Leukotriene B4 (LTB4, pg/mL)	131.38 ± 2.12	166.35 ± 2.05***	133.30 ± 4.12	181.51 ± 3.20***	128.18 ± 2.12	210.45 ± 2.25***
Prostaglandin (pg/mL)	42.66 ±	63.18 ±	38.23 ±	68.05 ±	38.45 ±	89.05 ±

	1.52	1.05***	1.52	2.08***	1.50	2.15***
Testis inflammatory markers						
Nitric oxide (NO, nmol/mg protein)	2.25 ± 0.02	4.14 ± 0.30***	2.45 ± 1.12	4.67 ± 1.02***	2.12 ± 0.42	8.05 ± 1.16***
Myeloperoxidase (MPO, U/min/mg protein)	1.25 ± 0.10	8.67 ± 0.41***	1.97 ± 0.45	9.96 ± 0.12***	2.11 ± 0.10	11.21 ± 0.15***
Lactic dehydrogenase (LDH, U/mg protein)	2.11 ± 0.51	6.28 ± 0.71***	3.01 ± 0.51	10.22 ± 0.81***	3.71 ± 0.50	12.23 ± 0.31***
Tumor necrosis factor-α (TNF-α, pg/mg protein)	4.25 ± 0.61	12.23 ± 1.01***	4.11 ± 0.43	14.34 ± 1.30***	5.27 ± 0.43	17.76 ± 1.20***
Testis lipid peroxidation products – Oxidative stress markers						
Conjugated dienes (nmol/mg protein)	35.26 ± 2.19	67.88 ± 2.91***	38.04 ± 3.10	74.11 ± 3.31***	40.01 ± 3.22	82.14 ± 2.11***
Lipid hydroperoxides (nmol/mg protein)	30.42 ± 2.08	67.04 ± 1.80***	32.12 ± 3.09	77.89 ± 3.11***	34.56 ± 1.09	83.45 ± 1.20***
Malondialdehyde (nmol/mg protein)	5.11 ± 1.41	20.00 ± 1.31***	6.23 ± 1.40	26.88 ± 1.05***	8.18 ± 1.40	32.99 ± 1.80***
Protein carbonyl (nmol/mg protein)	7.34 ± 0.10	11.36 ± 1.20***	7.12 ± 0.82	17.78 ± 1.20***	5.11 ± 0.76	24.33 ± 2.12***
Fragmented DNA (%)	7.99 ± 0.66	52.31 ± 3.01***	8.20 ± 0.61	63.45 ± 2.34***	8.40 ± 0.45	78.67 ± 2.34**

Testis levels of enzymatic and non-enzymatic antioxidants – Oxidative stress markers						
Reduced glutathione (GSH) (nmol/mg protein)	55.34 ± 0.32	31.1 ± 0.59***	53.33 ± 0.31	13.46 ± 0.34***	54.21 ± 0.83	11.73 ± 0.21***
Glutathione disulfide (GSSG, nmol/mg protein)	3.14 ± 1.03	12.34 ± 1.20***	4.46 ± 0.04	19.55 ± 1.30***	3.12 ± 0.11	34.12 ± 2.15***
GSH:GSSG ratio	17.62 ± 0.01	2.52 ± 1.02***	11.96 ± 1.03	0.69 ± 0.23***	17.38 ± 1.02	0.35 ± 0.03**
GST	13.76 ± 0.3	11.7 ± 0.44**	14.71 ± 0.21	10.21 ± 0.23***	14.13 ± 0.18	9.27 ± 0.06***
Superoxide dismutase (SOD)	57.15 ± 0.52	46.07 ± 0.51***	56.23 ± 0.24	24.83 ± 0.66***	54.22 ± 0.13	11.82 ± 0.27***
Catalase (CAT)	28.73 ± 0.47	25.81 ± 0.45***	28.73 ± 0.47	24.57 ± 0.58***	25.21 ± 0.03	14.88 ± 0.31***
Testis cell proliferation and differentiation, apoptotic and steroidogenic markers						
Tubule differentiation index (TDI %)	66.75 ± 1.12	44.00 ± 1.20***	63.00 ± 1.05	39.33 ± 0.91***	55.00 ± 0.05	22.09 ± 0.61***
Apoptotic index (AI, %)	4.46 ± 0.04	27.88 ± 1.15***	4.37 ± 0.02	48.99 ± 2.06***	5.62 ± 0.04	57.23 ± 0.38***
PCNA immunoreactivity ratio	52.98 ± 1.23	47.98 ± 1.28***	52.17 ± 1.1	38.99 ± 1.21***	50.09 ± 0.21	22.33 ± 0.72***
BAX immunoreactivity ratio	9.22	33	9.32	32.51	8.51	44.55

	± 0.68	± 0.78***	± 0.61	± 0.05***	± 0.48	± 0.89***
BCL-2 immunoreactivity ratio	69.89 ± 2.03	48.99 ± 2.12***	72.33 ± 2.12	47.66 ± 2.71***	67.77 ± 1.24	17.11 ± 1.23***
BAX/BCL-2 ratio	0.13 ± 0.01	0.67 ± 0.11**	0.12 ± 0.02	0.68 ± 0.03***	0.12 ± 0.11	2.60 ± 0.13***
StARimmunoreactivity ratio	77.89 ± 2.12	44.89 ± 2.42***	77.83 ± 1.08	33.98 ± 1.12***	75.09 ± 1.25	26.79 ± 1.88***
3β-HSDimmunoreactivity ratio	42.66 ± 1.16	34.18 ± 1.31**	42.43 ± 2.17***	24.66 ± 1.12***	40.31 ± 1.45	22.32 ± 1.21***
LHR immunoreactivity ratio	33.12 ± 1.92	22.78 ± 1.05***	28.19 ± 1.33	16.33 ± 1.38***	26.62 ± 1.33	5.99 ± 1.49***

Datas are presented as mean ± standard error mean (n = 5).PCNA/BCL-2/StAR/LHR/3β-HSD immunoreactivity ratio = Positively reacted cells/total number of cells×100;Abbreviations: **Standard diet**: standard pellet diet - protein-19.40%, carbohydrate-63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g; HDL: High density lipoprotein; LDL: Low density lipoprotein; PCNA: Proliferating cell nuclear antigen; BCL-2: B-cell lymphoma 2; BAX: Bcl-2-associated X protein; StAR: Steroidogenic acute regulatory protein; 3β-HSD: 3β-hydroxysteroid dehydrogenase; LHR: Luteinizing hormone receptor

Table 16: Long-term feeding effects of fermented pork fat diet (Sa-um) on hepatic insulin resistance, liver and kidney function and lipid profiles, in male Wistar albino rats exposed for 30,60 and 90 days

Parameter	Experimental groups					
	30 days		60 days		90 days	
	Standard diet	FPF- diet	Standard diet	FPF- diet	Standard diet	FPF- diet
Hepatic insulin resistance profiles						
Serum insulin level (ng/mL)	0.24 ± 0.02	1.52 ± 0.02***	0.88 ± 0.11	1.69 ± 0.31*	0.92 ± 0.12	2.21 ± 0.04***
Serum glucose level (mg/dL)	102.33 ± 3.16	330.23 ± 3.36***	108.9 ± 4.23	380.23 ± 2.33***	111.1 ± 3.13	5 40.23 ± 4.11***
Homeostasis model assessment of insulin resistance index (HOMA-IR)	109.15 ± 1.04	22.30 ± 2.01***	4.26 ± 1.03	28.56 ± 0.12***	4.55 ± 0.98	53.07 ± 1.02***
Pancreatic β-cell function (HOMA-	4.86	9.30	16.70	8.98	17.10	8.24

β, %)	± 1.09	± 0.99***	± 0.89	± 1.03***	± 1.09	± 0.99***
Liver and kidney function profiles						
Alanine aminotransferase (ALT, IU/mL)	14.65 ± 2.83	56.68 ± 5.18***	13.15 ± 2.11	85.25 ± 5.52***	14.93 ± 2.98	136.72 ± 2.56***
Aspartate aminotransferase (AST, IU/mL)	12.65 ± 2.83	46.68 ± 3.18***	13.19 ± 2.86	76.77 ± 5.52***	14.63 ± 2.88	138.72 ± 8.57**
AST/ALT ratio	0.86 ± 0.80	0.82 ± 1.03***	1.00 ± 0.03	0.90 ± 1.02***	0.98 ± 0.80	1.01 ± 0.01***
Alkaline phosphatase (ALP, IU/L)	5.45 ± 0.83	16.48 ± 1.18***	8.25 ± 2.86	44.63 ± 2.38***	35.25 ± 3.52	66.72 ± 4.57***
Urea (mg/dL)	14.73 ± 2.73	45.48 ± 3.25***	18.45 ± 2.66	68.25 ± 4.92***	22.63 ± 2.98	121.77 ± 7.57***
Blood urea nitrogen (BUN, mg/dL)	6.88 ± 1.06	21.21 ± 0.16***	8.61 ± 1.12	31.88 ± 0.89***	10.57 ± 0.21	56.87 ± 1.03***
Creatinine (mg/dL)	0.63 ± 0.06	2.68 ± 0.35***	0.75 ± 0.07	5.25 ± 0.92***	1.33 ± 0.08	12.42 ± 2.52***
BUN/Creatinine ratio	10.92 ± 0.01	7.92 ± 1.20***	11.49 ± 0.23	6.07 ± 0.12***	7.94 ± 0.03	4.58 ± 1.23***
Serum lipid profiles						

Total cholesterol (TC, mg/dL)	64.62 ± 1.16	122.04 ± 1.09***	87.48 ± 1.08	299.52 ± 2.38***	92.34 ± 2.21	484.7 ± 2.76***
Triacylglycerols (TAG, mg/dL)	42.48 ± 1.08	88.20 ± 1.36***	45.90 ± 1.39	154.98 ± 2.85***	50.04 ± 1.2	315.36 ± 1.28***
VLDL cholesterol (mg/dL)	19.30 ± 3.02	40.09 ± 4.36**	20.86 ± 3.15	70.44 ± 3.65***	22.74 ± 3.34	143.34 ± 2.0***
HDL cholesterol (mg/dL)	33.66 ± 3.66	15.84 ± 2.02*	34.02 ± 1.02	8.64 ± 1.08***	34.92 ± 3.98	4.50 ± 2.08***
LDL cholesterol (mg/dL)	11.77 ± 2.03	66.24 ± 2.18 ***	32.76 ± 3.06	220.50 ± 1.52 ***	34.74 ± 3.18	336.96 ± 1.57***
Leptin (ng/mL)	7.06 ± 0.19	14.15 ± 1.08***	8.22 ± 1.09	16.48 ± 1.35***	9.22 ± 1.32	19.78 ± 1.82***
Cardiac index (CI)	1.92 ± 0.12	7.70 ± 1.03***	2.58 ± 0.21	34.7 ± 0.17***	2.64 ± 0.23	107.71 ± 1.21***
Atherogenic index (AI)	30.96 ± 1.62	106.2 ± 1.23***	53.46 ± 0.23	290.88 ± 0.89***	57.42 ± 1.10	483.2 ± 0.99***
Coronary artery index (CAI)	0.34 ± 0.18	4.18 ± 0.13***	0.96 ± 0.12	0.12 ± 0.31*	0.99 ± 0.18	74.86 ± 0.16***

Datas are presented as mean ± standard error mean (n = 5).AST:ALT ratio = AST/ALT; Blood urea nitrogen = Serum urea × 0.467; VLDL = 0.2 × TAG; LDL = TC − (HDL + VLDL); CI = TC/HDL; AI = (total cholesterol−HDL)/HDL; CAI = LDL/HDL; HOMA-IR = [(Serum insulin level (ng/mL) × Serum glucose level (mg/dL)] / 22.5; HOMA-β = [20 × Insulin (ngl/mL)] / [Glucose (mg/dL) - 3.5] *100; AI = number of apoptotic cells/number of total cells × 100); PCNA/BCL-2/StAR/LHR/3β-HSD immunoreactivity ratio = Positively reacted cells/total number of cells×100. Abbreviations: **Standard diet**: standard pellet diet - protein-19.40%, carbohydrate 63.60%, fat-9.67% and total energy-15.48 kJ/g **FPF- diet**: Fermented pork medium fat diet - protein-19.40%, carbohydrate-63.60%, fat-30.10% and total energy-15.48 kJ/g; HDL: High density lipoprotein; LDL: Low density lipoprotein

5.1.7. Histological analysis and morphological changes of seminiferous tubules

Normal testis histological features including the compactness of seminiferous tubules (SFT) with complete stages of spermatogenesis (*) and full sperm mass in lumen (®) were more evident in the control diet **(FIG 3 A)** while testis structural disorganization, dearth of sperm mass in lumen (+), vacuolization (µ), and loss of compactness of the SFT were observed in FPF diet fed rats **(FIG 3 C and D).** The alterations in the SFT histo-architecture were more prominent with FPF-M diet tissue sections showing disrupted seminiferous tubules, emptiness of sperm mass in lumen (+), fragmented spermatocytes ($), apoptotic spermatocytes (#), delamination/disorientation of germinal epithelial cells (@), multinucleated germ cells (^), sloughing of germ cells (©), increased vacuolization within the tubules (µ), and in interstitial spaces start Leydig cell dystrophy **(FIG 3 E and F).** Drastic testis tissue structural alterations were detected in FPF-H diet as indicated by depletion of Leydig and Sertoli cells (**), wider interstitial space (%), vacuolated spermatocyte (β) and degeneration of Sertoli cells (→), prominent vacuolization, and extensive tubular degeneration along with disorientation of the different developing stages of spermatogenic and Sertoli cells **(FIG 3 G and H).** Long term high dietary FPF consumption imposed maximal testis tissue damage in FPF-M 60 days and 90 days to the magnitude of causing partial to complete tubular atrophy**.**

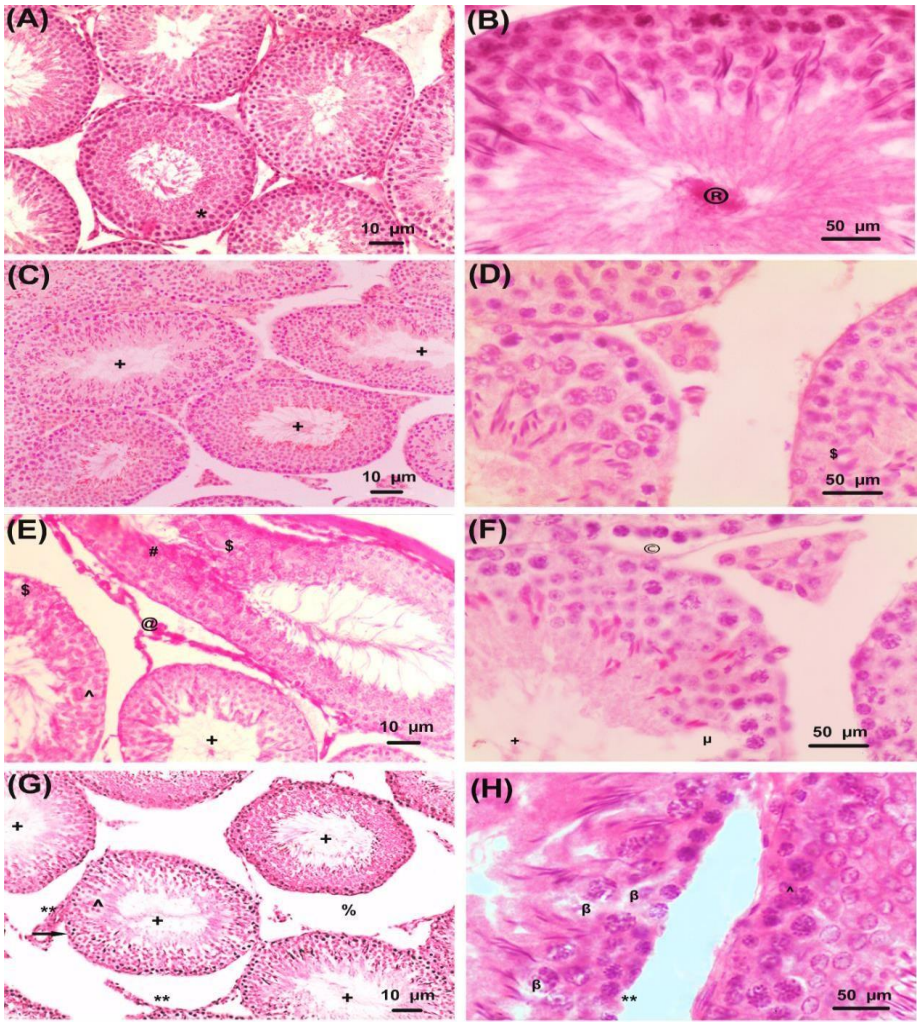


FIG 3: Histological analysis of testis (A-H) showing effects of high fat diet in control (A, B), FPF-M (C, D) for 30 days, FPF-M (E,F) for 60 days, FPF-M (G,H) for 90 days

The high testis tissue damage score of 1.00 -3.44 was registered in FPF diet group showing testicular atrophy of about 85 – 100%, where seminiferous tubules lined by one layer of cells with marked cellular necrosis and loss of spermatogenesis, with interstitial edema and hyperemia while 50 – 75% testicular atrophy was noticed in FPF (2.68 score) diet (**Table 17).** A significant decrease in the JTBS was observed in the FPF (9.20) 30 days, FPF (8.80) 60 days and FPF (7.30) 90 days compared with the control diet (9.90), signifying that the FPF diet induced progressive deterioration of testicular histo-architecture leading to disruption of seminiferous tubules, hypo-spermatogenesis, maturation arrest and testicular damage (Table 10). Long-term feeding of FPF diet resulted in severe morphological changes in the seminiferous tubules, i.e., a significant reduction in the MSTD (128 – 115), STEH (83.33 - 69.09), TAT (27.89-19.78) and an increase in IS (9.78-7.94), and TL (13.33 – 10.34) demonstrating FPF diet induced testicular tissue disintegrity and sperm loss (**Table 17)**. Further, a drastic decline in the germinal and interstitial cells was detected in the FPF supplemented diet comprising spermatogonia (0.69 – 0.96 fold), spermatocytes (0.22 – 0.77 fold), spermatids (0.48 – 1.05 fold), Sertoli cells (1.20 – 3.11 fold) and Leydig cells (0.43 – 1.02 fold) resulting in hypo-spermatogenesis and testicular dysfunction (**Table 17).**

Table 17: Effects of long-term feeding of fermented pork fat diet (Sa-um) on morphological changes of seminiferous tubules and quantification of germ and Leydig cellsin testis of male Wistar albino rats exposed for 30, 60 and 90 days

Parameters	Experimental groups					
	30 Days		60 days		90 Days	
	Standard diet	FPF-diet	Standard diet	FPF-diet	Standard diet	FPF-diet
Morphological changes of seminiferous tubules (10 random non-overlapping fields/treatment)						
Johnsen's mean testicularbiopsy score (JTBS)	9.90 ± 0.03	9.20 ± 0.02***	9.80 ± 0.02	8.80 ± 0.03***	9.40 ± 0.01	7.30 ± 0.02***
Mean diameter of seminiferoustubule (MSTD, µ	128.00 ± 0.12	125.01 ± 0.11***	127.01 ± 0.13	124.01 ± 0.11***	127.00 ± 0.09	115.00 ± 2.23***
Seminiferous tubuleepithelial height (STEH, µm)	83.33 ± 0.13	82.00 ± 0.11***	81.22 ± 0.12	79.98 ± 0.11***	80.99 ± 0.09	69.09 ± 1.01***
Interstitialspace (IS, µm)	9.78 ±	9.34 ± 0.06***	9.72 ±	9.11 ±	8.99 ±	7.94 ±

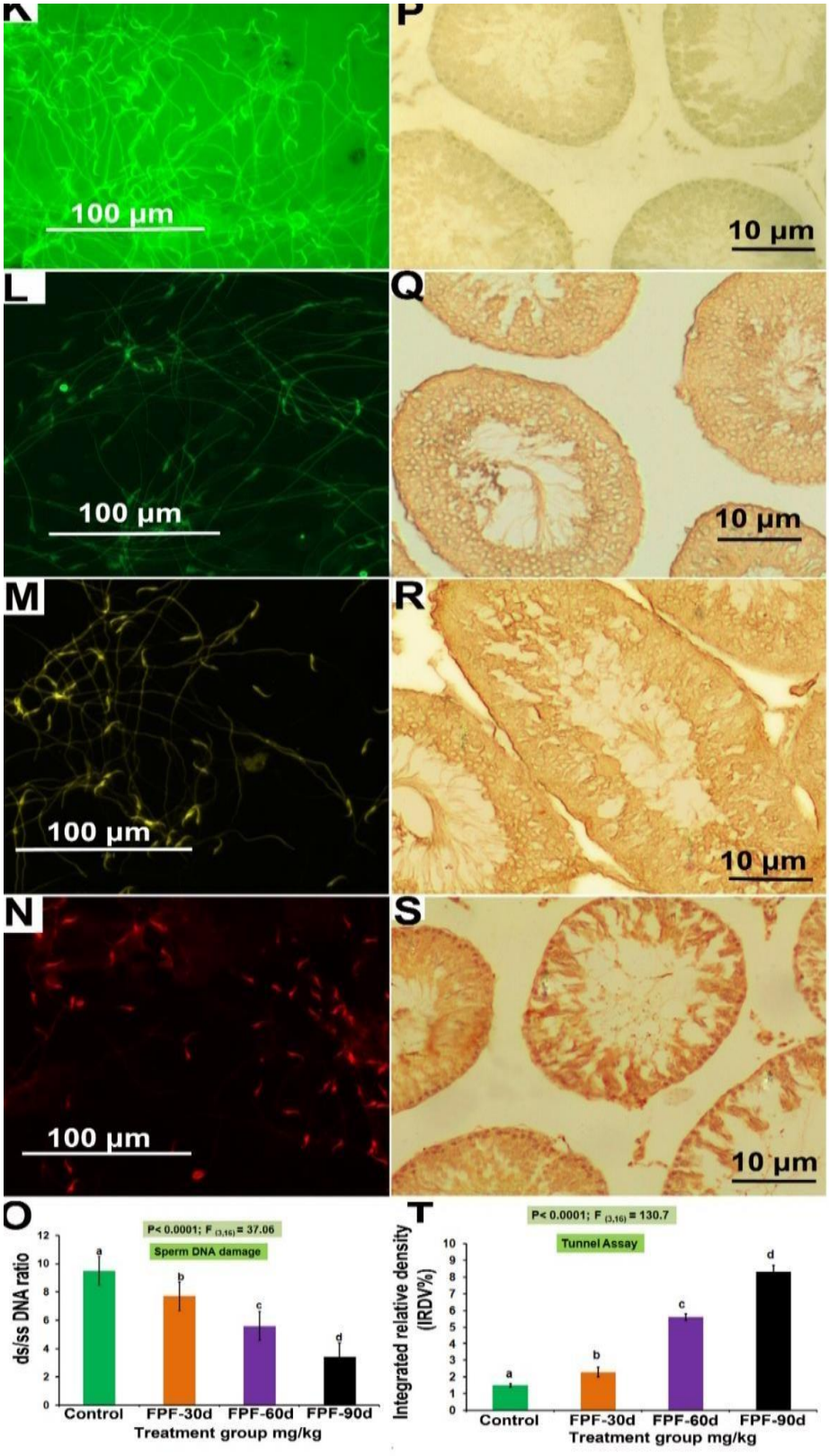
	0.04		0.04	0.02***	0.05	0.02***
Tunica albuginea thickness (TAT, µm)	27.89 ± 0.11	25.66 ± 0.12***	29.33 ± 0.98	23.44 ± 1.06**	25.45 ± 0.11	19.78 ± 1.11***
Tubular lumen (TL, µm)	13.33 ± 0.06	12.18 ± 0.08***	12.11 ± 0.06	11.89 ± 0.02**	12.37 ± 0.03	10.34 ± 0.07**
Testis tissue damage score	1.00 ± 0.11	2.33 ± 0.14***	1.67 ± 1.11	5.86 ± 0.12**	2.33 ± 0.23	3.44 ± 0.23***
Enumeration of germ cells and Leydig cells (10 random non-overlapping fields/treatment)						
No. of Spermatogonia cells	91.22 ± 2.12	80.30 ± 2.19*	88.22 ± 2.68	78.21 ± 3.22**	82.41 ± 4.22	58.30 ± 3.81**
No. ofspermatocytes	82.11 ± 2.72	71.50 ± 2.69*	79.2 ± 2.03	68.60 ± 2.05**	72.10 ± 3.14	60.30 ± 1.93**
No. of spermatids	142.22 ± 8.32	125.10 ± 6.88 ns	143.4 ± 4.63	122.30 ± 2.62**	140.30 ± 2.83	104.90 ± 8.02**

No. of Sertoli cells	9.21 ± 1.51	7.40 ± 0.88ns	1.02 ± 0.86	6.80 ± 0.11***	7.31 ± 2.01	2.21 ± 0.61*
No. of Leydig cells	9.70 ± 0.09	8.67 ± 0.08***	8.77 ± 0.25	7.23 ± 0.23**	7.84 ± 0.67	6.78 ± 0.09ns

Data are presented as mean ± standard error mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Datamarked with ***,**,* are statistically significant (p< 0.0001,p<0.005,p<0.001).Abbreviations: **Control diet**: standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g; **FPF diet**: Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g

5.1.8. Long-term feeding of FPF diets inhibited germ cell proliferation, differentiation, apoptosis and steroidogenesis

As assessed by the TUNEL assay, occurrence of an increased number of TUNEL-positive germ cells (dark brown stained, IRDV) was detected in the testis tissue sections of the FPF (2-8.8%) diet fed rats, as compared with no TUNEL-positive cells in the control group rats **(FIG 4)**. Acridine orange staining the dsDNA/ssDNA ratio was declined (0.01 – 0.12 fold, **FIG 4)** significantly in the FPF diet groups in a dose-dependent manner, witnessing a reduction in DNA integrity. A higher percentage of sperm DNA damage (40.33 – 69.02%) dose dependently observed in the FPF diet groups compared with the control diet group.


K
100 µm
P
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Q
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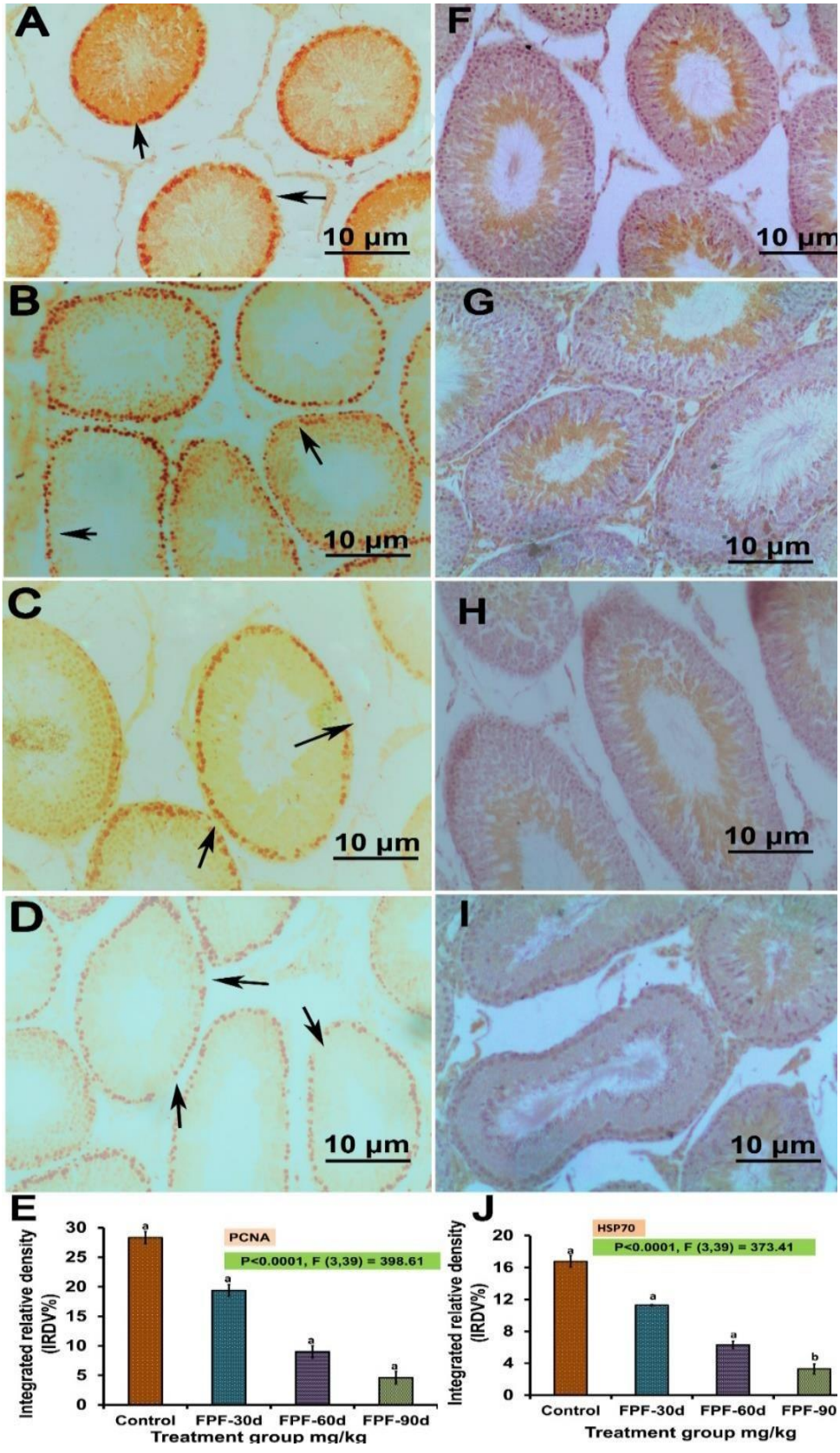
M
100 µm
R
10 µm
N
100 µm
S
10 µm
O
P< 0.0001; F (3,16) = 37.06
Sperm DNA damage
ds/ss DNA ratio
Control
FPF-30d
FPF-60d
FPF-90d
Treatment group mg/kg
T
P< 0.0001; F (3,16) = 130.7
Tunnel Assay
Integrated relative density (IRDV%)
Control
FPF-30d
FPF-60d
FPF-90d
Treatment group mg/kg

FIG 4: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 30,60 and 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with different letters (a, b, c, d) are statistically signifcant ($p<0.001$) if they do not share the same letters. Immunolocalization of AO (K-O), and Tunel Assay (P-T) in FPF diet supplemented rat testis tissue. Immunoreactivity quantifcation data (% area) of AO (O) and Tunel (T); Control diet standard pellet diet; FPF-M for 30 days; FPF-M for 60 days and FPF-H diet for 90 days

PCNA (proliferating cell nuclear antigen), a potent biomarker for proliferation in spermatogonia and spermatocytes (leptotene, zygotene and pachytene spermatocytes) has been extensively used for to immunohistochemical studies as long as HSP 70. Now days, it has been used as an early biomarker for detecting chemically induced testicular toxicity due to its DNA repair nature. In our study, we observed that FPF exposure in normal healthy rats significantly affected expression of PCNA protein in germ cells and spermatocytes of seminiferous tubule **(FIG 5).** In another side, normal control group showed well expression of PCNA and decrease of HSP 70 in nuclei of outer germinal layer (spermatogonia). Moreover, some staining in spermatocytes (prophase I) was also noticed in their nuclear content however, other cells involved in spermatogenesis like spermatids and spermatozoa were devoid of its expression.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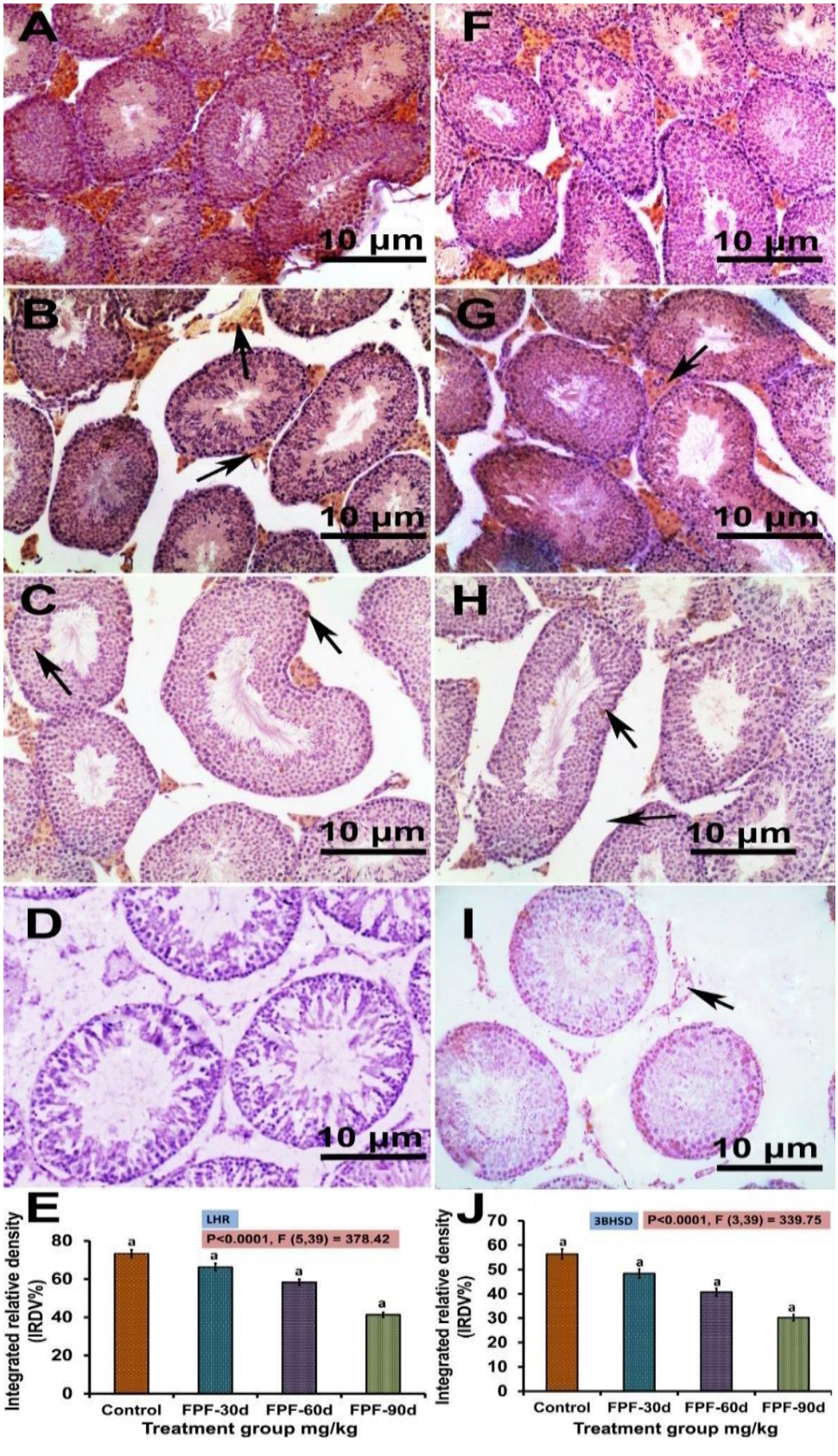
C
10 μm
H
10 μm
D
10 μm
I
10 μm
E
PCNA
P<0.0001, F (3,39) = 398.61
Integrated relative density (IRDV%)
Control
FPF-30d
FPF-60d
FPF-90d
Treatment group mg/kg
J
HSP70
P<0.0001, F (3,39) = 373.41
Integrated relative density (IRDV%)
Control
FPF-30d
FPF-60d
FPF-90
Treatment group mg/kg

FIG 5: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 30,60 and 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with diferent letters (a, b, c, d) are statistically signifcant ($p<0.001$) if they do not share the same letters. Immunolocalization of PCNA (A-E), and HSP 70(F-J) in FPF diet supplemented rat testis tissue. Immunoreactivity quantifcation data (% area) of PCNA (E) and HSP 70 (J); Control diet standard pellet diet; FPF-M for 30 days; FPF-M for 60 days and FPF-H diet for 90 days

3β-hydroxysteroid dehydrogenase (3β-HSD) is crucial enzyme in process of steroidogenesis that catalyzes the steroid into progesterone, 17αhydroxyprogesterone, androstenedione and testosterone from pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA) and androstenediol respectively. It is generally well located in gonads (testis and ovary) and cortical part of adrenal gland therefore involved in production of sex hormone and corticosteroids (glucocorticoids and mineralocorticoids) respectively. In mouse testis, immune histochemistry study revealed its expression in Leydig cells however in many other primate studies revealed its expression in Sertoli cells. It is well documented that expression of 3β-HSD is regulated by LH receptor hence activation of LH receptor stimulates more catalization of steroids into sex hormones and corticosteroids through steroidogenesis. Since LH secretion from anterior pituitary is necessary for stimulation of enzymes involved in steroid production therefore LH receptor plays a regulatory control over 3β-HSD expressions. In normal control group, the majority of 3β-HSD expression was documented in Leydig cells and rarely seen in other cells of seminiferous tubules. An interrupted steroidogenesis has been documented after HFD treatment to healthy normal mice as there was decline in expression of 3βHSD testicular tissue (**FIG 6**). LH receptors (LHR) or human chorionic gonadotropin (hCG) is well located in gonads (testis and ovary) and their accessory sex organs. Besides that, LHR expressions are also reported in brain and

placental tissue. LHR plays a major role in Leydig cells development and differentiation in the early phases of testicular cells development necessary for steroidogenesis. It shows regulatory control on hypothalamic pituitary gonadal (HPG) axis through high-affinity G protein coupled receptors (GPCRs). Moreover, in testis, cAMP independent messengers regulates activation of LHR, leading a controlled over steroidogenesis in Leydig cells. It is already reported in many study that LHR is highly susceptible to exogenous steroid present in numerous food leading into decline in altered steroidogenesis. In our study, we claimed that FPF diet being a steroid compound, altered the expression of LHR either via interfering in binding ability with steroid receptors or by various unknown mechanism. We observed that normal control group consist of a massive density of LHR positive stained Leydig cells in interstitial spaces of seminiferous tubules. However, FPF diet treatment to normal healthy mice significantly declined in LHR expression in dose dependent manner when compared to normal control group **(FIG 6).**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C
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H
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D
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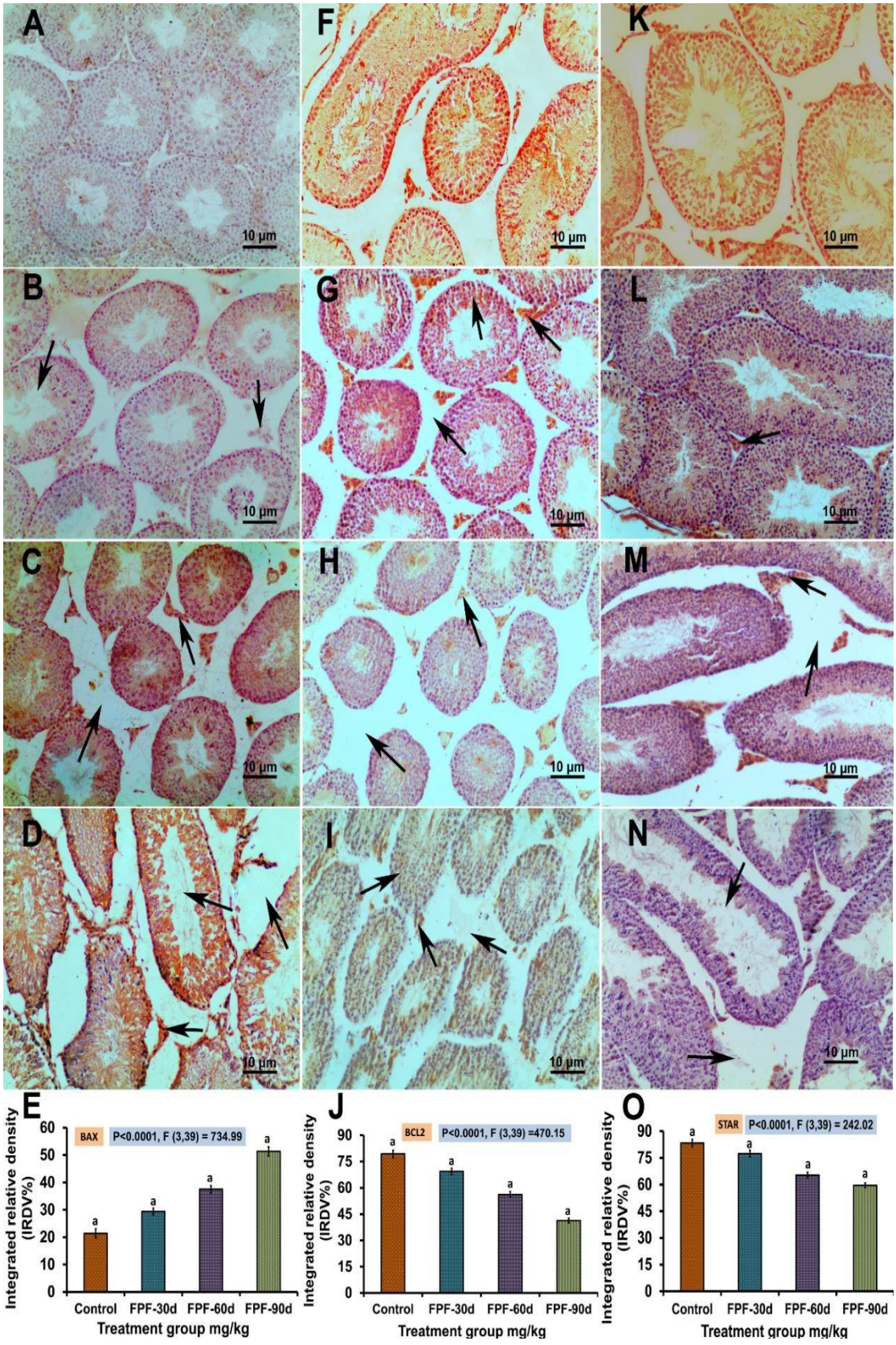
I
10 μm
E
LHR
P<0.0001, F (5,39) = 378.42
Integrated relative density (IRDV%)
80
60
40
20
0
a
a
a
a
Control
FPF-30d
FPF-60d
FPF-90d
Treatment group mg/kg
J
3BHSD
P<0.0001, F (3,39) = 339.75
Integrated relative density (IRDV%)
70
60
50
40
30
20
10
0
a
a
a
a
Control
FPF-30d
FPF-60d
FPF-90d
Treatment group mg/kg

FIG 6: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 30,60 and 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with different letters (a, b, c, d) are statistically significant ($p>0.001$) if they do not share the same letters. Immunolocalization of 3β-hydroxysteroid dehydrogenase (3β-HSD, F-I), and Lutenizing hormone receptor (LHR, A-D) in FPF diet supplemented rat testis tissue. Immunoreactivity quantification data (% area) of 3β-HSD (J) and LHR (E); Control diet standard pellet diet; FPF-M for 30 days; FPF-M for 60 days and FPF-H diet for 90 days

Bcl-2 family genes are known for their anti-apoptosis or pro-apoptosis property and play an essential role in process of mitochondrial apoptosis pathway. This gene is also involved in regulation of spermatogenesis as dead cells or damaged spermatozoa needs to remove from seminiferous tubules. Many other factors such as environmental toxicants, many cytotoxic agents (pesticides), excessive heat and radiation from different sources may increase the process of germ cell apoptosis in mammalian testis. Bcl-2 expression may directly altered by exposure of any cytotoxic compound leading into abnormal spermatogenesis in male testis. In normal mice testis, its prime location is germ cell, spermatocytes (primary and secondary) and developing spermatozoa. HFD increases the rate of apoptosis in testicular tissue of normal healthy mice possibly by up-regulating Bcl-2 expression. Immunohistochemical analysis of Bcl-2 protein revealed a significant Bcl-2 expression in testis of all HFD treated groups when compared to normal control group **(FIG 7)**. However, some Bcl-2 positive Leydig cells were also seen after FPF diet exposure. In testicular tissue, StAR (steroidogenic acute regulatory protein) is one of the most important enzymes in steroidogenic pathway controls cholesterol transfer to inner mitochondrial membrane through a series of event. It is primarily located in Leydig cells situated between interstitial spaces of seminiferous tubules and on head of developing spermatozoa. The expression of StAR is dependent on the concentration of Leydig cells. Like other steroidogenic enzyme involved in

steroidogenesis, StAR is also affected with exogenous steroids. In this study, we revealed that the expression of StAR in testicular Leydig cells was seriously affected with FPF diet treatment when compared to normal control **(FIG 7).** The immunohistochemical study of StAR in normal control group showed a positive expression in Leydig cells and in sperm masses towards lumen. In addition to that, it is concluded that higher doses of FPF seriously declined the StAR expression however, the lower doses were not that much effective than compared to normal control group.


A
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D
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N
10 μm
E
BAX
P<0.0001, F (3,39) = 734.99
Integrated relative density (IRDV%)
Control
FPF-30d
FPF-60d
FPF-90d
Treatment group mg/kg
J
BCL2
P<0.0001, F (3,39) =470.15
O
STAR
P<0.0001, F (3,39) = 242.02

FIG 7: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 30,60 and 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with different letters (a, b, c, d) are statistically signifcant ($p>0.001$) if they do not share the same letters**.** Immunolocalization of BAX (A-D), BCL2 (F-I) and STAR (K-N) in FPF diet supplemented rat testis tissue. Immunoreactivity quantifcation data (% area) of BAX (E) and BCL2 (J) and STAR (O); Control diet standard pellet diet; FPF-M for 30 days; FPF-M for 60 days and FPF-M diet for 90 days.

5.1.9. Western Blotting

The nuclear factor (NF)-kB is a primary regulator of inflammatory responses and may be linked to pathology associated with obesity. A central mediator of inflammatory and stress responses is the NF-kB family of transcription factors. As a response to foreign pathogens and general stressful insults, NF-kB is activated in most cell types. In addition, NF-kB activity is linked to cancer development through its regulation of apoptosis, cell proliferation, angiogenesis, metastasis and cell survival. TNF-a is mainly released from activated macrophages and can trigger a deleterious signaling cascade to induce the production of other pro-inflammatory cytokine Based on the crucial role of TNF-α in chronic low-grade inflammation, it has been used as markers to monitor the dynamic change of inflammation in responses to high-fat meals and exercise In the current study, we also demonstrated significant increase in plasma TNF- α in HFD rats. High levels of circulating TNF- α are believed to lead to severe inflammatory response, metabolic alteration, and insulin resistance**.** Caspase-3 and 9 belongs to cysteine proteases family and plays an essential role in process of apoptosis. It is well documented that abnormal formation of cell during spermatogenesis need to remove by a process of well-organized programmed cell death, apoptosis. Caspase-3 activation induces apoptosis by protein degradation within cell and stimulate Sertoli cell to phagocytes the cells. Moreover, abnormal activation of caspase-3 within cell may affect the process of sperm production as excessive death of germ cell affect fertility output. As a main and final

perpetrator of apoptosis, caspase-3 activation may affected by environmental condition (heat, radiation and pesticides) and other cytotoxic agents stimulates the proteases to induce morphological and physiological changes leading into cell death. FPF diet is widely used in pharmacology industry has been known for its protective effect against various kinds of diseases. However, in our study, it is concluded that FPF diet exposure in normal healthy mice induces testicular germ cell apoptosis through a series of events. Caspase-3 positive cells were observed mainly in germ cells and primary spermatocytes after FPF diet exposure. However, some spermatids and Leydig cell were also seen positive for caspase-3 protein expression. In our study, control group showed a light to moderate staining in germinal epithelial cells (spermatogenic cells series) and Leydig cells. Higher doses of FPF-H were more likely to affect germ cell survival and induce apoptosis of germinal cells and other cells produced during spermatogenesis. Leydig cells localized in interstitial compartment, responsible for production of testosterone were significantly affected by FPF diet administration leading into declined serum testosterone. Poly (ADP-ribose) polymerase (PARP) is a member of the enzyme family which is involved in DNA damage and repair. PARP can be activated by DNA strand breaks, irregular DNA structures or other post-translational modifications. Its activation can regulate the protein function, chromatin compaction and gene expression by modifying target proteins via poly (ADP-ribosyl) ation (PARP, a classic DNA repair enzyme, may participate in multiple cellular functions by modulating multiple target proteins via the modification of poly (ADP-ribosyl)ation **(FIG 8).**

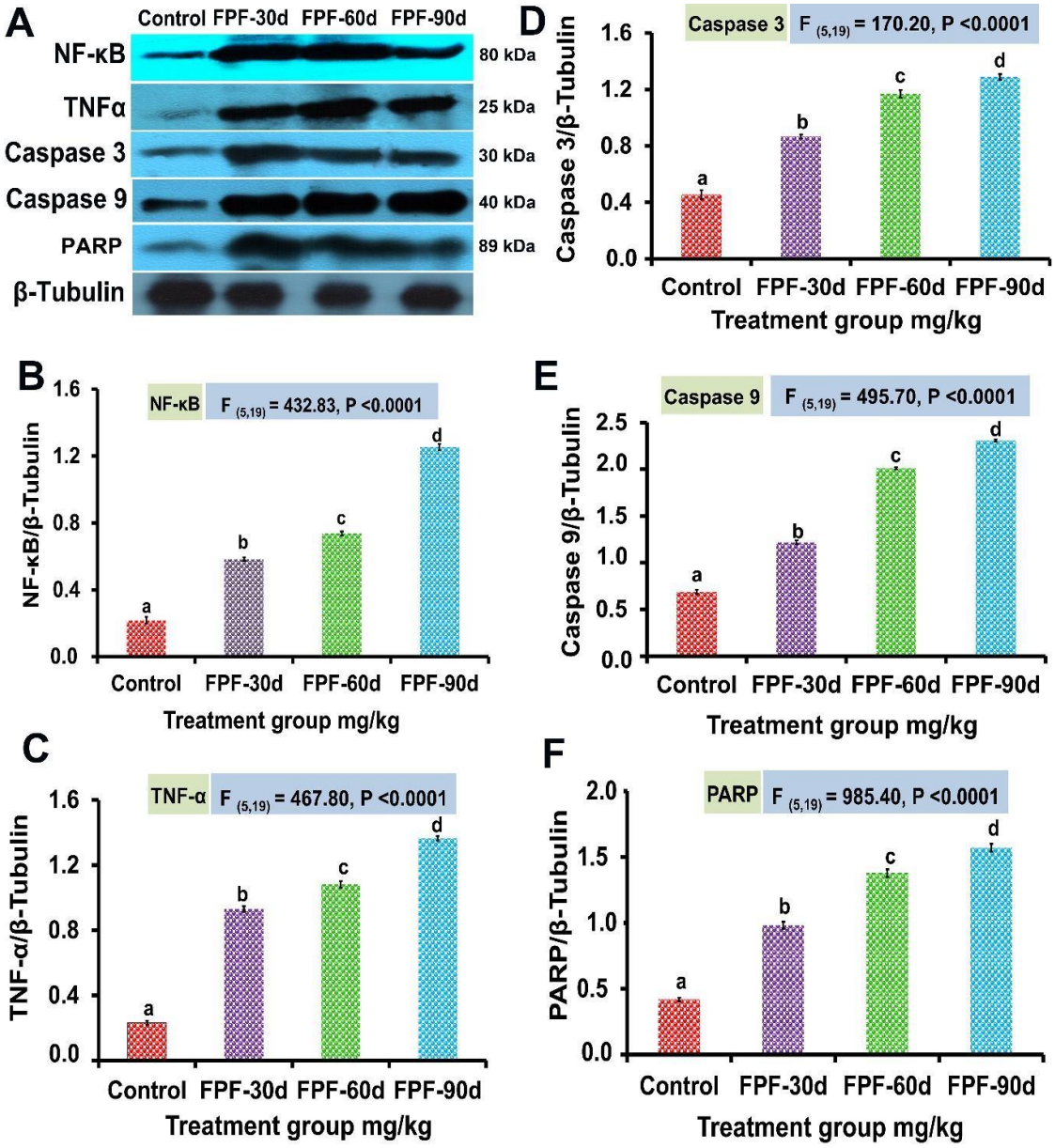

FIG 8: Representatives of Western blotting analysis (A-F) showing effects of high fat diet in control, FPF-M for 30 days, FPF-M for 60 days, FPF-M for 90 days

5.2. Long-term consumption of fermented pork fat-based diets differing in calorie, fat content, and fatty acid levels mediates oxidative stress, inflammation, redox imbalance, germ cell apoptosis, disruption of steroidogenesis, and testicular dysfunction in Wistar rats

5.2.1. FPF, diet composition, FAME and TAG analysis

The nutrient and mineral analysis of the FPF showed a pH of 6.58, 6.28% moisture content, 94.56% fat, and 4.56 mg/100 g sodium with a high calorifc value of 910.71 kcal/100 g (Table 1). The control diet contained 11% of the total calories from fat, with 10.45%, 12.55%, 13.12%, and 14.63% of calories originating from SFA, MUFA, PUFA-ω6, and PUFA-ω3 fatty acids, respectively. The FPF-L diet comprised 10% of total calories from fat, with 26.39%, 8.17%, 18.87%, and 8.51% of calories arising from SFA, MUFA, PUFA-ω6 and PUFA-ω3 fatty acids, respectively. The FPF-M diet consisted of 30% of total calories from fat, with 40.88%, 6.39%, 21.42%, and 6.89% of calories ascending from SFA, MUFA, PUFA-ω6, and PUFA-ω3 fatty acids, respectively. The FPF-H diet contained 60% of total calories from fat, with unequal parts of SFA (99.14%), MUFA (4.51%), PUFA-ω6 (28.37%), and PUFA-ω3 (2.85%). The total quantity of calories in the control diet was less than in the FPF-L, FPF-M and FPF-H diets **(Table 18).** Analysis of the FAME composition in the diets revealed that SFA (lauric, myristic, palmitic, and stearic acid: 2.52–9.49 fold) and PUFA-ω6 (linoleic, γ-linolenic, and arachidonic acid: 1.43–2.16 fold) levels were drastically elevated to high quantities in the FPF-H diet, followed by the FPF-M and FPF-L diets, compared to the control. In contrast, MUFA (palmitoleic, oleic, and vaccenic acid: 0.35 to 0.65 fold) and PUFA-ω3 (α-linolenic, eicosatrienoic, and eicosapentaenoic acid: 0.19 to 0.58 fold) levels were substantially reduced to a low quantity in the FPF-diets. The n-6:n-3 PUFA ratio (2.46–11.07-fold) was higher in FPF diets than in the control. The levels of twenty triacylglycerol species were also detected in higher quantities in the FPF-diets than in

the control, especially PLP (5.85–13.89- fold), POP (7.52–9.67-fold), PPO (3.18–6.87-fold), and LPO (2.11–2.87-fold) **(Table 18)**

Table 18: Analysis of nutrients and minerals composition in fermented pork fat (Sa-um), fat diet composition and their fatty acid methyl ester and triacylglycerol profiles

Nutrients and minerals analysis	
Nutrients and minerals	**Fermented pork fat (Sa-um)**
pH	6.58 ± 0.16
Moisture content (% by weight)	6.28 ± 1.85
Total ash content (% by weight)	0.56 ± 0.12
Crude fiber (% by weight)	Below detectable limit
Protein (mg/g)	6.45 ± 1.08
Carbohydrate (mg/g)	20.14 ± 1.62
Fat (mg/g)	945.65 ± 3.95
Calorific value (Kcals/100 g)	910.71 ± 3.16
Sodium (mg/100 g)	4.56 ± 0.28
Potassium (mg/100 g)	3.92 ± 0.16
Calcium (mg/100 g)	2.76 ± 0.32
Magnesium (mg/100 g)	0.48 ± 0.07
Iron (mg/100 g)	0.26 ± 0.04
Zinc (mg/100 g)	0.08 ± 0.01
Diet composition	

	Control diet	**FPF-L diet**	**FPF-M diet**	**FPF-H diet**
Protein (%)	24	20	20	20
Carbohydrate (%)	65	65	50	20
Fat (%)	11	15	30	60
Total energy (kJ/g)	12.56	14.21	17.5	23
Fatty acid methyl esters composition analysis (mg/g)				
Fatty acid methyl esters (FAME)	**Control diet**	**FPF-L diet**	**FPF-M diet**	**FPF-H diet**
Saturated fatty acids (SFA)				
C12, Lauric acid	15.67 ± 1.05	66.75 ± 2.45	82.36 ± 2.57	99.12 ± 2.28
C14, Myristic acid	12.52 ± 0.34	48.26 ± 0.02	70.51 ± 1.21	95.51 ± 1.82
C16, Palmitic acid	35.64 ± 1.48	79.20 ± 2.66	158.51 ± 3.75	516.85 ± 3.15
C18, Stearic acid	38.56 ± 1.38	68.75 ± 1.58	96.35 ± 1.22	278.86 ± 3.61
Monounsaturated fatty acids (MUFA)				
C16:1, Palmitoleic acid, ω7	63.17 ± 1.92	34.61 ± 1.96	28.61 ± 2.96	20.61 ± 1.96
C18:1, Oleic acid, ω9	40.05 ± 1.95	35.36 ± 1.56	26.12 ± 1.16	20.28 ± 1.08
C18:1, Vaccenic acid, ω7	21.23 ± 1.42	10.55 ± 0.75	9.26 ± 0.85	3.15 ± 0.15
Polyunsaturated fatty acids (PUFA)				

C18:2, Linoleic acid, ω6	30.60 ± 1.82	36.60 ± 1.82	44.66 ± 2.27	79.08 ± 1.81
C18:3, α-Linolenic acid, ω3	73.60 ± 1.49	41.75 ± 1.75	20.35 ± 1.22	10.78 ± 0.62
C18:3, γ-Linolenic acid, ω6	21.54 ± 1.10	39.35 ± 1.38	50.66 ± 1.80	78.62 ± 1.49
C20:3, Eicosatrienoic acid, ω3	56.78 ± 1.55	33.21 ± 1.81	39.65 ± 0.55	11.41 ± 0.71
C20:4, Arachidonic acid, ω6	78.54 ± 1.26	112.50 ± 2.51	117.85 ± 2.95	124.95 ± 1.05
C20:5, Eicosapentaenoic, ω3	15.65 ± 0.45	10.09 ± 0.66	7.98 ± 0.82	5.18 ± 0.25
SFA (mg/g)	104.45 ± 1.26	263.85 ± 2.48	408.82 ± 2.05	991.35 ± 3.16
MUFA (mg/g)	125.45 ± 1.18	81.65 ± 1.82	63.99 ± 1.65	45.08 ± 0.95
PUFA ω6 (mg/g)	131.18 ± 1.05	188.71 ± 1.42	214.20 ± 3.52	283.65 ± 2.44
PUFA ω3 (mg/g)	146.28 ± 1.58	85.05 ± 1.42	68.98 ± 0.52	28.45 ± 0.52
n-6 : n-3 PUFA ratio	0.90	2.22	3.11	9.97
Triacylglycerol composition analysis (mg/g)				
Triacylglycerol (TAG)	**Control diet**	**FPF-L diet**	**FPF-M diet**	**FPF-H diet**
1,2-palmitoyl-3-stearoyl-sn-glycerol, PPS	2.45 ± 0.36	16.15 ± 0.35	20.08 ± 0.45	24.78 ± 0.92
Trilinolein, LLL	2.66 ± 0.48	7.25 ± 0.71	12.33 ± 0.85	15.81 ± 0.95
1,2-linoleoyl-3-oleoyl-sn-glycerol, OLL	23.81 ± 0.18	45.62 ± 0.27	58.95 ± 0.04	64.12 ± 1.66
Palmitodilinolein,	35.85 ± 0.26	68.74 ± 0.75	81.16 ± 1.08	92.26 ± 1.84

PLL				
1,2-dioleyl-3-linolenoylglycerol, OOL	34.31 ± 0.74	70.55 ± 1.35	85.05 ± 0.68	92.19 ± 1.58
1-linolein-2-palmitin-3-olein, LPO	92.65 ± 1.66	195.72 ± 2.28	248.39 ± 3.65	266.75 ± 2.08 `
1,2-palmitoyl-3-linolein-sn-glycerol, PPL	27.88 ± 1.42	48.68 ± 1.52	60.92 ± 1.72	75.14 ± 1.28
1,2,3-trioleylglycerol, OOO	25.89 ± 1.28	41.95 ± 1.06	55.19 ± 1.88	58.44 ± 1.72
1,3-palmitoyl-2-oleoyl-sn-glycerol, POP	26.56 ± 3.75	199.75 ± 2.92	212.48 ± 2.11	256.90 ± 2.16
Dipalmitoyl-oleoyl-glycerol, PPO	32.85 ± 1.21	104.73 ± 1.02	156.18 ± 1.82	225.85 ± 1.05
Tripalmitoyl-glycerol, PPP	10.12 ± 0.36	18.14 ± 0.20	34.66 ± 0.51	50.12 ± 0.95
1-Stearoyl-2-oleoyl-3-oleoyl-glycerol, OOSt	16.37 ± 0.52	34.16 ± 0.85	42.48 ± 0.38	59.68 ± 0.15
1,3-palmitoyl-2-linoleoyl-sn-glycerol, PLP	20.55 ± 0.02	120.26 ± 1.02	194.25 ± 2.05	285.61 ± 2.91
Trinonadecanoyl-glycerol, NNN	3.05 ± 0.21	7.92 ± 0.41	16.42 ± 1.02	22.06 ± 1.15
1,2-oleoyl-3-sn-palmitoylglycerol, OOP	4.48 ± 0.05	8.15 ± 0.92	14.45 ± 1.35	20.33 ± 0.75

1,3-oleoyl-2-palmitoyl-sn-glycerol, OPO	6.06 ± 0.84	12.48 ± 0.88	29.38 ± 1.28	40.18 ± 1.16

Data on nutrient composition of the diet, FAME and TAG profiles are presented as mean ± standard error mean.

5.2.2. Long-term feeding of FPF diets (excessive calories, high fat content, and fatty acid levels) affected anthropometric and nutritional measures

Body weight gain (8% of initial weight), organ weights (especially wet weight of liver and fat), and the Lee index (> 3.0) are indices used to assess obesity, and food and calorie intake determines high fat diet-induced obesity. A significant ($p < 0.0001$) increase in food and water consumption (1.15–1.47 and 1.33–2.66-fold, **FIG 9A and B**), body weight (1.32–1.79-fold), liver weight (0.99–1.08-fold), and body fat mass distribution in the epididymal (2.80–5.13-fold), retroperitoneal (1.33–2.01-fold), perirenal (1.25–1.82-fold), mesenteric (2.28–3.38-fold), and dorsal subcutaneous fat (2.00–3.85-fold) were observed in rats fed a high fat diet (FPF-L, FPF-M, and FPF-H) than those fed the control diet **(Table 19).** In continuation, body mass index (BMI), specific rate of body mass gain, energy intake, and feed efficiency were observed to be 1.03–2.16 fold, 3.82–9.65 fold, 1.30–2.69 fold, and 1.36–1.61 fold respectively, higher in the rats fed the high fat diet compared to the control diet **(Table 19).** Lee index (1.01–1.24-fold, **FIG 9A**) and fasting blood glucose level (3.94–4.48-fold, Table 2) were also found to be increased in the FPF diets compared with the control diet ($p < 0.0001$), signifying the diabetic and obese condition of the rats due to long term feeding of the high fat diet. The weights of the kidney, testis, and accessory sex organs decreased (0.78–0.95 fold, 0.80–0.94 fold, and 0.53–0.93 fold, respectively; see Table S1and **FIG 9E–H**) in rats fed the high fat diet compared to the control diet, and the same trend

was observed in the rectal temperature and gonado-somatic index parameters (0.70–0.95 fold and 0.21–0.77 fold, respectively; see **FIG 9C and 9D**). As a whole, there was a significant ($p<0.0001$) increase in the absolute and relative liver weights, fasting blood glucose level, body fat mass, and Lee index, and a decrease in the absolute and relative kidney and reproductive organ weights, and rectal temperature in the three FPF diet groups compared with the control diet, further evidencing the high fat diet induced obesity and its pathological processes.

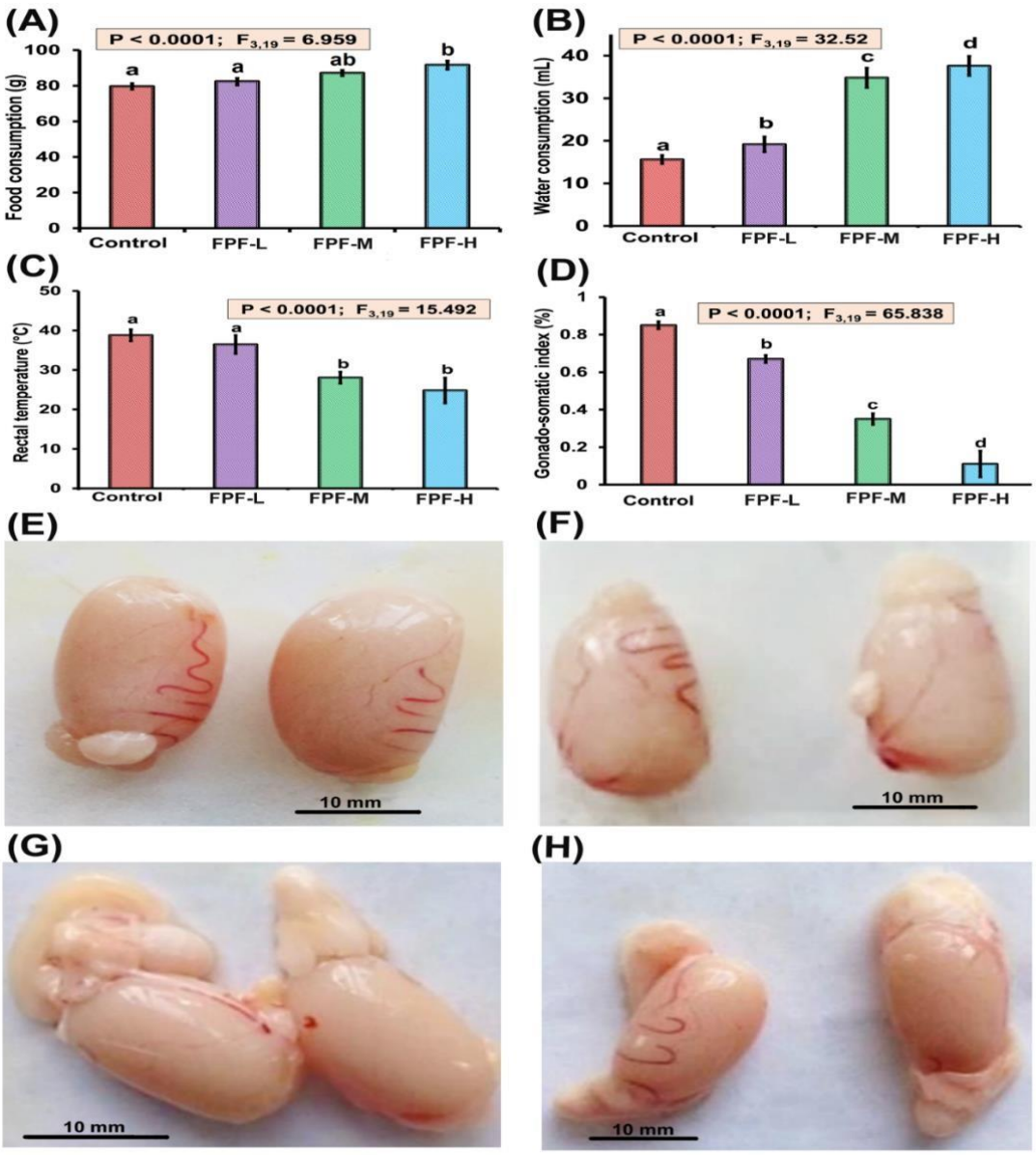

FIG 9: Long-term feeding effects of fermented pork fat diet (Sa-um) on food consumption (g) (**A**), water consumption (mL) (**B**), rectal temperature (°C) (**C**), and Gonadosomatic index (**D**) in male Wistar albino rats. Observation of reduction in the testis size and excess epididymal fat mass in FPF-L (**F**), FPF-M (G), and FPF-H (H) compared with the control diet (**E**). Values are mean of five observations (n=5). Statistical comparison was

performed using one-way ANOVA followed by Tukey's multiple comparison tests. Bar graph with different letter means statistically significant difference at $p < 0.05$ and similar letters are not significant. **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **FPF-H diet** Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23kJ/g

Table 19: Long-term feeding effects of fermented pork fat diet on body and organ weights, body fat mass distribution and food and calorie intake nutritional parameters in male Wistar albino rats

Parameter	Control diet	FPF-L diet	FPF-M diet	FPF-H diet	$F_{3,19}$	P value
Body and organ weight						
Body weight (g)	145.18 ± 4.51a	192.11 ± 3.39b	224.40 ± 2.92c	261.23 ± 3.87d	175.430	<0.0001
Liver (g)	6.17 ± 0.25a	8.12 ± 0.26b	10.18 ± 0.30c	12.04 ± 0.22d	96.101	<0.0001
Liver (g/100g)	4.24 ± 0.03a	4.23 ± 0.03a	4.53 ± 0.04b	4.60 ± 0.03b	34.543	<0.0001
Kidney (g)	1.15 ± 0.04a	1.09 ± 0.03a	1.06 ± 0.02b	0.90 ± 0.03c	12.00	0.0002
Kidney (g/100g)	0.79	0.56	0.47	0.34	37.825	<0.0001

	± 0.04a	± 0.03b	± 0.02b	± 0.03c		
Testis (g)	1.42 ± 0.03a	1.34 ± 0.03a	1.25 ± 0.04b	1.13 ± 0.03b	14.419	<0.0001
Testis (g/100g)	0.99 ± 0.03a	0.70 ± 0.03b	0.56 ± 0.02c	0.43 ± 0.03d	74.409	<0.0001
Seminal vesicles (g)	0.72 ± 0.02a	0.67 ± 0.03a	0.64 ± 0.02b	0.59 ± 0.02b	5.651	0.0078
Seminal vesicles (g/100g)	0.49 ± 0.02a	0.35 ± 0.03b	0.28 ± 0.02b	0.22 ± 0.02c	25.714	<0.0001
Vas deferens(g)	0.29 ± 0.02a	0.24 ± 0.02a	0.21 ± 0.01b	0.17 ± 0.02b	7.872	0.0019
Vas deferens (g/100g)	0.20 ± 0.02a	0.12 ± 0.02b	0.09 ± 0.01b	0.06 ± 0.01b	14.500	<0.0001
Cauda (g)	0.17 ± 0.02a	0.14 ± 0.02a	0.12 ± 0.01a	0.09 ± 0.02b	3.487	0.0405
Cauda (g/100g)	0.11 ± 0.02a	0.07 ± 0.01a	0.05 ± 0.02a	0.03 ± 0.01b	4.667	0.0158
Caput (g)	0.25 ± 0.02a	0.22 ± 0.01a	0.20 ± 0.02a	0.16 ± 0.02b	4.385	0.0196

Caput (g/100g)	0.17 ± 0.02a	0.11 ± 0.01a	0.09 ± 0.02b	0.06 ± 0.02b	6.641	0.0040
Body fat mass distribution						
Epididymal fat (mg/g)	1.80 ± 0.06a	5.05 ± 0.15b	6.38 ± 0.08c	9.25 ± 0.25d	402.41	<0.0001
Retroperitoneal fat (mg/g)	1.60 ± 0.09a	2.14 ± 0.12a	2.15 ± 0.18a	3.23 ± 0.24b	16.609	<0.0001
Perirenal fat (mg/g)	1.60 ± 0.04a	2.01 ± 0.04b	2.58 ± 0.02c	2.91 ± 0.05d	223.41	<0.0001
Mesenteric fat (mg/g)	0.60 ± 0.01a	1.37 ± 0.02b	1.08 ± 0.02c	1.97 ± 0.02d	1009.3	<0.0001
Dorsal sub-cutaneous fat (mg/g)	0.20 ± 0.01a	0.40 ± 0.01b	0.66 ± 0.01c	0.77 ± 0.02d	377.67	<0.0001
Food and calorie intake nutritional parameters						
Body mass index (BMI, g/cm^2)	5.32 ± 0.26a	5.53 ± 0.41a	6.51 ± 0.32a	11.53 ± 0.48b	59.915	<0.0001
Specific rate of body mass gain (g/kg)	5.07 ± 0.76a	19.39 ± 1.25b	36.90 ± 1.82c	48.95 ± 2.16d	147.24	<0.0001
Energy intake (kJ/day)	1012.59 ± 3.68a	1316.98 ± 5.16b	1850.63 ± 4.82c	2728.26 ± 5.28d	24778	<0.0001

Feed efficiency (FE; %)	2.44 ± 0.13a	3.95 ± 0.16b	4.02 ± 0.25b	3.34 ± 0.22b	13.963	<0.0001

Datas are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Data marked with different letters (a, b, c, d) are statistically significant ($p < 0.0001$) if they do not share the same letters. **Relative organ weight** (g/100g) = {[organ weight/body weight] × 100}, **BMI** = body weight (g) / length^2 (cm^2), **Specific rate of body mass gain** (g/kg) = dM / M dt, where dM represents the gain of body weight during dt = t_2 - t_1 and M is the rat body weight at t_1, **Energy intake** (kJ/day) = mean food consumption x dietary metabolizable energy, **Feed efficiency** (FE; g gained/kcal) = (mean body weight gain (g) 100) / total energy intake (kcal) .**Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **FPF-H diet** Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23 kJ/g

5.2.3. Long-term feeding of FPF diets altered serum FAME profles

Serum levels of SFA (lauric, myristic, palmitic, and stearic acid: 2.21–3.14 fold) and PUFA-ω6 (linoleic, γ-linolenic, and arachidonic acid: 1.48–2.66 fold) were signifcantly ($p<0.0001$) increased, while MUFA (myristic, palmitic, palmitoleic, stearic, and oleic acid: 0.21–0.48 fold) and PUFA-ω3 (α-linolenic, eicosatrienoic, and eicosapentaenoic acid: 0.30–0.64 fold) levels were signifcantly ($p<0.0001$) decreased in the serum of rats fed with FPF-diets compared to the control-diet group **(Table 2),** which is implicated with the induction of obesity and onset of chronic infammation and oxidative stress in rats. Our study demonstrated that the n-6:n-3 PUFA ratio levels were significantly ($p<0.0001$) increased in the FPF-diets to the tune of 1.48–2.66 fold, respectively

(Table 1), which is associated with dietary intake-mediated changes in the production of infammatory mediators and regulators toward a pro-infammatory profile.

5.2.4. Long-term feeding of FPF diets instigated insulin resistance, altered hepato-renal function markers, elevated serum lipid profles, activated testis oxidative stress and depleted of antioxidant reserve

Compared to the control-diet group, FPF-diet groups showed a signifcant ($p<0.0001$) elevation in serum glucose e (3.94–4.48-fold) and insulin (2.93–3.64-fold) levels, which further directed toward insulin resistance and pancreatic defect in β-cell function as indicated by a signifcant increase in the HOMA-IR (11.57–16.63-fold) and a reduction in HOMA-β (0.72–0.79-fold) than the control-diet group **(Table 20)**

Serum ALT (2.19–4.55 fold), AST (2.99–5.16 fold), ALP (2.92–4.50 fold), urea (3.15–5.28 fold), creatinine (2.23–3.36 fold), AST:ALT ratio (1.13–1.36 fold), and BUN:Creatinine ratio (1.41–1.56 fold) were signifcantly ($p<0.001$) increased in the three FPF-diet groups compared with the control-diet group due to metabolic syndrome induced by a high-fat diet, resulting in hepatic and renal damage **(Table 20)**

The serum TC, TAG, VLDL, LDL, and leptin levels in the serum of rats fed a FPF-diet increased significantly ($p<0.0001$) by 3.42–3.92, 3.37–4.00, 34.29–47.27, 5.86–7.02 fold, respectively, and HDL level (0.84–0.45 fold) decreased compared with the control-diet group, indicating disturbances in lipid metabolism such as hypercholesterolemia and hyperleptinemia, which are associated with the pathogenesis of insulin resistance, obesity, atherosclerosis, and cardiovascular disease by elevating cardiovascular risk factors (cardiac index, atherogenic index, and coronary artery index: 4.00–8.75, 5.98–13.34, 6.97–15.39 fold, respectively) **(Table 20)**

Long-term feeding of FPF diets to rats for 90 consecutive days induced oxidative stress in the testis, as validated by a signifcant ($p<0.0001$) increase in the concentrations of conjugated dienes (1.66–2.05 fold), lipid hydroperoxides (2.32–2.69 fold),

malondialdehyde (2.15–3.05 fold), protein carbonyl (2.02–4.39 fold), and fragmented DNA (8.95–13.82 fold) compared with the control diet **(Table 20).**

5.2.5. Long-term feeding of FPF diets repressed serum reproductive hormones

FPF diet-induced impairment of spermatogenesis and male reproduction was refected in the levels of male reproductive hormones, namely, testicular (**FIG 2 A**, 0.42–0 and serum (**FIG 10 B**, 0.34–0.69 fold) testosterone, and serum LH (**FIG 2 E**, 0.32–0.64 fold), which showed a signifcant ($p<0.0001$, decrease in FPF diets, while the serum estradiol (**FIG 10 C** , 1.03–1.66 fold) and FSH (**FIG 10** D, 1.02–1.28 fold) levels were signifcantly ($p<0.0001$) increased compared to control. Enzymatic catalase (0.54–0.87 fold), (The non-enzymatic (GSH: 0.27–0.72 fold, **Table 20**) antioxidant reserves were drastically depleted ($p<0.0001$) in the FPF diets compared to the control diet, indicating that antioxidant enzymes are responsible for reducing the redox imbalance and that these antioxidants were suppressed due to the intensifcation of oxidative stress in the testis caused by FPF diet supplementation. Furthermore, the GSH:GSSG ratio (reduced-to-oxidized glutathione ratio), which is a benchmark of the mitochondrial or cellular redox state, was signifcantly ($p<0.0001$) lower (0.27–0.72 fold) for GSH, but higher (4.03–7.68 fold) for GSSG in the FPF diet groups, signifying that oxidative stress in the testis occurred in groups supplemented with dietary FPF than in the control diet. The increased GSH:GSSG ratio in the testis ($p<0.0001$) of rats fed a FPF diet suggests that the rats were exposed to oxidative stress **(Table 20).**

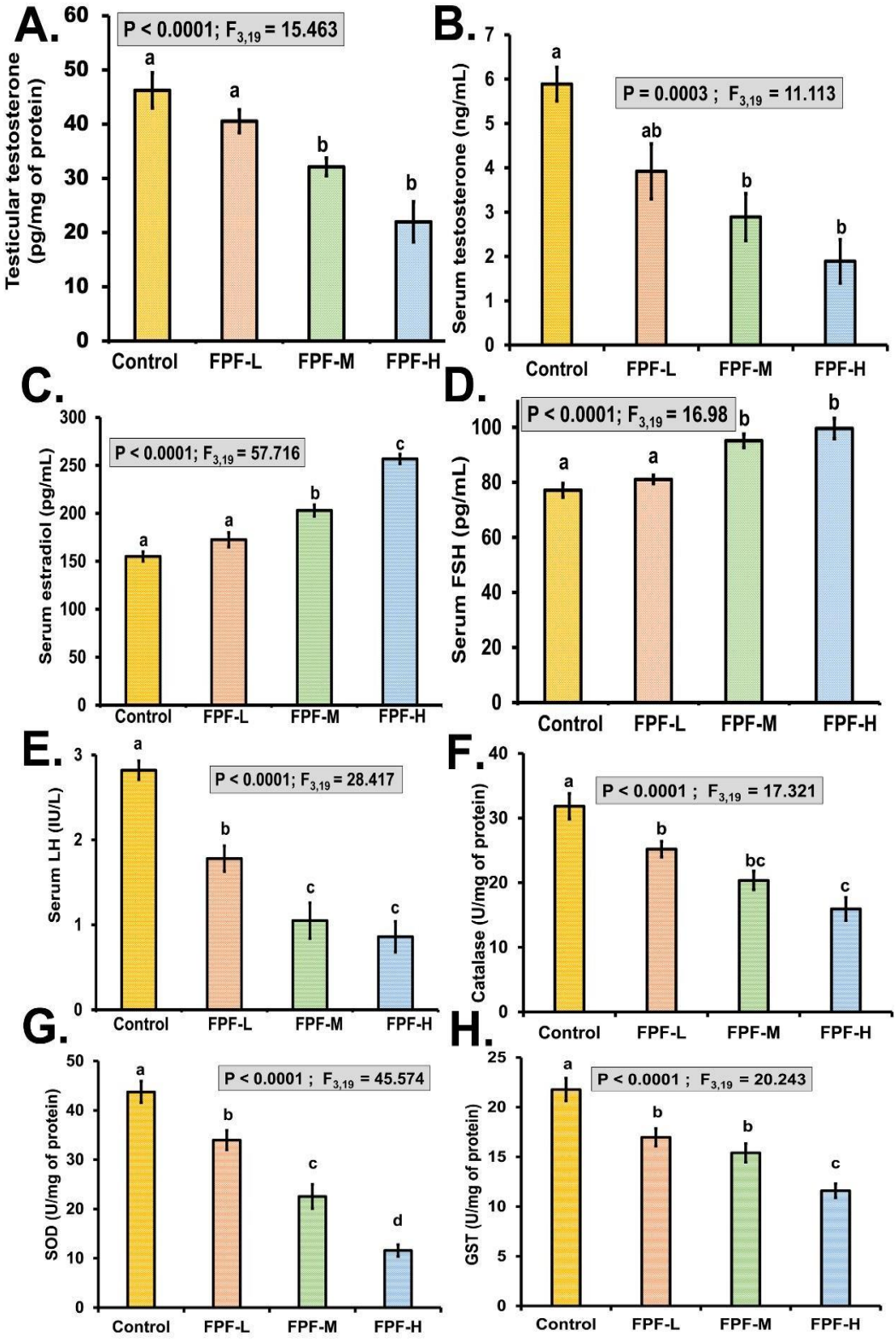
A.
P < 0.0001; F3,19 = 15.463
Testicular testosterone (pg/mg of protein)
Control
FPF-L
FPF-M
FPF-H
B.
P = 0.0003 ; F3,19 = 11.113
Serum testosterone (ng/mL)
C.
P < 0.0001; F3,19 = 57.716
Serum estradiol (pg/mL)
D.
P < 0.0001; F3,19 = 16.98
Serum FSH (pg/mL)
E.
P < 0.0001; F3,19 = 28.417
Serum LH (IU/L)
F.
P < 0.0001 ; F3,19 = 17.321
Catalase (U/mg of protein)
G.
P < 0.0001 ; F3,19 = 45.574
SOD (U/mg of protein)
H.
P < 0.0001 ; F3,19 = 20.243
GST (U/mg of protein)

FIG 10: Assessment of sex hormones and antioxidant enzyme levels in the serum and testis of male Wistar albino rats exposed to fermented pork fat diet (Saum) for 90 days. Values were the mean of fve observations (n=5). Statistical comparison was performed using one-way ANOVA followed by Tukey's multiple comparison tests, and a bar graph with diferent letters indicating statistical signifcance at $p<0.001$; similar letters indicate that there is no signifcant diference. A Testicular testosterone level (pg/ mg of protein); B Serum testosterone level (ng/mL); C Serum estradiol level (pg/mL); D Serum follicle stimulating hormone level (pg/mL); E Serum luteinizing hormone level (IU/L); F Catalase (CAT, U/mg of protein) level; G Superoxide dismutase (SOD, U/mg of protein) level; H Glutathione S transferase (GST, U/mg of protein) level; Control diet standard pellet diet; FPF-L diet Fermented pork low fat diet; FPF-M diet Fermented pork medium fat diet; FPF-H diet Fermented pork high fat diet

Table 20 : Long-term feeding effects of fermented pork fat diet (Sa-um) on hepatic insulin resistance, liver and kidney function and lipid profiles, serum fatty acid methyl esters composition and testicular inflammatory, oxidative stress, apoptotic and steroidogenic markers in male Wistar albino rats exposed for 90 days

Parameters	Control diet	FPF-L diet	FPF-M diet	FPF-H diet	$F_{3,19}$	P value
Hepatic insulin resistance profiles						
Serum insulin level (ng/mL)	0.45 ± 0.02a	1.32 ± 0.08b	1.48 ± 0.12b	1.64 ± 0.35b	7.858	0.0019
Serum glucose level (mg/dL)	105.40 ± 3.46a	416.05 ± 4.25b	450.35 ± 5.05c	472.64 ± 4.56d	920.48	<0.0001
Homeostasis model assessment of insulin resistance index (HOMA-IR)	2.11 ± 0.26a	24.41 ± 1.74b	29.62 ± 2.95bc	34.45 ± 2.85cd	27.196	<0.0001
Pancreatic β-cell function (HOMA-β, %)	8.83 ± 0.34a	6.40 ± 0.15b	6.62 ± 0.28b	6.99 ± 0.42b	12.478	0.0002
Liver and kidney function profiles						
Alanine aminotransferase (ALT, IU/mL)	13.15 ± 2.86a	28.85 ± 1.96b	42.88 ± 3.36c	59.89 ± 1.83d	59.597	<0.0001
Aspartate aminotransferase (AST, IU/mL)	23.15 ± 2.26a	69.25 ± 2.52b	95.33 ± 1.71c	119.56 ± 2.11d	361.19	<0.0001
AST/ALT ratio	1.76 ± 0.02a	2.40 ± 0.01b	2.22 ± 0.01c	2.00 ± 0.03d	204.36	<0.0001
Alkaline phosphatase (ALP, IU/L)	85.58 ± 2.09a	250.27 ± 3.52b	314.18 ± 2.55c	385.60 ± 2.22d	2328.2	<0.0001
Urea (mg/dL)	39.50 ± 1.92a	124.73 ± 1.37b	173.87 ± 1.85c	208.82 ± 2.26d	1530.2	<0.0001

Blood urea nitrogen (BUN, mg/dL)	18.45 ± 1.66a	58.25 ± 2.92 b	81.25 ± 2.68c	97.52 ± 2.30d	197.98	<0.0001
Creatinine (mg/dL)	0.95 ± 0.07a	2.12 ± 0.10b	2.85 ± 0.13c	3.20 ± 0.25c	41.934	<0.0001
BUN/Creatinine ratio	19.42 ± 1.10a	27.47 ± 1.81b	28.49 ± 1.48b	30.47 ± 2.15b	8.353	0.0014
Serum lipid profiles						
Total cholesterol (TC, mg/dL)	87.48 ± 1.58a	299.52 ± 2.68b	323.90 ± 2.46c	343.28 ± 2.72d	2438.1	<0.0001
Triacylglycerols (TAG, mg/dL)	45.90 ± 1.39a	154.98 ± 1.85b	163.70 ± 1.44c	183.71 ± 2.25d	83.548	<0.0001
VLDL cholesterol (mg/dL)	2.78 ± 0.94a	95.35 ± 0.98b	106.31 ± 0.84b	131.43 ± 0.60c	14.429	<0.0001
HDL cholesterol (mg/dL)	34.02 ± 1.02a	28.64 ± 1.17b	24.65 ± 1.00c	15.54 ± 1.26d	49.382	<0.0001
LDL cholesterol (mg/dL)	44.28 ± 1.64a	259.86 ± 2.09b	286.51 ± 2.74c	311 ± 2.98d	2562.2	<0.0001
Leptin (ng/mL)	5.06 ± 0.69a	11.35 ± 1.58b	16.48 ± 1.35bc	19.78 ± 1.82c	20.349	<0.0001
Cardiac index (CI)	1.35 ± 0.74a	5.41 ± 0.80b	6.64 ± 1.19b	11.82 ± 1.25c	62.066	<0.0001
Atherogenic index (AI)	1.58 ± 0.29a	9.45 ± 1.02b	12.10 ± 1.54b	21.09 ± 2.00c	35.818	<0.0001
Coronary artery index (CAI)	1.30 ± 0.12a	9.07 ± 1.00b	11.62 ± 1.13b	20.01 ± 1.25c	61.716	<0.0001
Serum fatty acid methyl esters composition (mg/g)						
Saturated fatty acids (SFA)						
C12, Lauric acid	11.81 ± 0.26a	42.26 ± 2.08b	64.45 ± 2.72c	87.18 ± 2.35d	15.678	<0.0001
C14, Myristic acid	14.26 ± 0.16a	45.12 ± 0.36b	67.05 ± 1.21c	80.58 ± 1.62d	11.479	0.0003
C16, Palmitic acid	141.20 ± 7.94a	294.41 ± 6.28b	368.61 ± 5.96c	386.75 ± 6.38c	103.79	<0.0001
C18, Stearic acid	41.66 ± 5.28a	79.55 ± 6.02b	117.56 ± 3.06c	126.82 ± 7.03c	39.420	<0.0001
Monounsaturated fatty acids (MUFA)						
C16:1, Palmitoleic acid, ω7	65.19 ± 2.38a	25.38 ± 3.18b	20.74 ± 2.03c	15.41 ± 1.26d	34.480	<0.0001
C18:1, Oleic acid, ω9	275.18 ± 4.16a	130.42 ± 8.58b	108.02 ± 6.35c	56.05 ± 6.95d	66.331	<0.0001
C18:1, Vaccenic acid, ω7	19.45 ± 1.38a	12.10 ± 0.75ab	7.80 ± 0.92b	1.95 ± 0.10c	13.845	<0.0001
Polyunsaturated fatty acids (PUFA)						
C18:2, Linoleic acid, ω6	131.44 ± 6.28a	175.38 ± 5.82b	230.85 ± 3.36c	310.84 ± 8.21d	28.208	<0.0001
C18:3, α-Linolenic acid, ω3	91.58 ± 0.84a	47.36 ± 0.28b	36.05 ± 0.36b	23.28 ± 0.16c	50.864	<0.0001
C18:3, γ-Linolenic acid, ω6	18.66 ± 1.50a	41.64 ± 1.75b	61.35 ± 2.10c	80.25 ± 2.41d	24.871	<0.0001
C20:3, Eicosatrienoic acid, ω3	30.95 ± 1.75a	25.84 ± 1.91ab	16.38 ± 1.77b	12.60 ± 5.90c	320.19	<0.0001
C20:4, Arachidonic acid, ω6	27.36 ± 5.01a	46.88 ± 3.64b	61.44 ± 3.03c	85.66 ± 3.28d	61.815	<0.0001

C20:5, Eicosapentaenoic, ω3	20.62 ± 0.92a	18.22 ± 0.36ab	16.35 ± 0.10b	6.11 ± 0.26c	16.559	<0.0001
SFA (mg/g)	210.93 ± 16.78a	466.34 ± 13.45b	621.67 ± 12.82c	685.33 ± 15.58d	78.882	<0.0001
MUFA (mg/g)	361.85 ± 10.95a	172.86 ± 9.95b	138.90 ± 10.45c	75.64 ± 12.05d	61.108	<0.0001
PUFA ω6 (mg/g)	179.62 ± 10.66a	265.99 ± 11.20b	354.92 ± 10.56c	478.67 ± 9.85d	117.27	<0.0001
PUFA ω3 (mg/g)	145.65 ± 5.62a	92.75 ± 3.75b	69.90 ± 1.82c	43.52 ± 1.26d	123.06	<0.0001
n-6 : n-3 PUFA ratio	1.23 ± 0.04a	1.83 ± 0.06b	2.44 ± 0.03c	3.29 ± 0.08d	247.84	<0.0001
Serum inflammatory markers						
Interleukin-1 (pg/mL)	13.18 ± 1.72a	39.65 ± 2.38b	48.55 ± 3.26bc	56.48 ± 3.20c	48.060	<0.0001
Interleukin-6 (pg/mL)	16.54 ± 1.96a	28.25 ± 1.88b	37.89 ± 2.10c	48.22 ± 1.45d	52.682	<0.0001
Interleukin-10 (pg/mL)	46.15 ± 1.45a	30.28 ± 2.95b	22.38 ± 1.49bc	17.72 ± 1.90c	37.411	<0.0001
Leukotriene B4 (LTB4, pg/mL)	128.38 ± 4.12a	156.35 ± 4.05b	179.51 ± 3.70c	203.45 ± 4.25d	63.259	<0.0001
Prostaglandin (pg/mL)	38.66 ± 1.52a	53.18 ± 1.45b	68.05 ± 2.08c	81.05 ± 2.95d	77.178	<0.0001
Testis inflammatory markers						
Nitric oxide (NO, nmol/mg protein)	1.45 ± 0.92a	3.12 ± 0.35b	4.68 ± 1.32c	6.05 ± 2.16d	34.063	<0.0001
Myeloperoxidase (MPO, U/min/mg protein)	1.95 ± 0.18a	8.85 ± 0.48b	9.56 ± 0.72b	10.15 ± 0.95b	34.706	<0.0001
Lactic dehydrogenase (LDH, U/mg protein)	3.05 ± 0.58a	7.14 ± 0.75b	9.62 ± 0.82bc	11.49 ± 0.92c	22.018	<0.0001
Tumor necrosis factor-α (TNF-α, pg/mg protein)	5.25 ± 0.65a	10.58 ± 1.02b	11.72 ± 1.32b	13.18 ± 1.26b	9.969	<0.0006
Testis lipid peroxidation products – Oxidative stress markers						
Conjugated dienes (nmol/mg protein)	38.64 ± 3.16a	64.12 ± 2.90b	72.90 ± 3.30bc	78.95 ± 3.18c	31.994	<0.0001
Lipid hydroperoxides (nmol/mg protein)	32.52 ± 3.09a	75.55 ± 1.84b	80.26 ± 3.81bc	87.68 ± 2.40c	74.252	<0.0001
Malondialdehyde (nmol/mg protein)	8.38 ± 1.46a	18.05 ± 1.35b	22.60 ± 1.44bc	25.55 ± 1.85c	23.851	<0.0001
Protein carbonyl (nmol/mg protein)	5.12 ± 0.82a	10.36 ± 1.22a	16.78 ± 1.20b	22.48 ± 2.33b	25.301	<0.0001
Fragmented DNA (%)	5.39 ± 0.66a	48.28 ± 3.16b	61.75 ± 3.64c	74.50 ± 3.25d	105.39	<0.0001
Testis levels of non-enzymatic antioxidants – Oxidative stress markers						
Reduced glutathione (GSH) (nmol/mg	73.94 ± 4.56a	53.56 ± 3.25b	35.15 ± 2.95c	20.38 ± 2.66d	45.591	<0.0001

protein)						
Glutathione disulfide (GSSG, nmol/mg protein)	3.14 ± 0.06a	12.64 ± 1.28b	19.55 ± 1.38c	24.12 ± 2.75c	30.010	<0.0001
GSH:GSSG ratio	23.57 ± 1.05a	4.28 ± 0.23b	1.83 ± 0.09c	0.86 ± 0.02c	394.97	<0.0001
Testis cell proliferation and differentiation, apoptotic and steroidogenic markers						
Tubule differentiation index (TDI %)	51.58 ± 1.05a	28.42 ± 1.25b	21.15 ± 0.95c	14.75 ± 0.66d	258.09	<0.0001
Apoptotic index (AI, %)	2.59 ± 0.04a	22.22 ± 1.15b	44.71 ± 2.06c	56.17 ± 3.38d	133.79	<0.0001
PCNA immunoreactivity ratio	46.58 ± 1.85a	31.16 ± 1.28b	25.95 ± 1.36b	18.39 ± 0.72c	77.552	<0.0001
BAX immunoreactivity ratio	8.56 ± 0.68a	28.02 ± 1.05b	32.55 ± 1.16b	54.55 ± 1.58c	263.72	<0.0001
BCL-2 immunoreactivity ratio	79.56 ± 2.63a	49.95 ± 2.32b	40.61 ± 2.71c	18.84 ± 0.94b	123.57	<0.0001
BAX/BCL-2 ratio	0.11 ± 0.02a	0.56 ± 0.04a	0.80 ± 0.03a	2.90 ± 0.26b	6.930	<0.0033
StAR immunoreactivity ratio	78.54 ± 2.68a	48.76 ± 2.42b	29.05 ± 1.92c	20.54 ± 1.88c	130.92	<0.0001
3β-HSD immunoreactivity ratio	42.43 ± 2.63a	31.28 ± 1.30b	20.48 ± 1.19c	18.62 ± 1.52c	39.281	<0.0001
LHR immunoreactivity ratio	28.19 ± 1.92a	19.70 ± 1.75b	10.15 ± 1.38c	1.08 ± 1.49c	50.663	<0.0001

Datas are presented as mean ± standard error mean (n = 5). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p < 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p > 0.05$**AST:ALT ratio** = AST/ALT, **Blood urea nitrogen** = Serum urea × 0.467, **VLDL** = 0.2 × TAG, **LDL** = TC − (HDL + VLDL), **CI** = TC/HDL, **AI** = (total cholesterol−HDL)/HDL, **CAI** = LDL/HDL, **HOMA-IR** = [(Serum insulin level (ng/mL) × Serum glucose level (mg/dL)] / 22.5, **HOMA-β** = [20 × Insulin (ngl/mL)] / [Glucose (mg/dL) - 3.5] *100, **AI** = number of apoptotic cells/number of total cells × 100), PCNA/BCL-2/StAR/LHR/3β-HSD immunoreactivity ratio = Positively reacted cells/total number of cells×100, **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented

pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **FPF-H diet** Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23 kJ/g, **VLDL** Very low-density lipoprotein, **HDL** High density lipoprotein, **LDL** Low density lipoprotein,PCNA Proliferating cell nuclear antigen, **BCL-2** B-cell lymphoma 2, **BAX** Bcl-2-associated X protein, **StAR** Steroidogenic acute regulatory protein, **3β-HSD** 3β-hydroxysteroid dehydrogenase, **LHR** Luteinizing hormone receptor

5.2.6. Long-term FPF feeding affected sperm morphology and sperm parameters

Long-term feeding of FPF diets triggered sperm dysfunction not only reducing sperm number, but alsodiminishing the viable and motile spermatozoa. A significantdecrease($p< 0.05$) was observed in the sperm functional indices in terms of sperm motility (13.48 – 24.72%, **FIG 10 B),** daily sperm production (12.42 – 22.41%, **FIG 11 C),** sperm concentration (cauda: 7.84 – 31.02%, **FIG 11 D** and caput: 17.05 – 33.42%, **FIG 10 E**) and sperm transit time (cauda: 15.68 – 21.61%,**FIG 11 F** and caput: 17.61 – 26.14%, **FIG 10 E)** in the FPF diets fed rat groups compared to the control. Additionally, the FPF dietinduced effects werevalidated on sperm morphological characteristics. A higher prevalence of sperm anomalies detected in the FPF diet groups compared to the control group. The quantitative scoring showed that the most common sperm head anomalies (7.06 – 21.08%) were amorphous, banana, and detached head, whereas coiled and broken forms were observed as tail abnormalities (5.91 – 16.68%) **(Table 21).** The high number of abnormally denatured ssDNA (yellowish orange/red fluorescent, AO+) was observed in the FPF-H followed by FPF-M and FPF-L diet group compared with the control diet (normal native dsDNA, green fluorescent, AO−, **FIG 11 H).** The dsDNA/ssDNA ratio was declined (0.05 – 0.24 fold, **FIG 11 H)** significantly in the FPF diet groups in a dose-dependent manner, witnessing a reduction in DNA integrity. A higher percentage of sperm DNA damage (26.56 – 38.45%)dose dependently observed in the FPF diet groups compared with the control diet group.

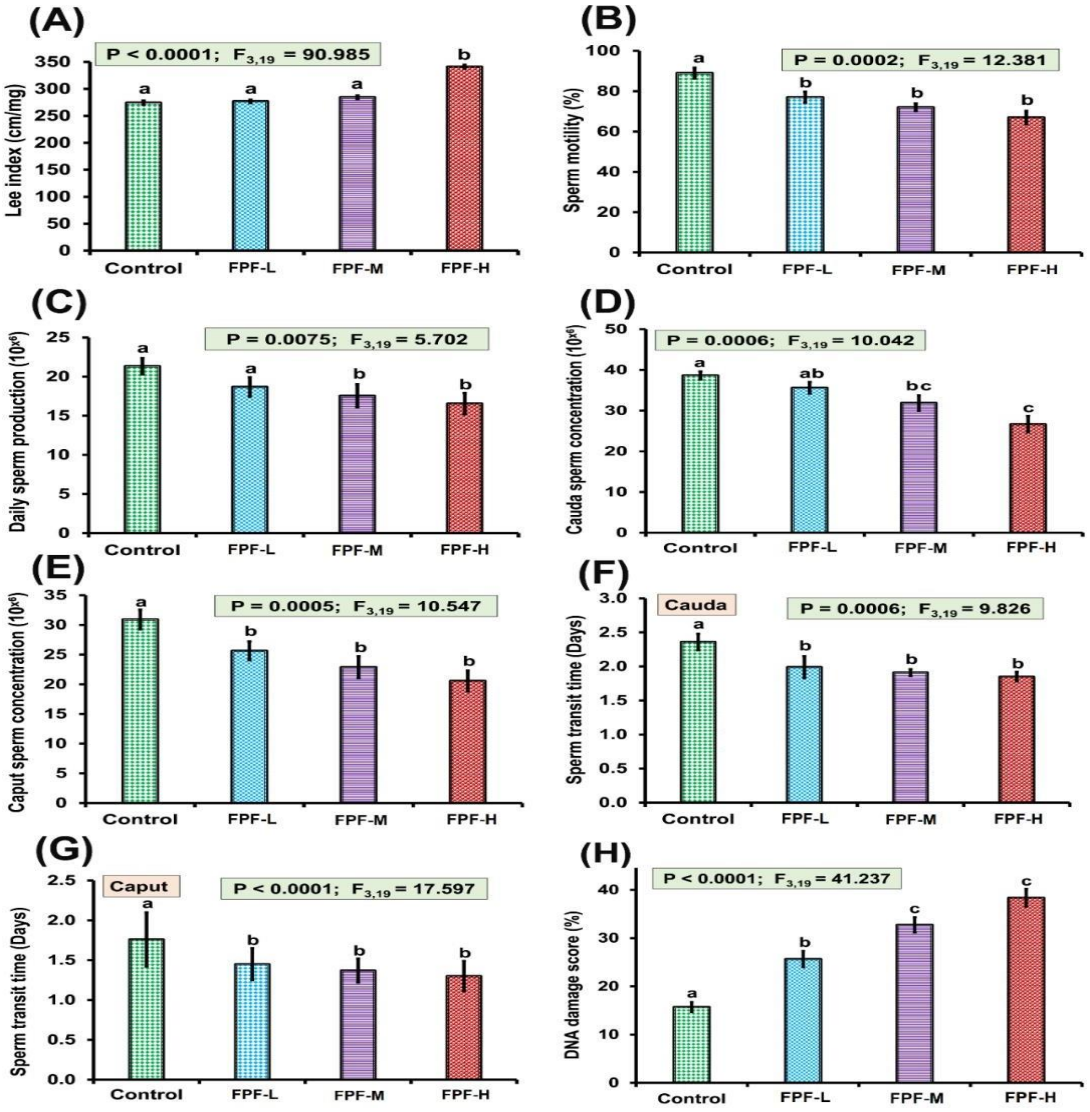

FIG 11: Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p < 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p > 0.05$. **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **FPF-H diet**

Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23 kJ/g

Table 21: Long-term feeding effects of fermented pork fat diet on sperm morphology in male Wistar albino rats

Sperm morphology	Control diet	FPF-L diet	FPF-M diet	FPF-H diet	$F_{3,19}$	P value
Sperm morphology – Normal (%)						
Normal phenotypes	90.05 ± 0.89a	87.02 ± 1.22a	66.57 ± 0.93b	62.08 ± 0.89c	182.92	<0.0001
Sperm morphology - Head abnormality (%)						
Amorphous	2.79 ± 0.04a	2.23 ± 0.12b	4.42 ± 0.14c	6.49 ± 0.03d	400.81	<0.0001
Banana head	2.13 ± 0.05a	2.46 ± 0.22a	5.66 ± 0.02b	8.71 ± 0.04c	606.26	<0.0001
Detached head	1.21 ± 0.15a	2.37 ± 0.07b	5.78 ± 0.13c	6.49 ± 0.25d	247.24	<0.0001
Total of head abnormalities	6.13 ± 0.03a	7.06 ± 0.04b	15.86 ± 0.03c	21.08 ± 0.06d	995.01	<0.0001
Sperm morphology - Tail abnormality (%)						
Coiled tail	1.67 ± 0.04a	3.03 ± 0.08b	2.77 ± 0.04c	8.36 ± 0.06d	2715	<0.0001
Broken tail	1.72 ±	2.88 ±	4.83 ±	8.32 ±	17.426	<0.0001

	0.11a	0.61a	0.84b	0.91b		
Total of tail abnormalities	3.39 ± 0.36a	5.91 ± 0.67a	7.16 ± 0.59b	16.68 ± 0.91b	76.365	<0.0001

Data are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p < 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p > 0.05$.The sperm abnormality was weighed as percent abnormality = {[No. of abnormal sperm/Total no. of sperm] × 100}.**Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **FPF-H diet** Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23 kJ/g

5.2.7. Long-term FPF feeding disrupted testis histoarchitecture

Normal testis histologicalfeatures including the compactness of seminiferous tubules (SFT) with complete stages of spermatogenesis (*) and full sperm mass in lumen (®)were more evident in the control diet (**FIG 12 A and B**) while testis structuraldisorganization, dearth of sperm mass in lumen (+), vacuolization (µ), and loss of compactness of the SFTwere observed in FPF-L diet fed rats (**FIG 12 C and D**). The alterations in the SFT histo-architecture were more prominent with FPF-Mdiet tissue sections showing disrupted seminiferous tubules, emptiness of sperm mass in lumen (+), fragmented spermatocytes ($), apoptotic spermatocytes (#), delamination/disorientation of germinal epithelial cells (@), multinucleated germ cells (^), sloughing of germ cells (©), increased vacuolization within the tubules (µ), and in interstitial spaces start Leydig cell dystrophy (**FIG 12 E and F**). Drastic testis tissue structural alterations were detected in FPF-H diet as indicated by depletion of Leydig and Sertoli cells (**), wider interstitial space (%), vacuolated spermatocyte (β) and degeneration of Sertoli cells (→), prominent vacuolization, and extensive tubular degeneration along with disorientation of the different developing stages of spermatogenic and Sertoli cells (**FIG 12 G and H**). Long term high dietary FPF consumption imposed maximal testis tissue damage in FPF-M and FPF-H to the magnitude of causing partial to complete tubular atrophy.

The high testis tissue damage score of 4.22 and 5.71 was registered in FPF-M and FPF-H diet group showing testicular atrophy of about 75 – 100%, where seminiferous tubules lined by one layer of cells with marked cellular necrosis and loss of spermatogenesis, with interstitial edema and hyperemia while 50 – 75% testicular atrophy was noticed in FPF-L (2.68 score) diet (Table 3). A significant decrease in the JTBS was observed in the FPF-H (4.66), FPF-M (5.11) and FPF-L (7.83) compared with the control diet (9.21), signifying that the FPF diet induced progressive deterioration of testicular histo-architecture leading to disruption of seminiferous tubules, hypo-spermatogenesis, maturation arrest and testicular damage (**Table 22**). Long-term feeding of FPF diet resulted in severe morphological changes in the seminiferous tubules, i.e., a significant reduction in the MSTD (33.08 – 46.92%), STEH (15.09 – 37.21%), TAT (20.94 – 56.73%) and an increase inIS

(31.22 – 59.41%), and TL (13.68 – 48.50%) demonstrating FPF diet induced testicular tissue disintegrity and sperm loss (TableS3). Further, a drastic decline in the germinal and interstitial cells was detected in the FPF supplemented diet comprising spermatogonia (0.63 – 0.96 fold), spermatocytes (0.34 – 0.79 fold), spermatids (0.48 – 1.05 fold), Sertoli cells (0.48 – 0.95 fold) and Leydig cells (0.57 – 1.13 fold) resulting in hypo-spermatogenesis and testicular dysfunction (**Table 22**).

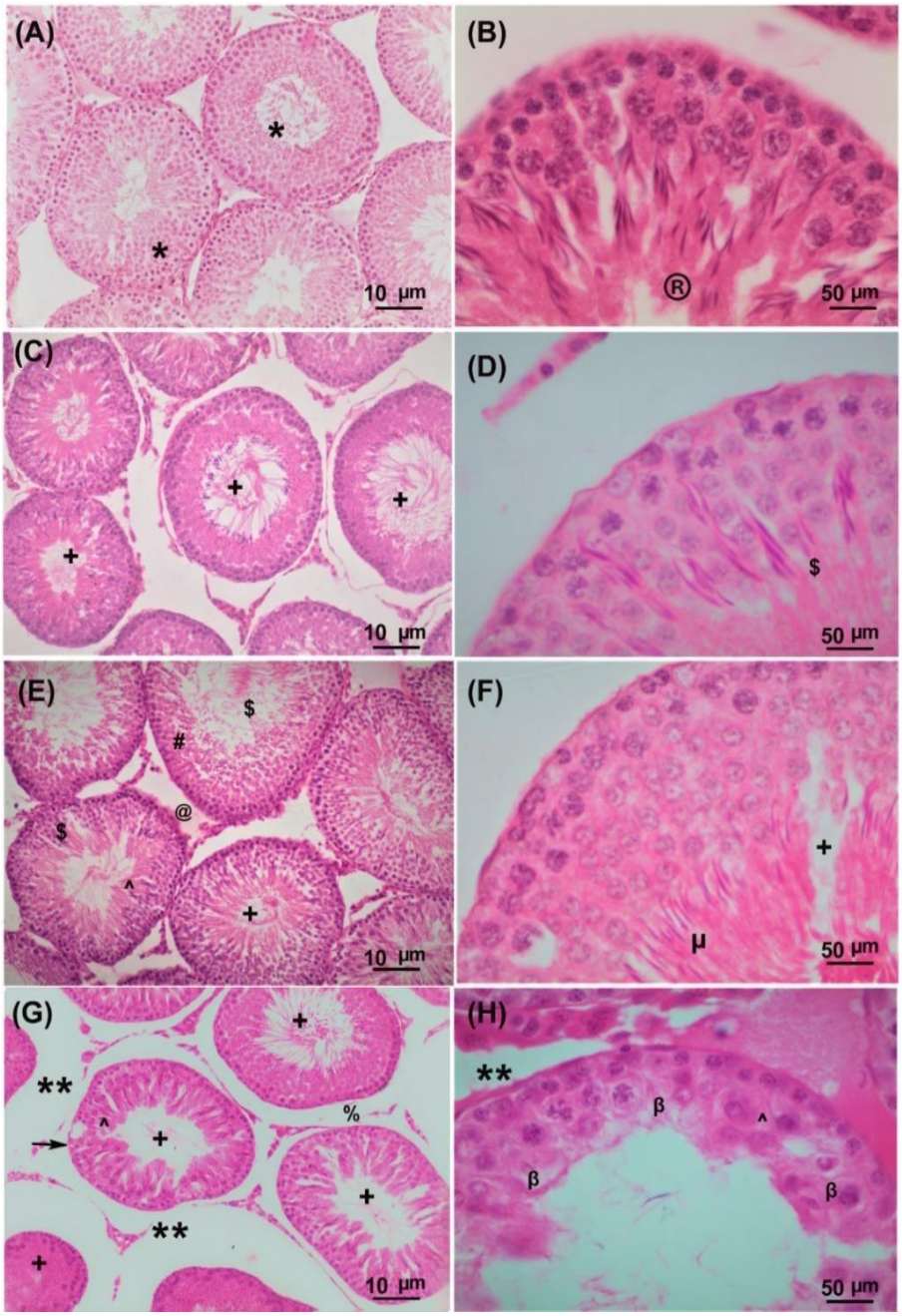

FIG 12: Histological analysis of the testis (A-H)) showing effects of high fat diet in control (A,B), FPF-L (C,D), FPF-M (E,F), FPF-H (G,H)for90days

Table 22: Effects of long-term feeding of fermented pork fat diet (Sa-um) on morphological changes of seminiferous tubules and quantification of germ and Leydig cells in testis of male Wistar albino rats exposed for 90 days

Parameters	Control diet	FPF-L diet	FPF-M diet	FPF-H diet	$F_{3, 39}$	P value
Morphological changes of seminiferous tubules (10 random non-overlapping fields/treatment)						
Johnsen's mean testicular biopsy score (JTBS)	9.21 ± 0.45a	7.83 ± 0.62a	5.11 ± 0.19b	4.66 ± 0.52b	21.292	<0.0001
Mean diameter of seminiferous tubule (MSTD, µm)	251.85 ± 2.34a	168.52 ± 1.38b	140.22 ± 2.18c	133.67 ± 2.28c	681.45	<0.0001
Seminiferous tubule epithelial height (STEH, µm)	83.91 ± 1.87a	71.25 ± 2.88a	78.54 ± 1.44a	52.69 ± 3.85b	25.907	<0.0001
Interstitial space (IS, µm)	9.88 ± 0.45a	8.33 ± 0.68a	5.83 ± 0.46b	4.01 ± 0.33b	27.565	<0.0001
Tunica albuginea thickness (TAT, µm)	24.31 ± 1.43a	19.22 ± 1.68b	15.73 ± 0.89b	10.52 ± 0.13c	23.766	<0.0001
Tubular lumen (TL, µm)	10.66 ± 0.46a	8.98 ± 1.21a	6.36 ± 0.84b	5.49 ± 0.78b	10.878	<0.0001
Testis tissue damage score	0a	2.68 ± 0.23b	4.22 ± 0.11c	5.71 ± 0.43d	95.195	<0.0001
Enumeration of germ cells and Leydig cells (10 random non-overlapping fields/treatment)						
No. of Spermatogonia cells	40.46 ± 1.38a	38.84 ± 2.61a	22.18 ± 1.69b	25.66 ± 3.27b	15.258	<0.0001
No. of spermatocytes	67.89 ± 2.81a	53.76 ± 2.19a	39.38 ± 1.45b	22.84 ± 3.78b	41.544	<0.0001
No. of spermatids	65.92 ± 2.81a	69.08 ± 3.46a	50.10 ± 4.49b	31.84 ± 1.44c	27.728	<0.0001
No. of Sertoli cells	11.45 ±	10.88 ±	7.66 ±	5.48 ±	25.135	<0.0001

	0.49a	0.83a	0.11b	0.56c		
No. of Leydig cells	6.85 ± 0.59a	7.76 ± 0.34a	4.33 ± 0.77b	3.89 ± 0.19b	13.079	<0.0001

Data are presented as mean ± standard error mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p < 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p > 0.05$.**Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **FPF-H diet** Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23 kJ/g

5.2.8. Long-term FPF feeding inhibited germ cell proliferation, differentiation, apoptosis and steroidogenesis

In the testis tissue sections of the control diet group, the spermatogonia and early-stage spermatocytes were strongly positive with PCNA staining **(FIG 13 K),** whereas there were significantly fewer PCNA-positive germinal cells (0.18 – 0.42 fold, **FIG 13 L and O)** in the FPF diet group. The PCNA ratio decreased significantly up to 32.60 – 60.86% in FPF diet groups than the control indicated the inhibition of germ cell proliferation in testis tissue and testis atrophy. Further, FPF diet contributed to defective spermatogonial differentiation that was reflected as a significant decrease in the TDI (44.90 – 71.40%, the status of type B spermatogonial differentiation into spermatocyte and spermatids) compared with the control diet (Table 2). As assessed by the TUNEL assay, occurrence of an increased number of TUNEL-positive germ cells (dark brown stained, IRDV) was detected in the testis tissue sections of the FPF-H (7.2%), FPF-M (3.9%) and FPF-L (2.3%) diet fed rats, as compared with no TUNEL-positive cells in the control group rats **(FIG 13 Fand J).** The rate of germ cell apoptosis (measured by TUNELassay) was computed in terms of apoptotic index (AI). Increased incidence of apoptotic index (apoptotic germ cells) was observed in the FPF-H (56.17%), FPF-M (44.71%) and FPF-L (22.22%) diet group than the control diet group (2.59%) indicating activation of apoptosis and induction of DNA damage in the germ cells**.**

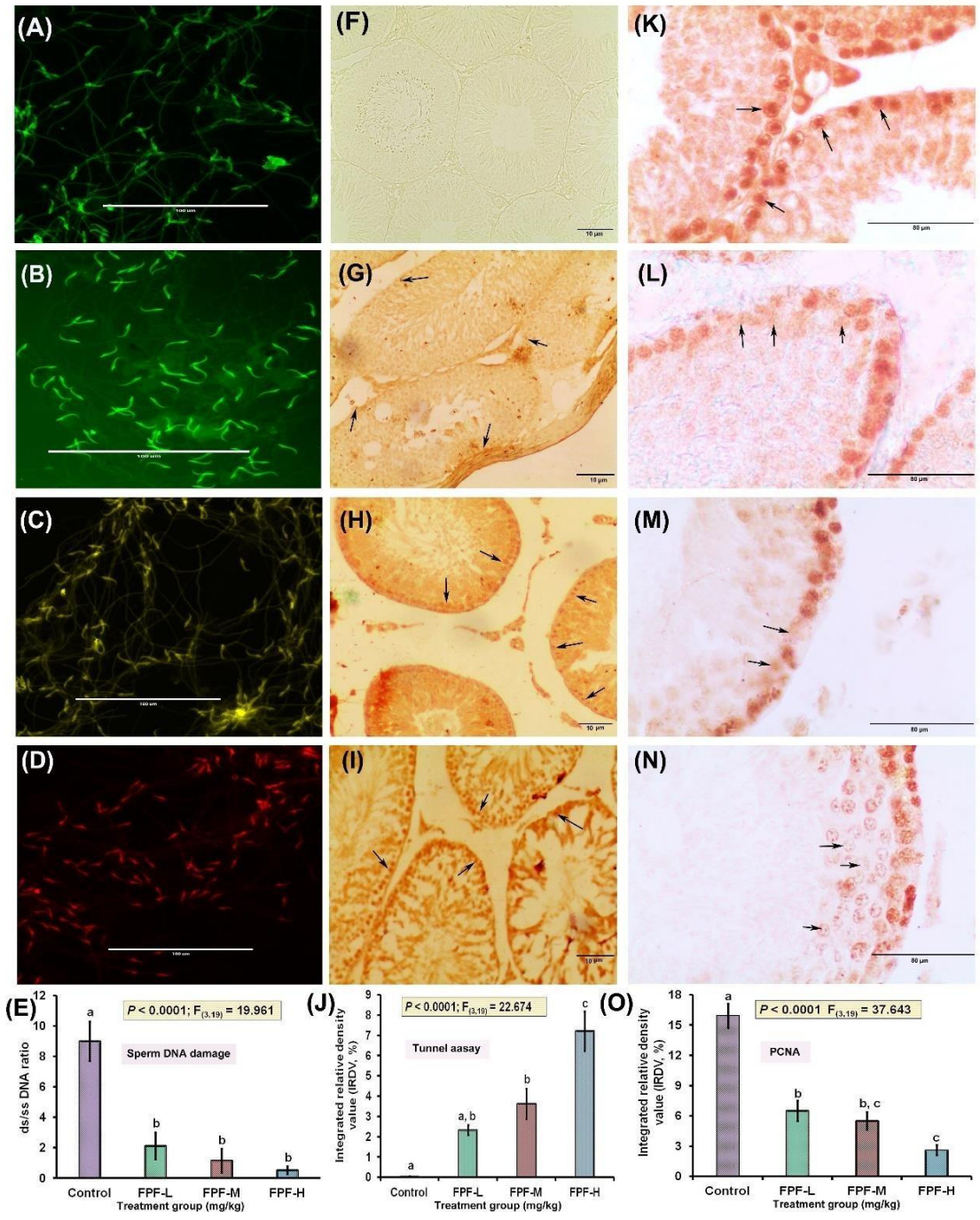

FIG 13: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with different letters (a, b, c, d,e,f) are statistically signifcant (p>0.001) if they do not share the same letters.

Immunolocalization PCNA (A-F) in FPF diet supplemented rat testis tissue and PEITC. Immunoreactivity quantifcation data (% area) of sperm DNA damage €, Tunel assay (J) and PCNA (O); Control diet standard pellet diet; FPF-M and PEITC.

Bcl-2 family genes are known for their anti-apoptosis or pro-apoptosis property and play an essential role in process of mitochondrial apoptosis pathway. This gene is also involved in regulation of spermatogenesis as dead cells or damaged spermatozoa needs to remove from seminiferous tubules. Many other factors such as environmental toxicants, many cytotoxic agents (pesticides), excessive heat and radiation from different sources may increase the process of germ cell apoptosis in mammalian testis. Bcl-2 expression may directly altered by exposure of any cytotoxic compound leading into abnormal spermatogenesis in male testis. In normal mice testis, its prime location is germ cell, spermatocytes (primary and secondary) and developing spermatozoa. HFD increases the rate of apoptosis in testicular tissue of normal healthy mice possibly by up-regulating Bcl-2 expression. Immunohistochemical analysis of Bcl-2 protein revealed a significant Bcl-2 expression in testis of all HFD treated groups when compared to normal control group **(FIG 13)**. However, some Bcl-2 positive Leydig cells were also seen after FPF diet exposure. In testicular tissue, StAR (steroidogenic acute regulatory protein) is one of the most important enzymes in steroidogenic pathway controls cholesterol transfer to inner mitochondrial membrane through a series of event. It is primarily located in Leydig cells situated between interstitial spaces of seminiferous tubules and on head of developing spermatozoa. The expression of StAR is dependent on the concentration of Leydig cells. Like other steroidogenic enzyme involved in steroidogenesis, StAR is also affected with exogenous steroids. In this study, we revealed that the expression of StAR in testicular Leydig cells was seriously affected with FPF diet treatment when compared to normal control **(FIG 14).** The immunohistochemical study of StAR in normal control group showed a positive expression in Leydig cells and in sperm masses towards lumen. In addition to that, it is concluded that higher doses of FPF seriously declined the StAR expression

however, the lower doses were not that much effective than compared to normal control group.Intense BAX immunostaining (2.8 – 3.9 fold,**FIG 14 A and E**), and high BAX(69.45 – 84.31%)and BAX/BCL-2 (0.56 – 2.90) immunoreactivity ratios (Table2) were observed in the testis of FPF-fed rats,whereasmild tofaint BCL-2 (0.36 – 0.61 fold, **FIG 14 F and J),** StAR (0.45 – 0.87 fold, **FIG 14 K and O**), The immunoreactivity ratio was significantly decreased in BCL-2 (37.22 – 76.32%), StAR (37.92 – 73.85%), defining germ cells, Leydig cells, and Sertoli cells undergoing cell death, inhibition of cholesterol transfer within mitochondria leading to testis apoptosis and disruption of spermatogenesis and steroidogenesis.

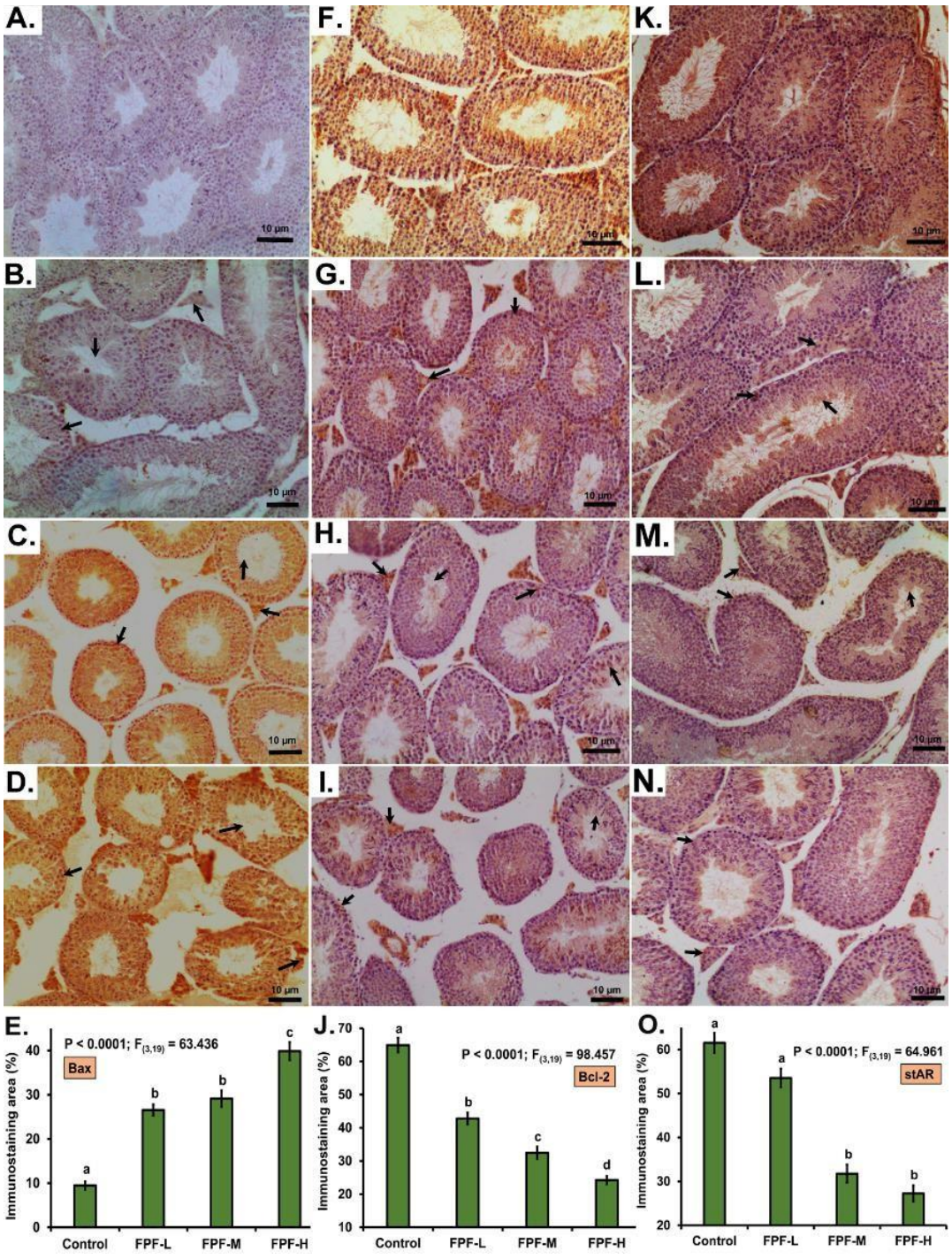

FIG 14: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with diferent letters (a,

b, c, d,) are statistically signifcant (p>0.001) if they do not share the same letters. Immunoreactivity quantifcation data (% area) of BAX (E), BCL2 (J) and stAR (O); Control diet standard pellet diet; FPF-L, FPF-M and FPF-H

3β-hydroxysteroid dehydrogenase (3β-HSD) is crucial enzyme in process of steroidogenesis that catalyzes the steroid into progesterone, 17αhydroxyprogesterone, androstenedione and testosterone from pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA) and androstenediol respectively. It is generally well located in gonads (testis and ovary) and cortical part of adrenal gland therefore involved in production of sex hormone and corticosteroids (glucocorticoids and mineralocorticoids) respectively. In mouse testis, immune histochemistry study revealed its expression in Leydig cells however in many other primate studies revealed its expression in Sertoli cells. It is well documented that expression of 3β-HSD is regulated by LH receptor hence activation of LH receptor timulates more catalization of steroids into sex hormones and corticosteroids through steroidogenesis. Since LH secretion from anterior pituitary is necessary for stimulation of enzymes involved in steroid production therefore LH receptor plays a regulatory control over 3β-HSD expressions. In normal control group, the majority of 3β-HSD expression was documented in Leydig cells and rarely seen in other cells of seminiferous tubules. An interrupted steroidogenesis has been documented after HFD treatment to healthy normal mice as there was decline in expression of 3βHSD testicular tissue (**FIG 15**). LH receptors (LHR) or human chorionic gonadotropin (hCG) is well located in gonads (testis and ovary) and their accessory sex organs. Besides that, LHR expressions are also reported in brain and placental tissue. LHR plays a major role in Leydig cells development and differentiation in the early phases of testicular cells development necessary for steroidogenesis. It shows regulatory control on hypothalamic pituitary gonadal (HPG) axis through high-affinity G protein coupled receptors (GPCRs). Moreover, in testis, cAMP independent messengers regulates activation of LHR, leading a controlled over steroidogenesis in Leydig cells. It is already reported in many study that LHR is highly susceptible to exogenous steroid present in numerous food leading into decline in altered

steroidogenesis. In our study, we claimed that FPF diet being a steroid compound, altered the expression of LHR either via interfering in binding ability with steroid receptors or by various unknown mechanism. We observed that normal control group consist of a massive density of LHR positive stained Leydig cells in interstitial spaces of seminiferous tubules. However, FPF diet treatment to normal healthy mice significantly declined in LHR expression in dose dependent manner when compared to normal control group **(FIG 15).** 3β-HSD (0.29 – 0.48 fold, **FIG 15 A and E)**, and LHR (0.01 – 0.94 fold, **FIG 15 F and J**) immunostaining. 3β-HSD (26.28 – 56.12%), and LHR (30.11 – 96.16%) compared with the control group (**FIG 15**)

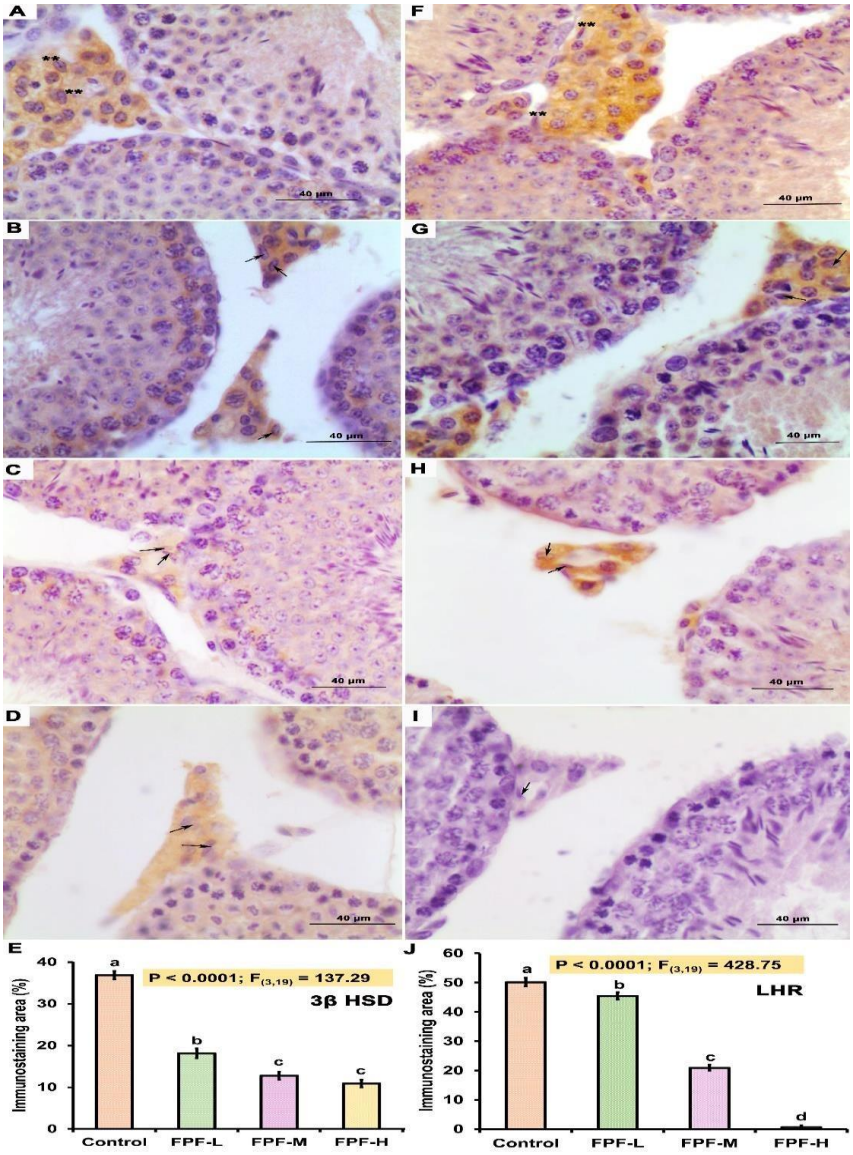

FIG 15: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet for 90 days. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with diferent letters (a, b, c, d,) are statistically signifcant (p>0.001) if they do not share the same letters.. Immunoreactivity quantifcation data (% area) of 3β HSD (E) and LHR (J); Control diet standard pellet diet; FPF-L, FPF-M and FPF-H.

Univariate and multivariate analyses

5.2.9. Long-term FPF feeding disturbs the anthropometrical, physiological and metabolic profiles

ANOVA statistical analysis supported that long-term feeding of FPF diet significantly ($F_{20,83}$ = 23.54 and P=6.051E-20) altered the serum lipid profiles, serum testosterone, Lee index, sperm quality, sperm functional indices, lipid peroxidation, and antioxidant levels (**Table 23**). Additionally, a strong correlation was observed between the FPF diet and anthropometrical, physiological, and metabolic parameters, confirming that the FPF diet caused oxidative stress, inflammation, lipid metabolism disorder, obesity, depletion of reproductive hormones and antioxidant enzyme reserves and reproductive dysfunction **(Table 23)**.Correlation studies between serum FAME levels and inflammatory and oxidative stress markers substantiated the association of obesity and metabolic syndrome in FPF diet fed rats **(Table 23).**This finding suggests an important role of SFA, MUFA and PUFAω6 for the severity of inflammation and oxidative stress in FPF diet fed rats under obese conditions.

Table 23: One way Analysis of Variance (ANOVA) showing the interaction among Lee index, sperm quality and functional indices, serum lipid profiles, serum testosterone, lipid peroxidation, and antioxidant levels upon long-term feeding effects of fermented pork fat diet in male Wistar albino rats exposed for 90 days

	LI	SM	DSP	CSC	CPC	CSTT	CPTT	DNAD	TC	TAG	VLDL	HDL	LDL	Tes	MDA	CAT	SOD	GST	GSH	HA	TA
LI	**0**	*.0002*	*0002*	*0.0002*	*0.0002*	*0.0002*	*.0002*	*0.0002*	*0.0003*	*0.0002*	*0.0002*	*.0002*	*0.0001*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*
SM	**11.78**	**0**	*0002*	*0.005*	*0.001*	*0.005*	*.0001*	*0.0001*	*0.0002*	*0.0001*	*0.0002*	*0.0002*	*0.0003*	*0.0001*	*0.0002*	*.0002*	*0.0002*	*.0002*	*.0002*	*.0002*	*.0002*
DSP	**24.95**	**13.12**	**0**	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0002*	*0.0004*	*0.0002*	*0.0002*	*0.0002*	*0.005*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
CSC	**14.11**	**32.32**	**10.79**	**0**	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0002*	*0.0005*	*0.0002*	*0.0002*	*0.0002*	*0.0001*	*0.005*	*0.005*	*0.005*	*0.005*	*0.005*	*0.005*	*0.005*
CPC	**32.55**	**42.76**	**10.35**	**44.33**	**0**	*0.0001*	*0.0001*	*0.0001*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
CSTT	**45.79**	**14.01**	**10.89**	**68.52**	**32.42**	**0**	*0.0001*	*0.0001*	*0.0002*	*0.0003*	*0.0002*	*0.0002*	*0.0002*	*0.0001*	*0.0001*	*0.0001*	*0.005*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
CPTT	**32.82**	**14.04**	**10.92**	**47.15**	**62.72**	**23.01**	**0**	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0002*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.001*	*0.005*
DNAD	**24.38**	**12.60**	**10.51**	**27.44**	**36.89**	**31.41**	**44.10**	**0**	*0.0002*	*0.0001*	*0.0002*	*0.0002*	*0.0002*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
TC	**18.78**	**11.47**	**14.59**	**13.86**	**14.24**	**15.48**	**15.51**	**14.07**	**0**	*0.0001*	*0.0002*	*.0002*	*0.0002*	*0.0002*	*0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*	*0.0002*
TAG	**27.55**	**17.76**	**12.35**	**56.58**	**20.03**	**32.45**	**32.75**	**18.34**	**12.24**	**0**	*0.0001*	*.9979*	*0.0002*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.005*	*0.0001*	*0.0001*

VLDL	**12.24**	**20.56**	**32.66**	**48.68**	**23.11**	**35.53**	**35.83**	**21.42**	**11.93**	**33.08**	**0**	***.9879***	***0.0002***	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.0001***
HDL	**14.51**	**22.73**	**15.89**	**40.49**	**38.45**	**21.28**	**41.31**	**13.05**	**14.27**	**19.65**	**22.73**	**0**	***0.0002***	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.001***	***0.005***
LDL	**14.07**	**27.70**	**10.83**	**30.03**	**10.48**	**11.72**	**11.75**	**10.31**	**3.765**	**84.73**	**81.65**	**10.44**	**0**	***0.0002***	***0.0002***	***0.0002***	***0.0002***	***0.0002***	***0.0002***	***0.0002***	***0.001***
Tes	**15.71**	**33.92**	**20.80**	**59.72**	**11.54**	**48.76**	**21.77**	**13.23**	**15.46**	**31.57**	**34.65**	**11.92**	**11.63**	**0**	***0.0001***	***0.0001***	***0.0001***	***0.001***	***0.001***	***0.005***	***0.001***
MDA	**15.81**	**44.02**	**39.37**	**69.15**	**12.21**	**32.28**	**41.80**	**14.23**	**15.53**	**32.51**	**35.65**	**12.92**	**11.73**	**19.97**	**0**	***0.0001***	***0.0001***	***0.0001***	***0.005***	***0.0001***	***0.001***
CAT	**14.59**	**32.80**	**19.54**	**48.25**	**39.26**	**12.02**	**12.33**	**20.81**	**14.28**	**20.42**	**23.51**	**17.75**	**10.52**	**11.15**	**12.15**	**0**	***0.0001***	***0.0001***	***0.0001***	***0.0001***	***0.0001***
SOD	**14.39**	**32.61**	**33.06**	**28.43**	**15.94**	**14.01**	**14.31**	**10.98**	**14.08**	**18.44**	**21.52**	**12.06**	**10.32**	**13.13**	**14.13**	**19.82**	**0**	***0.0001***	***0.001***	***0.0001***	***0.0001***
GST	**15.01**	**53.23**	**19.33**	**90.65**	**46.33**	**77.83**	**40.85**	**63.21**	**14.71**	**24.67**	**27.75**	**15.16**	**10.94**	**29.08**	**19.05**	**42.41**	**62.23**	**0**	***0.0001***	***0.001***	***0.0001***
GSH	**13.42**	**51.63**	**16.65**	**69.09**	**51.34**	**23.76**	**24.06**	**36.53**	**13.11**	**46.91**	**11.77**	**10.96**	**23.43**	**22.88**	**23.88**	**31.73**	**97.52**	**15.97**	**0**	***0.0001***	***0.005***
HA	**15.23**	**63.44**	**12.87**	**31.17**	**67.39**	**56.77**	**59.78**	**24.28**	**14.92**	**26.77**	**29.85**	**21.23**	**11.15**	**48.02**	**27.99**	**63.47**	**83.29**	**21.06**	**18.08**	**0**	***0.001***
TA	**15.48**	**73.69**	**32.68**	**43.68**	**42.45**	**31.71**	**34.72**	**10.93**	**15.17**	**29.28**	**32.36**	**36.29**	**11.48**	**22.95**	**32.92**	**48.53**	**30.84**	**46.12**	**20.59**	**25.06**	**0**

Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. F value is shown in regular font while p-value is shown in italics font. Statistically significant values between variables are shown as highlighted in italics ($p < 0.05$). One way ANOVA: $F_{20,83} = 23.54$ and p=6.051E-20, Levene's test for homogeneity of variance: p=1.17E-05, Welch F test: F =59.65 and p=4.76E-15. **LI** Lee index, **SM** Sperm motility, **DSP** Daily sperm production, **CSC** Cauda sperm concentration, **CPC** Caput sperm concentration, **CSTT** Cauda sperm transit time, **CPTT** Caput sperm transit time, **DNAD** DNA damage score, **TC** Serum total Cholesterol, **TAG** Serum triacylglycerides, **VLDL** Very low density lipoprotein, **HDL** High density lipoprotein, **LDL** Low density lipoprotein, **Tes** Serum testosterone, **MDA** Lipid peroxidation (malondialdehyde levels), **CAT** Catalase, **SOD** Super oxide dismutase, **GST** Glutathione S transferase, **GSH** Reduced glutathione, **HA** Sperm head abnormalities, **TA** Sperm tail abnormalities

Table 24: Correlation analysis showing the interaction among Lee index, sperm quality and functional indices, serum lipid profiles, serum testosterone, lipid peroxidation, and antioxidant levels upon long-term feeding effects of fermented pork fat diet (Sa-um) in male Wistar albino rats exposed for 90 days

	LI	SM	DSP	CSC	CPC	CSTT	CPTT	DNAD	TC	TAG	VLDL	HDL	LDL	Tes	MDA	CAT	SOD	GST	GSH	HA	TA
LI	0	*0.262*	*0.275*	*0.092*	*0.252*	*0.394*	*0.353*	*0.216*	*0.235*	*0.056*	*0.346*	*0.075*	*0.283*	*0.232*	*0.039*	*0.113*	*0.142*	*0.166*	*0.050*	*0.133*	*0.011*
SM	-0.738	0	*0.000*	*0.060*	*0.001*	*0.019*	*0.010*	*0.007*	*0.012*	*0.084*	*0.008*	*0.062*	*0.003*	*0.002*	*0.119*	*0.046*	*0.036*	*0.013*	*0.104*	*0.120*	*0.227*
DSP	-0.725	1.000	0	*0.066*	*0.001*	*0.017*	*0.008*	*0.008*	*0.014*	*0.092*	*0.007*	*0.069*	*0.003*	*0.003*	*0.128*	*0.051*	*0.039*	*0.017*	*0.112*	*0.124*	*0.240*
CSC	-0.908	0.940	0.935	0	*0.051*	*0.144*	*0.117*	*0.031*	*0.073*	*0.005*	*0.112*	*0.004*	*0.080*	*0.042*	*0.012*	*0.001*	*0.006*	*0.029*	*0.007*	*0.027*	*0.093*
CPC	-0.749	0.999	0.999	0.949	0	*0.026*	*0.016*	*0.003*	*0.018*	*0.076*	*0.013*	*0.056*	*0.006*	*0.000*	*0.108*	*0.037*	*0.027*	*0.014*	*0.093*	*0.102*	*0.223*
CSTT	-0.606	0.981	0.983	0.856	0.974	0	*0.002*	*0.047*	*0.028*	*0.176*	*0.002*	*0.144*	*0.011*	*0.032*	*0.226*	*0.123*	*0.105*	*0.055*	*0.207*	*0.224*	*0.338*
CPTT	-0.647	0.990	0.992	0.883	0.984	0.998	0	*0.032*	*0.019*	*0.146*	*0.000*	*0.117*	*0.005*	*0.020*	*0.192*	*0.098*	*0.082*	*0.040*	*0.175*	*0.192*	*0.303*
DNAD	0.784	-0.993	-0.992	-0.969	-0.997	-0.953	-0.968	0	*0.028*	*0.055*	*0.029*	*0.039*	*0.018*	*0.002*	*0.081*	*0.021*	*0.012*	*0.013*	*0.068*	*0.070*	*0.198*
TC	0.765	-0.988	-0.986	-0.927	-0.982	-0.972	-0.981	0.972	0	*0.083*	*0.018*	*0.061*	*0.007*	*0.016*	*0.121*	*0.062*	*0.058*	*0.010*	*0.110*	*0.165*	*0.184*
TAG	0.944	-0.916	-0.908	-0.995	-0.924	-0.824	-0.854	0.945	0.917	0	*0.141*	*0.002*	*0.102*	*0.065*	*0.004*	*0.011*	*0.021*	*0.037*	*0.003*	*0.045*	*0.054*
VLDL	0.654	-0.992	-0.993	-0.888	-0.987	-0.998	-1.000	0.971	0.982	0.859	0	*0.112*	*0.004*	*0.018*	*0.186*	*0.093*	*0.077*	*0.037*	*0.168*	*0.185*	*0.297*
HDL	-0.925	0.938	0.931	0.996	0.944	0.856	0.883	-0.961	-0.939	-0.998	-0.888	0	*0.077*	*0.046*	*0.011*	*0.007*	*0.015*	*0.023*	*0.008*	*0.050*	*0.066*
LDL	0.717	-0.997	-0.997	-0.920	-0.994	-0.989	-0.995	0.982	0.993	0.898	0.996	-0.923	0	*0.008*	*0.142*	*0.065*	*0.054*	*0.017*	*0.127*	*0.154*	*0.238*
Tes	-0.768	0.998	0.997	0.958	1.000	0.968	0.980	-0.998	-0.984	-0.935	-0.982	0.954	-0.992	0	*0.095*	*0.031*	*0.022*	*0.010*	*0.082*	*0.094*	*0.204*
MDA	0.961	-0.881	-0.872	-0.988	-0.892	-0.774	-0.808	0.919	0.879	0.996	0.814	-0.989	0.858	-0.905	0	*0.021*	*0.034*	*0.064*	*0.001*	*0.039*	*0.048*

CAT	-0.887	0.954	0.949	0.999	0.963	0.877	0.902	-0.979	-0.938	-0.989	-0.907	0.993	-0.935	0.969	-0.979	0	*0.002*	*0.023*	*0.014*	*0.028*	*0.112*
SOD	-0.858	0.964	0.961	0.994	0.973	0.895	0.918	-0.988	-0.942	-0.979	-0.923	0.985	-0.946	0.978	-0.966	0.998	0	*0.022*	*0.025*	*0.029*	*0.140*
GST	-0.834	0.987	0.983	0.971	0.986	0.945	0.960	-0.987	-0.990	-0.963	-0.963	0.977	-0.983	0.990	-0.936	0.977	0.978	0	*0.055*	*0.099*	*0.134*
GSH	-0.950	0.896	0.888	0.993	0.907	0.793	0.825	-0.932	-0.890	-0.997	-0.832	0.992	-0.873	0.918	-0.999	0.986	0.975	0.945	0	*0.032*	*0.058*
HA	0.867	-0.880	-0.876	-0.973	-0.898	-0.776	-0.808	0.930	0.835	0.955	0.815	-0.950	0.846	-0.906	0.961	-0.972	-0.971	-0.901	-0.968	0	*0.167*
TA	0.989	-0.773	-0.760	-0.907	-0.777	-0.662	-0.698	0.802	0.816	0.946	0.703	-0.934	0.762	-0.796	0.952	-0.888	-0.860	-0.866	-0.942	0.833	0

Correlation (r) value is shown in regular font while p-value is shown in italics font. Statistically significant values between variables are shown as highlighted in regular font ($p < 0.05$).**LI** Lee index, **SM** Sperm motility, **DSP** Daily sperm production, **CSC** Cauda sperm concentration, **CPC** Caput sperm concentration, **CSTT** Cauda sperm transit time, **CPTT** Caput sperm transit time, **DNAD** DNA damage score, **TC** Serum total Cholesterol, **TAG** Serum triacylglycerides, **VLDL** Very low density lipoprotein, **HDL** High density lipoprotein, **LDL** Low density lipoprotein, **Tes** Serum testosterone, **MDA** Lipid peroxidation (malondialdehyde levels), **CAT** Catalase, **SOD** Super oxide dismutase, **GST** Glutathione S transferase, **GSH** Reduced glutathione, **HA** Sperm head abnormalities, **TA** Sperm tail abnormalities.

5.2.10. MANOVA: PCA, cluster analysis, DCA

FPF diet fed rats demonstrated a significant increase in blood glucoselevel and reduction in rectal body temperature,which is evidenced from the negative correlation and regression relationship observed between fasting serum blood glucoselevel and rectal body temperature (adjusted r^2 = 0.7486; y = 103.07 – 0.03579x, **FIG 16**).

The PCA loading plot generated two principal component axes based on thelong-term FPF feeding effects on oxidative stress, inflammation, redox imbalance, germ cell apoptosis, steroidogenesis and testicular dysfunction. PCA 1 accounted for 97.89% variance and eigenvalue of 28157.6resolved mainly by FPF diet and PCA 2 with 1.84% variance and eigenvalue of 531.69 determined by the control dietindicating oxidative stress and inflammatory mediated deregulation oftesticular germ cell apoptosis and steroidogenesis is critical in male reproductive impairment due to FPF dietinduced obesity.The remote and secluded variables in the plot,i.e., FPF-H, FPF-M, and FPF-L diet, indicate a significantcorrelation between the variables (**FIG 16).**

The cluster analysis (Euclidean similarity distance measure) separatedthe FPF diet groups into two major clusters according to their fat content and high calories in diet with the no protection high-risk group (FPF-H diet treated rats) inone sub-cluster under cluster group 1, medium-risk group(FPF-M and FPF-L diet treated rats)in the second sub-cluster of cluster group 1, and no risk control diet group by the cluster group 2. The cluster analysis clearly visualized an intimate linkage between FPF-induced oxidative stress, inflammation, defects inspermatogenesis, dysregulationof steroidogenesis and apoptosis as the potential causes (**FIG 16**).

The detrended analysis eliminates the effects of accruingdata sets from a course and predicting only the absolutedeviations in values and to permit possible cyclical arrays to berecognized. The DCA plot alienatedthe FPF dietin a hierarchicalfashion as FPF-H, FPF-M, and FPF-L based onFPF diet induced testicular oxidative stress, inflammation, germ cell apoptosis, and disrupted steroidogenesis and spermatogenesis in rats (**FIG 16**).

The PCA scoreplot, Euclidean distance measure, and DCAanalyses showed a distinctive separation of FPF-H, FPF-M, and FPF-L dietvariables in a hierarchicalfashion, indicating a significantvariation between the FPFdiets in relation to high calorie, fat content and FAME levels and clearly visualized an intimate linkage between FPF-diet inducedmetabolic syndrome,oxidative stress, and inflammation are the potential causes for obesity, dysregulationof steroidogenesis and apoptosis, impairment in spermatogenesis, and sperm dysfunction in rats upon long-term FPF-diet feeding(**FIG 16 B and D**).

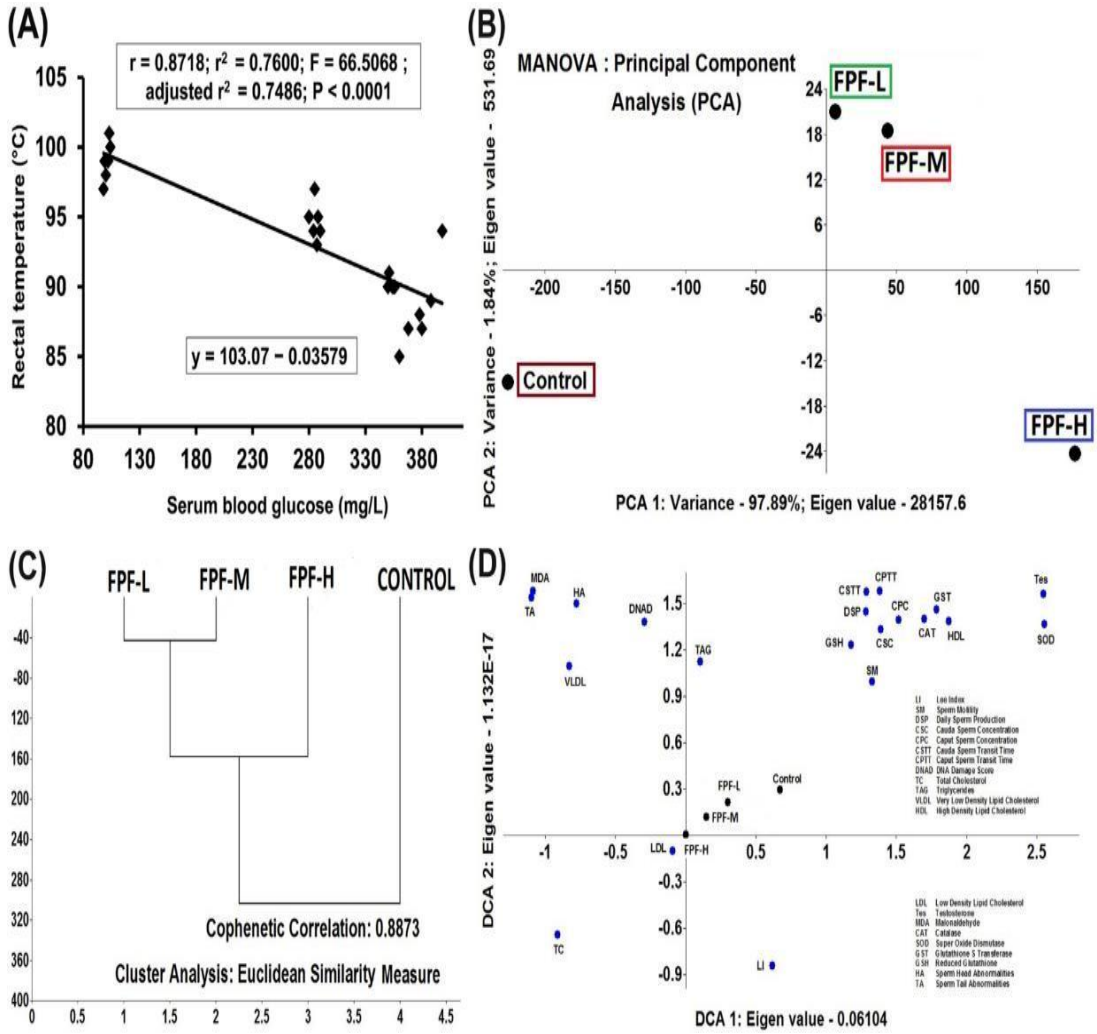

FIG 16: Correlation and regression and multivariate (MANOVA) analyses. **A** Relationship between serum blood glucose level and rectal temperature in fermented pork fat (FPF) supplemented adult rats. **B** Assessment of inhibitory potential of FPF in male adult rats by principal component analysis (PCA). **C** Euclidean similarity measure by cluster analysis to catalogue the FPF mediated testicular dysfunction in adult rats. **D** Detrended correspondence analysis (DCA) differentiates the FPF diet mediated effects in a hierarchical fashion predicting the male reproductive impairment. **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF-L diet** Fermented pork low fat diet - protein-20%, carbohydrate-70%, fat-10% and total energy-14.21 kJ/g, **FPF-M diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total

energy-17.5 kJ/g, **FPF-H diet** Fermented pork high fat diet - protein-20%, carbohydrate-20%, fat-60% and total energy-23 kJ/g. **LI** Lee index, **SM** Sperm motility, **DSP** Daily sperm production, **CSC** Cauda sperm concentration, **CPC** Caput sperm concentration, **CSTT** Cauda sperm transit time, **CPTT** Caput sperm transit time, **DNAD** DNA damage score, **TC** Serum total Cholesterol, **TAG** Serum triacylglycerides, **VLDL** Very low density lipoprotein, **HDL** High density lipoprotein, **LDL** Low density lipoprotein, **Tes** Serum testosterone, **MDA** Lipid peroxidation (malondialdehyde levels), **CAT** Catalase, **SOD** Super oxide dismutase, **GST** Glutathione S transferase, **GSH** Reduced glutathione, **HA** Sperm head abnormalities, **TA** Sperm tail abnormalities

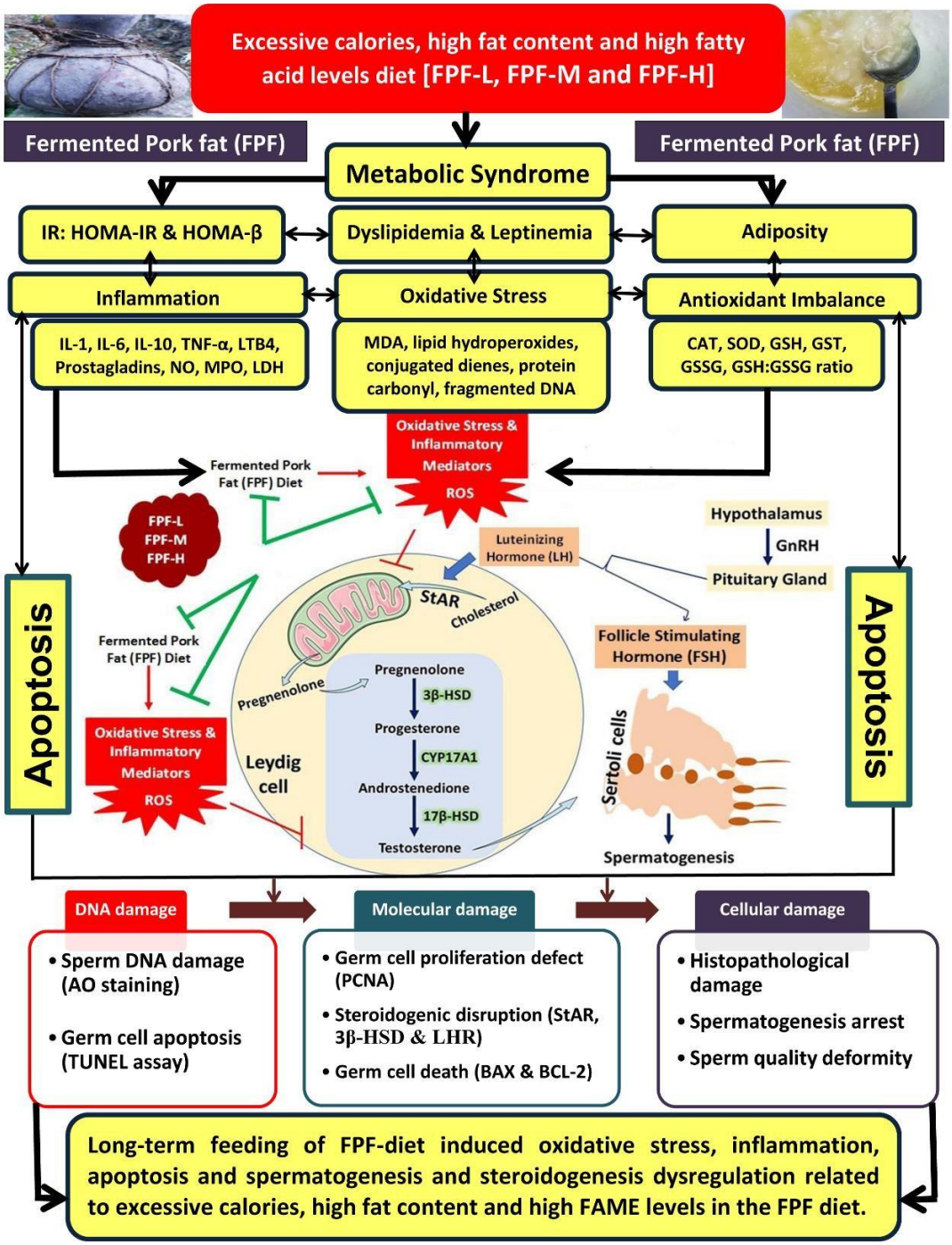
Excessive calories, high fat content and high fatty acid levels diet [FPF-L, FPF-M and FPF-H]
Fermented Pork fat (FPF)
Fermented Pork fat (FPF)
Metabolic Syndrome
IR: HOMA-IR & HOMA-β
Dyslipidemia & Leptinemia
Adiposity
Inflammation
Oxidative Stress
Antioxidant Imbalance
IL-1, IL-6, IL-10, TNF-α, LTB4, Prostagladins, NO, MPO, LDH
MDA, lipid hydroperoxides, conjugated dienes, protein carbonyl, fragmented DNA
CAT, SOD, GSH, GST, GSSG, GSH:GSSG ratio
Oxidative Stress & Inflammatory Mediators
ROS
Fermented Pork Fat (FPF) Diet
FPF-L
FPF-M
FPF-H
Hypothalamus
GnRH
Pituitary Gland
Luteinizing Hormone (LH)
StAR
Cholesterol
Pregnenolone
Pregnenolone
3β-HSD
Progesterone
CYP17A1
Androstenedione
17β-HSD
Testosterone
Leydig cell
Follicle Stimulating Hormone (FSH)
Sertoli cells
Spermatogenesis
Apoptosis
Apoptosis
DNA damage
• Sperm DNA damage (AO staining)
• Germ cell apoptosis (TUNEL assay)
Molecular damage
• Germ cell proliferation defect (PCNA)
• Steroidogenic disruption (StAR, 3β-HSD & LHR)
• Germ cell death (BAX & BCL-2)
Cellular damage
• Histopathological damage
• Spermatogenesis arrest
• Sperm quality deformity
Long-term feeding of FPF-diet induced oxidative stress, inflammation, apoptosis and spermatogenesis and steroidogenesis dysregulation related to excessive calories, high fat content and high FAME levels in the FPF diet.

FIG 17: Schematic representation of long-term feeding of pork fermented fat (FPF) diet leading to metabolic syndrome, oxidative stress, inflammation, testicular atrophy, sperm defects, impairment in germ cell proliferation, dysregulation of steroidogenesis and germ cell apoptosis in rats

5.3. Alleviative effect of PEITC on HFD induced reproductive toxicity

5.3.1. Long-term FPF feeding afflicted anthropometrical and nutritional measures and alleviation of PEITC

Body weight gain (7.8% of initial weight), organ weights (especially wet weight of liver and fat) are the indices to assess obesity and food and calorie intake determine the high fat diet induced obesity. FPF diet-fed rats showed a significant increase in food consumption (fold), water consumption (fold), bodyweight (1.2 – 2.34 fold), sperm motility (fold), blood glucose level (fold) and rectal temperature (fold) **(FIG 18 A-F)** respectively and liver (1.07 – 1.28 fold) weight, body fat mass distribution epididymal (1.01 – 2.73 fold), retroperitoneal (1.08 – 5.44 fold), perirenal (1.05 – 2.66 fold), m3esenteric (2.28 – 3.11 fold), and dorsal sub-cutaneous fat (1.21 – 3.05 fold)], body mass index (BMI, 1.02 – 2.23 fold), specific rate of body mass gain (3.82 – 7.77 fold), energy intake (1.44 – 2.69 fold), feed efficiency (1.36 – 3.45 fold) (**Table 25**), whereas, decrease in the kidney (0.21 – 0.25 fold), testis (0.14 – 0.94 fold) were observed compared with the control diet and PEITC groups. Increment in the absolute and relative liver weights, fasting blood glucose level, body fat mass, Lee index and decrement in the absolute and relative kidney and reproductive organ weights, rectal temperature in the FPF diet groups compared with the control diet further evidenced the high fat diet induced obesity and its pathological processes ameliorated by PEITC **(Table25).**

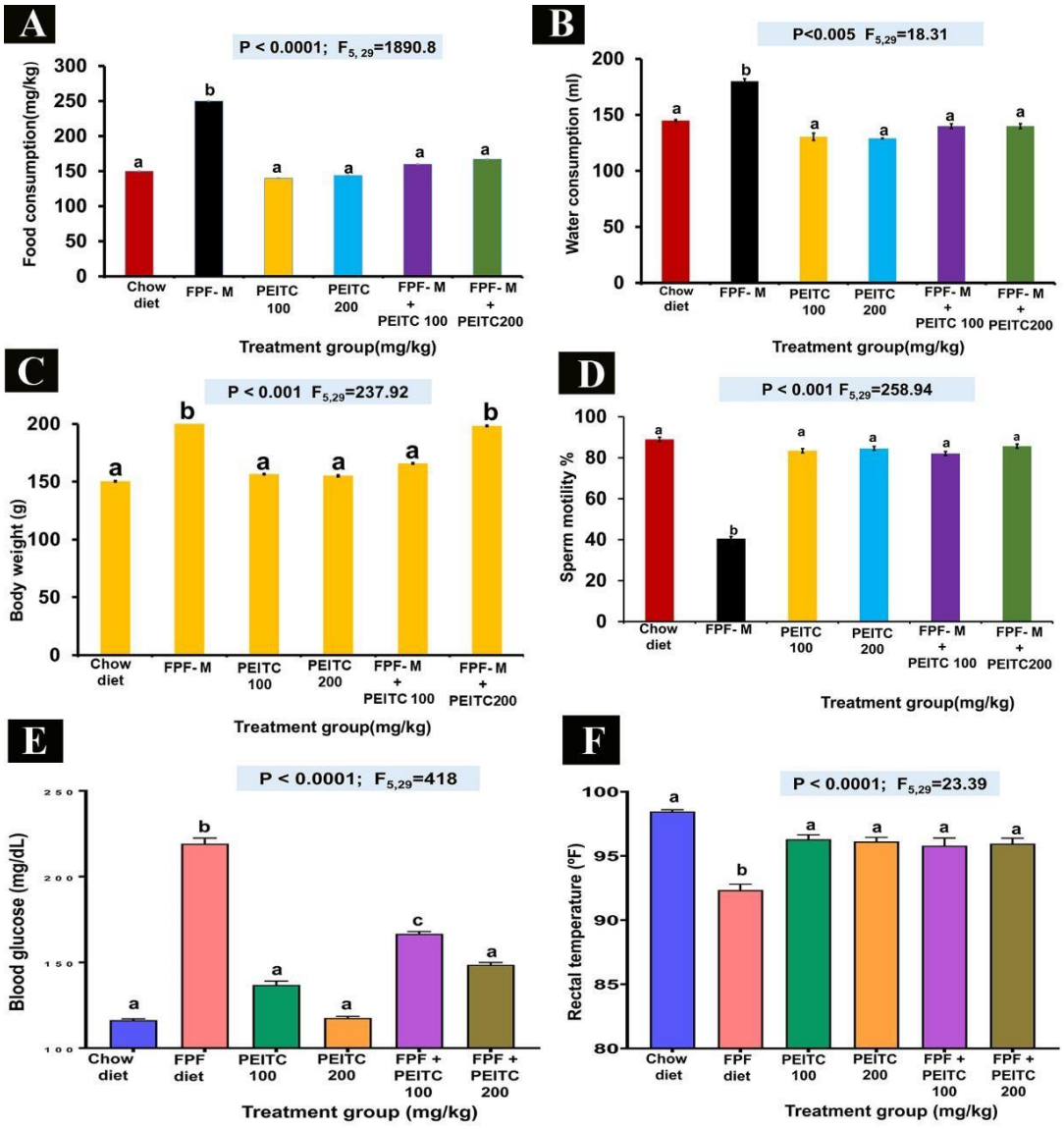

FIG 18: Long-term FPF feeding afflicted anthropometrical and nutritional measures **(A-F)**

A) Food consumption(mg/kg) **B)** Water consumption (ml) **C)** Body weight(g) **D)** Sperm motility% **E)** Blood glucose (mg/dL) **F)** Rectal temperature (°F)

Table 25: Long term feeding effects of fermented pork fat diet on body and organ weights, body fat mass distribution and food and calorie intake nutritional parameters in male Wistar albino rats

	Experimental groups							
Parameters	**Control-Diet**	**FPF-M**	**P100**	**P200**	**FPF-M+ P100**	**FPF-M+ P200**	**F 5,29**	**P value**
Body weight(g)	150.20 ± 0.30a	230.00 ± 0.30b	156.30 ± 0.20c	142.60 ± 0.40d	165.80 ± 0.20e	198.20 ± 0.40f	11701	<0.0001
Testis (g)	0.70 ± 0.03a	0.30 ± 0.12b	0.60 ± 0.09c	0.55 ± 0.03d	0.42 ± 0.09e	0.33 ± 0.02f	42.612	<0.0001
Testis (g/100g)	0.65 ± 0.03a	0.53 ± 0.03b	0.43 ± 0.07c	0.37 ± 0.03d	0.28 ± 0.05e	0.22 ± 0.04f	13.019	<0.0001
Liver (g)	8.12 ± 0.05a	7.56 ± 0.42b	7.26 ± 0.04c	7.12 ± 0.18c	7.12 ± 0.44c	6.33 ± 0.08d	5.037	<0.0001

Liver (g/100g)	6.33 ± 0.08a	5.83 ± 0.02b	5.63 ± 0.28c	5.44 ± 0.04d	5.30 ± 0.03d	5.38 ± 0.14d	8.126	<0.0001
Kidney (g)	0.42 ± 0.03a	0.36 ± 0.05b	0.36 ± 0.05b	0.28 ± 0.02c	0.28 ± 0.05c	0.26 ± 0.01d	2.661	<0.0001
Kidney (g/100g)	0.28 ± 0.04a	0.27 ± 0.03b	0.26 ± 0.03c	0.21 ± 0.04d	0.21 ± 0.04d	0.14 ± 0.08e	0.42	<0.82
Seminal vesicles (g)	0.36 ± 0.04a	0.30 ± 0.04b	0.30 ± 0.04b	0.28 ± 0.05c	0.26 ± 0.04d	0.20 ± 0.02e	1.79	<0.0001
Seminalvesicles (g/100g)	0.24 ± 0.04a	0.23 ± 0.03b	0.22 ± 0.03c	0.21 ± 0.02d	0.21 ± 0.03d	0.19 ± 0.03e	0.32	<0.001
Vas deferens (mg)	0.20 ± 0.04a	0.16 ± 0.04b	0.16 ± 0.04b	0.14 ± 0.03c	0.13 ± 0.04d	0.12 ± 0.01e	3.58	<0.0001
Vas deferens	0.14	0.12	0.11	0.14	0.11	0.10	0.35	<0.0001

(g/100g	± 0.04a	± 0.02b	± 0.02c	± 0.02d	± 0.02c	± 0.04a		
Cauda (g)	0.22 ± 0.03a	0.20 ± 0.04b	0.18 ± 0.04c	0.18 ± 0.03c	0.14 ± 0.03d	0.12 ± 0.01e	3.94	<0.0001
Cauda (g/100g)	0.15 ± 0.03a	0.14 ± 0.12b	0.13 ± 0.03c	0.17 ± 0.02d	0.13 ± 0.03c	0.11 ± 0.04a	0.13	<0.98
Caput (g)	0.26 ± 0.04a	0.23 ± 0.04b	0.18 ± 0.04c	0.18 ± 0.03c	0.16 ± 0.04d	0.14 ± 0.05e	1.23	<0.32
Caput (g/100g)	0.28 ± 0.04a	0.23 ± 0.03b	0.22 ± 0.02c	0.14 ± 0.02d	0.11 ± 0.02e	0.10 ± 0.04e	6.48	<0.06
ody fat mass distribution								
Epididymal fat (mg/g)	1.63 ± 0.03a	4.45 ± 0.04b	1.78 ± 0.01c	1.61 ± 0.02a	3.76 ± 0.04d	3.12 ± 0.01e	1400	<0.0001
Retroperitoneal	1.23	6.67	2.11	1.34	2.89	2.11	4920	<0.0001

fat (mg/g)	± 0.01a	± 0.03b	± 0.02c	± 0.04a	± 0.02d	± 0.04c		
Perirenal fat (mg/g)	1.12 ± 0.04a	4.89 ± 0.01b	2.65 ± 0.03c	1.65 ± 0.02d	2.76 ± 0.02e	2.54 ± 0.04c	2008.5	<0.0001
Mesenteric fat (mg/g)	0.18 ± 0.02a	3.86 ± 0.02b	1.89 ± 0.01c	1.22 ± 0.03d	2.12 ± 0.02e	1.98 ± 0.02c	3375.5	<0.0001
Dorsal sub-cutaneous fat (mg/g)	0.23 ± 0.04a	1.87 ± 0.02b	0.46 ± 0.04c	0.44 ± 0.03c	1.01 ± 0.01d	0.67 ± 0.03c	386.97	<0.0001
Food and calorie intake nutritional parameters								
Body mass index (BMI, g/cm^2)	5.54 ± 0.04a	6.77 ± 0.03b	5.34 ± 0.03a	5.42 ± 0.02a	6.20 ± 0.04b	5.80 ± 0.04c	259.26	<0.0001
Specific rate of body mass gain (g/kg)	5.77 ± 0.03a	48.99 ± 0.02b	33.89 ± 0.04c	32.47 ± 0.03d	36.55 ± 0.03e	35.78 ± 0.03f	21752	<0.0001
Energy intake	1014.50	28892.00	1019.80	1000.90	1234.60	1218.60	1.136	<0.0001

(kJ/day)	± 0.04a	± 0.03b	± 0.03c	± 0.04d	± 0.03e	± 0.03f		
Feed efficiency (FE; %)	14.80 ± 0.02a	7.96 ± 0.03b	15.32 ± 0.02c	14.24 ± 0.04a	13.42 ± 0.03d	16.26 ± 0.04e	9041.2	<0.0001

Datas are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Datamarked with a,b,c,d,e,f are statistically significant ($p< 0.0001$,$p<0.005$,$p<0.001$) **Relative organ weight**(g/100g) = {[organ weight/body weight] × 100},**BMI** = body weight (g) / length^2 (cm^2), **Specific rate of body mass gain** (g/kg) =dM / M dt, where dM represents the gain of body weight during dt = t_2 - t_1 and M is the rat body weight at t_1,**Energy intake** (kJ/day) = mean food consumption x dietary metabolizable energy, **Feed efficiency** (FE; g gained/kcal) = (mean body weight gain (g) 100) / total energy intake (kcal) **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **P** Phenethyl isothiocyanate-100mg/kg and 200mg/kg

5.3.2. Long term feeding effects on Sperm morphology and sperm parameters

Sperm morphology analysis revealed a significant increase in sperm abnormalities in rats treated for 60 days. Sperm were classified as normal and abnormal sperms i.e., tail (amorphous, banana, detached) abnormality and head (coil or broken) abnormality. As shown in **Table 26**, the normal sperms were found maximum in control groups and abnormality increased with FPF diets treated groups .Normal phenotypes in FPF diets compared to control (1.05-1.44 fold), total head abnormalities (1.25-3.11 fold) and total tail abnormalities (1.22-4.66 fold) were observed in **(Table 26**)

Long-term feeding of FPF diets triggered sperm dysfunction not only reducing sperm number, but also diminishing the viable and motile spermatozoa. A significant decrease ($p< 0.05$) was observed in the sperm functional indices in terms of sperm number (1.03-1.24 fold), daily sperm production (1.14-1.88), sperm concentration (cauda: 1.19-1.56 and caput: 1.35-1.55) in the FPF diets fed rat groups compared to the control.

Table 26: Long-term feeding effects of fermented pork fat diet on sperm morphology of rats and amelioration of PEITC

Sperm morphology	Control-Diet	FPF-M	P 100	P200	FPF-M+ P100	FPF-M+ P200	$F_{5,29}$	P value
Sperm morphology – Normal (%)								
Normal phenotypes	96.75 ± 0.05a	66.7 ± 0.04b	90.59 ± 0.04c	92.07 ± 0.04d	71.01 ± 0.06e	91.84 ± 0.06c	65941	<0.0001
Sperm morphology - Head abnormality (%)								
Amorphous	1.08 ± 0.05a	7.6 ± 0.04b	2.52 ± 0.05c	2.02 ± 0.06d	2.82 ± 0.04c	6.82 ± 0.06e	2861.7	<0.0001
Banana head	1.25 ± 0.06a	7.99 ± 0.04b	1.38 ± 0.02c	1.66 ± 0.06d	4.63 ± 0.11e	1.26 ± 0.12c	1279.4	<0.0001
Detached head	1.08 ± 0.05a	5.78 ± 0.05b	3.79 ± 0.03c	2 ± 0.07d	4.96 ± 0.01e	1.11 ± 0.07a	1546.8	<0.0001
Total of head abnormalities	3.41 ± 0.03a	21.37 ± 0.04b	7.69 ± 0.03c	5.68 ± 0.03d	12.41 ± 0.03e	9.19 ± 0.06f	27703	<0.0001
Sperm morphology - Tail abnormality (%)								
Coiled tail	0.91 ± 0.04a	2.77 ± 0.06b	1.54 ± 0.04c	1.83 ± 0.03d	5.4 ± 0.01e	2.26 ± 0.08b	1050	<0.0001
Broken tail	0.96 ± 0.03a	9.94 ± 0.04b	1.5 ± 0.04c	1.37 ± 0.06d	6.39 ± 0.03e	1.31 ± 0.07c	6184.3	<0.0001
Total of tail abnormalities	1.54 ± 0.04 a	12.71 ± 0.03b	3.04 ± 0.04c	3.2 ± 0.03c	11.79 ± 0.03d	3.57 ± 0.02e	23037	<0.0001

Datas were presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p< 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p> 0.05$. The sperm abnormality was weighed as percent abnormality = {[No. of abnormal sperm/Total no. of sperm] × 100} **Control diet** - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF diet** Fermented pork fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g **P** Phenethyl isothiocyanate-100mg/kg and 200mg/kg

Table 27: Long-term feeding effects of fermented pork fat diet on sperm parameters of rats

Parameters	**Experimental groups**							
Sperm count	**Control- diet**	**FPF-M**	**P100**	**P200**	**FPF-M+ P100**	**FPF-M+ P200**	**$F_{5,29}$**	**P value**
Sperm number ($\times 10^6$)	146.20 ± 1.22a	117.50 ± 3.12b	125.20 ± 1.08c	138.30 ± 4.60d	121.70 ± 4.14e	141.40 ± 2.49f	14.56	0.0001
Sperm number ($\times 10^6$/g)	368.47 ± 9.84a	205.16 ± 4.22b	207.21 ± 6.58c	297.22 ± 7.22d	206.81 ± 5.10e	313.14 ± 4.59f	113.67	0.0001
DSP ($\times 10^6$/testis/day)	27.98 ± 0.54a	14.83 ± 0.56b	24.26 ± 0.08c	23.38 ± 0.75d	18.84 ± 0.43e	24.52 ± 0.44f	86.31	0.0001
DSPr ($\times 10^6$/testis/day/g)	69.44 ± 16.30a	25.60 ± 2.11b	41.01 ± 3.37c	36.68 ± 0.32d	32.11 ± 2.53e	50.15 ± 4.38f	32.99	0.0001
Epididymis								
Caput								
Sperm number ($\times 10^6$)	753.2 ±	483.00 ±	655.80 ±	728.48 ±	515.67 ±	554.31 ±	62.31	0.0001

	15.36a	16.45b	16.87c	15.70d	14.22e	2.11f		
Sperm number (×10⁶/g)	341.3 ± 31.04a	88.86 ± 2.40b	212.00 ± 50.50c	236.90 ± 45.26d	97.27 ± 18.31e	201.93 ± 9.83f	8.86	0.0001
Sperm transit time (days)	1.8 ± 0.06a	1.48 ± 0.02b	1.47 ± 0.01b	1.29 ± 0.06c	1.11 ± 0.06d	1.11 ± 0.02d	35.72	0.0001
Cauda								
Sperm number (×10⁶)	756.20 ± 10.05a	483.00 ± 6.45b	629.80 ± 6.07c	589.34 ± 7.56d	532.98 ± 6.11e	632.20 ± 9.41f	147.40	0.0001
Sperm number (×10⁶/g	389.20 ± 2.82a	79.57 ± 1.44b	216.50 ± 4.32c	214.50 ± 4.32d	81.60 ± 1.23e	254.10 ± 3.55f	1318.2	0.0001
Sperm transit time (days)	1.94 ± 0.08a	1.35 ± 0.01b	1.64 ± 0.08c	1.45 ± 0.05b	1.37 ± 0.08b	1.87 ± 0.08a	13.94	0.0001

Datas are presented as mean ± standard error mean (n = 5 rats/treatment). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Datamarked with symbols are statistically significant ($p< 0.0001$,$p<0.005$,$p<0.001$). **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **P**Phenethylisothiocyanate-100mg/kgand200mg/kg

5.3.3. Long-term FPF feeding altered serum FAME profiles and amelioration of PEITC

Serum SFA (lauric, myristic, palmitic, stearic acid: 1.99 – 3.32 fold) and PUFA-ω6 (linoleic, γ-linolenic, arachidonic acid: 1.48 – 2.66 fold) levels were increased significantly while MUFA (myristic, palmitic, palmitoleic, stearic, oleic acid: 0.11 – 0.44fold) and PUFA-ω3 (α-linolenic, eicosatrienoic,and eicosapentaenoic acid: 0.11 – 0.54 fold) levels were decreased significantly in the serum of rats fed with FPF-diets and PEITC compared to the control-diet group **(Table 28)** implicated with the induction of obesity and onset of chronic inflammation and oxidative stress in rats. Our study demonstrated that the n-6:n-3 PUFA ratio levels were increased significantly in the FPF-diets to the tune of 2,01 – 2.69 fold, which is associated with dietary intake-mediated change in the production of inflammatory mediators and regulators toward a pro-inflammatory profile.

5.3.4. Long-term feeding of FPF diets instigated insulin resistance, altered hepato-renal function markers, elevated serum lipid profles, activated testis oxidative stress and depleted of antioxidant reserve

Compared to the control-diet group, FPF-diet groups showed significant elevation in serum glucose (1.21 – 5.02 fold) and insulin (1.11 – 4.02 fold) levels induced by FPF-diets further redirected toward insulin resistance and pancreatic defect in β-cell function as indicated by a significant increase in the HOMA-IR (4.49 –99.41 fold) and a reduction in HOMA-β (5.44 – 6.34 fold) than the control-diet group **(Table28).**

Serum ALT (1.66 – 3.56 fold), AST (2.34 – 4.55 fold), ALP (1.89 – 4.23 fold), urea (2.11 – 6.34 fold), creatinine (2.12 – 3.36 fold), AST:ALT ratio (1.13 – 2.12 fold) and BUN: Creatinine ratio (0.89 – 1.56 fold) were significantly increased in the three FPF-diet groups compared with the control-diet group because of metabolic syndrome resulting in hepatic and renal damage **(Table 28).**

The serum TC, TAG, VLDL, LDL and leptin levels in the serum of rats fed a FPF-diets increased significantly by 3.42 – 3.92, 3.37 – 4.00, 34.29 – 47.27, 5.86 – 7.02fold, respectively, and HDL level (0.84 – 0.45 fold) decreased compared with the control-diet group, indicating disturbance in lipid metabolism like hypercholesterolemia and hyperleptinemia which are associated with the pathogenesis of insulin resistance, obesity, atherosclerosis and cardiovascular disease by elevation in cardiovascular risk factors (CI, AI and CAI: 4.00 –8.75, 5.98 – 13.34, 6.97 – 15.39 fold, respectively**) (Table 28).**

Long-term feeding of FPF diets to rats for 90 consecutive days induced oxidative stress in the testis validated with a significant increase in the conjugated dienes (1.66 – 2.05 fold), lipid hydroperoxides (2.32 – 2.69 fold), malondialdehyde (2.15 – 3.05 fold), protein carbonyl (2.02 – 4.39 fold) and fragmented DNA (8.95 – 13.82 fold)concentration in the testis compared with the control diet (Table 2).The enzymatic (CAT:0.54 – 0.87 fold, SOD:0.26 – 0.78 fold, GST:0.53 – 0.78 fold) and non-enzymatic (GSH: 0.27 – 0.72 fold, Table 2) antioxidant reserves were depleted drastically in the FPF diets compared to the control diet, substantiating antioxidant enzymes are responsible for reducing the redox imbalance and these antioxidants were suppressed due to the intensification of oxidative stress in testis caused by FPF diet supplementation. Further, GSH:GSSG ratio (reduced-to-oxidized glutathione ratio) is a benchmark of the mitochondrial or cellular redox state and observed that the GSH level is significantly lower (0.27 – 0.72 fold), but that the GSSG level is higher (4.03 – 7.68 fold) in FPF diet groups, signifying that oxidative stress in the testis occurs in groups supplemented with dietary FPF than in a control diet. The increased GSH:GSSG in the testis of rats fed a FPF diet persuade the rats to oxidative stress **(Table 28).**

The PCNA ratio decreased significantly up to (33.23-77.55) in FPF diet groups than the control indicated the inhibition of germ cell proliferation in testis tissue and testis atrophy **(Table 28).** Further, FPF diet contributed to defective spermatogonial differentiation that was reflected as a significant decrease in the TDI (53.44 – 55.43), the status of type B spermatogonial differentiation into spermatocyte and spermatids) compared with the control diet **(Table 28).** Intense BAX immunostaining (6.77 – 23%), and BAX/BCL-2 (0.08 – 0.57)

immunoreactivity ratios **(Table 28**) were observed in the testis of FPF-fed rats, whereas mild to faint BCL-2 (40.12-81.22,), StAR (48.76-68.54O), 3β-HSD (35-44.08), and LHR (21.89-33.44) immunostaining. The immunoreactivity ratio was significantly decreased in BCL-2 (37.22 – 76.32), StAR (37.92 – 73.85), 3β-HSD (26.28 – 56.12), and LHR (30.11 – 96.16) compared with the control groups defining germ cells, Leydig cells, and Sertoli cells undergoing cell death, inhibition of cholesterol transfer within mitochondria leading to testis apoptosis and disruption of spermatogenesis and steroidogenesis.

FPF-diets promoted inflammation, which is evidenced through a significant increase in the serum and testis pro-inflammatory markers, including IL-1 (3.02 – 4.28 fold), IL-6 (1.71 – 2.92 fold), LTB4 (1.22 – 1.58 fold), prostaglandin (1.38 – 2.09 fold), NO (2.15 – 4.17 fold), MPO (4.54 – 5.21 fold), LDH (2.34 – 3.77 fold) and TNF-α (2.02 – 2.51 fold) and a significant decline in the anti-inflammatory marker, IL -10(0.38 – 0.66 fold) compared with the control-diet (**Table 28).**

Table 28: Long-term feeding effects of fermented pork fat diet (Sa-um) on hepatic insulin resistance, liver and kidney function and lipid profiles, serum fatty acid methyl esters composition and testicular inflammatory, oxidative stress, apoptotic and steroidogenic markers in male Wistar albino rats and amelioration of PEITC

Parameters	CHOW-Diet	FPF-M	P100	P200	FPF- M+ P100	FPF-M+ P200	$F_{5,29}$	P value
Hepatic insulin resistance profiles								
Serum insulin level (ng/mL)	0.35 ± 0.02a	6.89 ± 0.03b	1.41 ± 0.12c	0.39 ± 0.05a	1.37 ± 0.03d	1.32 ± 0.05d	1690.1	<0.0001
Serum glucose level (mg/dL)	100.20 ± 0.46a	503.20 ± 1.25b	111.3 ± 5.05c	109.3 ± 2.356d	212.9 ± 1.21e	121.3 ± 2.12f	3840.9	<0.0001
Homeostasis model assessmentof insulin resistance index (HOMA-IR)	1.55 ± 0.21a	154.09 ± 1.32b	6.97 ± 1.05bc	1.89 ± 1.05cd	12.96 ± 0.27e	7.11 ± 0.15f	5384.8	<0.0001
Pancreatic β-cell function (HOMA-β, %)	7.23 ± 0.30a	27.57 ± 0.10b	26.15 ± 0.22c	7.37 ± 0.32d	13.08 ± 0.44e	22.41 ± 0.21f	1050.7	0.0002
Liver and kidney function profiles								
Alanine aminotransferase (ALT, IU/mL)	13.15 ± 3.86a	59.89 ± 2.06b	22.88 ± 3.36c	17.89 ± 0.83d	28.85 ± 0.98e	24.55 ± 1.22f	49.27	<0.0001
Aspartate aminotransferase (AST, IU/mL)	23.15 ±	119.56 ±	35.33 ±	25.67 ±	29.25 ±	28.89 ±	701.18	<0.0001

	1.26a	2.02b	1.21c	1.11d	0.21e	1.89e		
AST/ALT ratio	1.76 ± 0.02a	1.99 ± 0.01b	1.54 ± 0.01c	1.43 ± 0.03d	1.01 ± 0.03e	1.17 ± 0.08f	90.20	<0.0001
Alkaline phosphatase (ALP,IU/L)	78.65 ± 2.04a	376 ± 2.02b	90.78 ± 1.20c	82.33 ± 1.05d	250.27 ± 3.02e	114.18 ± 0.55f	7302.00	<0.0001
Urea (mg/dL)	39.50 ± 1.92a	208.82 ± 2.06b	73.87 ± 1.15c	41.23 ± 1.20d	44.73 ± 1.37e	43.23 ± 2.11e	1567.5	<0.0001
Blood urea nitrogen (BUN, mg/dL)	20.02 ± 1.16a	87.52 ± 2.10b	31.31 ± 2.08c	21.90 ± 1.09d	38.25 ± 2.92e	32.09 ± 2.02f	154.58	<0.0001
Creatinine (mg/dL)	0.95 ± 0.05a	5.45 ± 0.10b	2.85 ± 0.03c	1.23 ± 0.25c	3.20 ± 0.04d	2.12 ± 0.03c	204.61	<0.0001
BUN/Creatinine ratio	21.07 ± 1.02a	16.05 ± 1.81b	10.98 ± 1.01c	17.80 ± 1.32d	11.95 ± 0.22e	15.13 ± 0.12 f	11.72	0.0014
Serum lipid profiles								
Total cholesterol (TC, mg/dL)	77.48 ± 1.38a	313.28 ± 1.22b	123.90 ± 1.46c	88.9 ± 2.72d	199.52 ± 0.98e	167 ± 1.21f	2991.30	<0.0001
Triacylglycerols (TAG, mg/dL)	48.90 ± 1.39a	183.71 ± 1.23b	56.77 ± 1.44c	50.99 ± 1.02d	154.98 ± 1.85e	113.4 ± 0.23f	2042.5	<0.0001
VLDL cholesterol (mg/dL)	9.78 ± 0.14a	36.74 ± 0.18b	11.35 ± 0.80c	10.19 ± 0.21d	30.99 ± 2.11e	22.68 ± 1.89f	106.91	<0.0001
HDL cholesterol (mg/dL)	34.02	13.23	27.89	30.44	29.89	27.65	27.96	<0.0001

	± 1.22a	± 1.17b	± 1.00c	± 1.36d	± 0.98d	± 2.12c		
LDL cholesterol (mg/dL)	33.68 ± 1.04a	263.31 ± 1.02b	84.66 ± 1.22c	48.27 ± 0.21d	138.64 ± 0.98e	116.67 ± 1.23f	6763.9	<0.0001
Leptin (ng/mL)	6.09 ± 0.19a	22.34 ± 2.11b	14.43 ± 1.21c	7.48 ± 1.30d	18.99 ± 1.21e	12.30 ± 1.80f	19.60	<0.0001
Cardiac index (CI)	2.27 ± 0.14a	23.67 ± 0.20b	4.44 ± 0.12c	2.92 ± 0.34a	6.67 ± 0.89d	6.02 ± 1.12e	172.74	<0.0001
Atherogenic index (AI)	1.27 ± 0.12a	22.67 ± 1.05b	3.44 ± 1.04c	1.92 ± 2.00a	5.67 ± 0.21d	5.03 ± 0.12e	61.72	<0.0001
Coronary artery index (CAI)	0.99 ± 0.10a	19.90 ± 1.00b	3.03 ± 1.03c	1.58 ± 1.20c	4.63 ± 0.60d	4.21 ± 0.22d	76.93	<0.0001
Serum fatty acid methyl esters composition (mg/g)								
Saturated fatty acids (SFA)								
C12, Lauric acid	13.31 ± 0.26a	99.18 ± 2.30b	24.45 ± 2.02c	17.22 ± 1.12a	42.26 ± 2.08d	33.44 ± 0.23e	398.60	<0.0001
C14, Myristic acid	16.33 ± 0.16a	82.33 ± 0.22b	46 ± 0.36c	22. 8 ± 0.89d	67.05 ± 1.21e	55.67 ± 1.08f	1049.1	0.0003
C16, Palmitic acid	138.09 ± 5.94a	377.04 ± 6.38b	177.89 ± 5.06c	151.22 ± 3.44d	284.41 ± 4.33e	276.08 ± 4.05f	355.14	<0.0001
C18, Stearic acid	51.22 ±	289.02 ±	83.44 ±	61.22 ±	137.23 ±	122 ±	388.15	<0.0001

	5.18a	4.22b	3.45c	6.02d	4.03e	3.06f		
Monounsaturated fatty acids (MUFA)								
C16:1, Palmitoleic acid, ω7	67.09 ± 0.89a	15.89 ± 1.26b	34.48 ± 2.11c	61.22 ± 1.22d	25.87 ± 3.18e	20.71 ± 0.89f	145.53	<0.0001
C18:1, Oleic acid, ω9	245.9 ± 4.16a	56.05 ± 3.95b	106.7 ± 6.35c	66.331 ± 2.31d	100.8 ± 3.34e	128.42 ± 3.58f	273.80	<0.0001
C18:1, Vaccenic acid, ω7	22.3 ± 1.38a	3.24 ± 0.10b	16.7 ± 0.23c	13.84 ± 1.21d	9.80 ± 0.92e	12.10 ± 1.33f	41.11	<0.0001
Polyunsaturated fatty acids (PUFA)								
C18:2, Linoleic acid, ω6	131.44 ± 3.23	314.8 ± 2.21b	141.22 ± 2.11c	136.77 ± 1.23d	230.85 ± 3.36e	175.38 ± 5.82f	471.10	<0.0001
C18:3, α-Linolenic acid, ω3	98.78 ± 0.84a	23.12 ± 0.21b	96.05 ± 2.12c	94.56 ± 2.12d	46.22 ± 0.28e	50.86 ± 2.11f	439.13	<0.0001
C18:3, γ-Linolenic acid, ω6	26.12 ± 1.50a	86.21 ± 2.12b	31.21 ± 2.10c	21.00 ± 0.89d	41.64 ± 1.75e	24.87 ± 0.56f	233.74	<0.0001
C20:3, Eicosatrienoic acid, ω3	42.33 ± 1.75a	7.99 ± 1.04b	24.78 ± 0.45c	32.00 ± 2.11d	16.38 ± 0.12e	24.89 ± 0.12f	96.86	<0.0001
C20:4, Arachidonic acid, ω6	30.00 ± 3.01a	85.12 ± 1.23b	41.81 ± 2.12c	30.33 ± 1.12d	61.44 ± 3.03e	46.88 ± 3.64c	68.89	<0.0001
C20:5, Eicosapentaenoic, ω3	22.11 ± 0.12a	4.33 ± 0.26b	17.22 ± 0.10c	16.55 ± 1.22d	12.33 ± 0.98e	18.02 ± 0.36f	84.45	<0.0001

SFA (mg/g)	218.95 ± 4.60a	847.57 ± 2.70b	331.78 ± 2.10c	252.46 ± 3.30d	330.95 ± 2.10e	487.19 ± 3.10f	5631.3	<0.0001
MUFA (mg/g)	335.29 ± 4.5a	75.18 ± 3.95b	67.88 ± 5.45c	141.39 ± 6.57d	136.47 ± 4.56e	161.23 ± 3.45f	398.88	<0.0001
PUFAω6 (mg/g)	187.56 ± 2.10a	486.13 ± 2.12b	214.23 ± 1.23c	188.10 ± 3.85d	333.93 ± 1.22e	247.13 ± 0.21f	3034.80	<0.0001
PUFAω3(mg/g)	163.22 ± 1.02a	35.44 ± 1.22b	138.05 ± 0.22c	114.11 ± 0.23d	74.93 ± 0.23e	93.77 ± 0.11f	4637.5	<0.0001
n-6 : n-3 PUFA ratio	1.14 ± 0.04a	13.72 ± 0.02b	1.55 ± 0.03a	1.64 ± 0.03a	4.45 ± 0.12c	2.63 ± 1.10d	113.43	<0.0001
Serum inflammatory markers								
Interleukin-1 (pg/mL)	14.67 ± 1.72a	77.76 ± 1.20b	39.65 ± 2.38c	17.16 ± 2.11d	48.06 ± 1.22e	48.55 ± 1.09f	189.97	<0.0001
Interleukin-6 (pg/mL)	17.22 ± 1.96a	52.68 ± 1.21b	28.15 ± 1.12c	21.22 ± 1.12d	43.33 ± 1.45e	37.89 ± 2.10f	77.74	<0.0001
Interleukin-10 (pg/mL)	47.23 ± 1.42a	19.90 ± 1.90b	26.45 ± 1.49bc	47.11 ± 1.09d	30.18 ± 2.45e	37.41 ± 1.12f	45.10	<0.0001
Leukotriene B4 (LTB4, pg/mL)	126.78 ± 1.12a	200.89 ± 4.25b	161.22 ± 1.10c	159.51 ± 1.70d	176.35 ± 4.05e	163.25 ± 2.09f	79.42	<0.0001
Prostaglandin (pg/mL)	39.03 ±	87.09 ±	55.67 ±	44.67 ±	77.17 ±	41.09 ±	86.44	<0.0001

	2.52a	2.95b	2.08c	2.31d	1.23e	1.45f		
Testis inflammatory markers								
Nitric oxide (NO, nmol/mg protein)	2.33 ± 0.92a	13.12 ± 0.35b	5.54 ± 0.98a	3.05 ± 0.99a	7.75 ± 1.21c	4.06 ± 2.11a	10.86	<0.0001
Myeloperoxidase (MPO, U/min/mg protein)	1.23 ± 0.18a	34.70 ± 1.12b	9.12 ± 0.72c	5.67 ± 0.56d	10.15 ± 0.95e	8.76 ± 0.48c	255.61	<0.0001
Lactic dehydrogenase (LDH, U/mg protein)	4.12 ± 0.58a	23.12 ± 1.12b	9.02 ± 0.82c	7.76 ± 0.78d	11.49 ± 0.12e	7.14 ± 0.75d	77.22	<0.0001
Tumor necrosis factor-α (TNF-α, pg/mg protein)	6.33 ± 0.61a	23.18 ± 1.21b	10.89 ± 1.12c	9.96 ± 0.12d	13.34 ± 1.32e	10.51 ± 1.02c	33.77	<0.0001
Testis lipid peroxidation products – Oxidative stress markers								
Conjugated dienes(nmol/mg protein)	39.89 ± 3.16a	88.23 ± 3.10b	52.90 ± 2.30c	43.21 ± 1.21d	64.12 ± 2.11e	61.21 ± 0.89f	58.26	<0.0001
Lipid hydroperoxides(nmol/mg protein)	35.22 ± 2.01a	92.34 ± 1.30b	50.41 ± 3.21bc	41.22 ± 2.01d	75.55 ± 1.84e	61.23 ± 1.22f	112.80	<0.0001
Malondialdehyde(nmol/mg protein)	2.56 ± 0.12a	5.42 ± 0.08b	3.24 ± 0.05c	3.60 ± 0.04d	4.40 ± 0.07e	4.04 ± 0.08f	145.03	<0.0001
Protein carbonyl(nmol/mg protein)	4.66 ± 0.22a	22.08 ± 2.01b	16.21 ± 1.11c	8.97 ± 0.21d	13.23 ± 2.12e	10.36 ± 1.22f	19.51	<0.0001
Fragmented DNA (%)	7.09 ± 0.16	58.28 ± 3.10	17.65 ± 2.32	11.75 ± 3.04	21.34 ± 1.23	13.46 ± 1.25	76.16	<0.0001

Testis levels of non-enzymatic antioxidants – Oxidative stress markers								
Reduced glutathione (GSH) (nmol/mg protein)	16.68 ± 0.48a	5.16 ± 0.29b	9.78 ± 0.41c	11.90 ± 0.62d	7.26 ± 0.32e	9.54 ± 0.31f	87.26	<0.0001
Glutathione disulfide(GSSG, nmol/mg protein)	4.14 ± 0.16a	24.12 ± 2.75b	8.12 ± 1.38c	5.67 ± 0.23d	11.64 ± 1.28e	8.78 ± 0.12c	27.77	<0.0001
GSH:GSSG ratio	4.02 ± 1.12a	0.20 ± 0.02b	1.20 ± 1.19c	2.09 ± 0.02d	0.62 ± 0.10b	1.08 ± 0.89c	3.25	0.022
Testis cell proliferation and differentiation, apoptotic and steroidogenic markers								
Tubule differentiation index (TDI %)	55.43 ± 1.05a	26.09 ± 0.25b	49.08 ± 0.35c	52.33 ± 0.26d	51.09 ± 0.09e	53.44 ± 0.21e	506.72	<0.0001
Apoptotic index (AI, %)	3.30 ± 0.04a	77.89 ± 1.05b	9.47 ± 3.23c	5.56 ± 2.38d	11.23 ± 0.78e	10.98 ± 0.04f	276.73	<0.0001
PCNA immunoreactivity ratio	77.55 ± 1.21a	18.39 ± 0.72b	27.45 ± 1.36c	72.34 ± 2.12d	44.55 ± 1.85e	33.23 ± 1.28f	265.30	<0.0001
BAX immunoreactivity ratio	6.77 ± 0.18a	59.55 ± 1.58b	19.77 ± 1.22c	11.22 ± 1.22d	34.00 ± 1.16e	23.00 ± 1.05f	275.37	<0.0001
BCL-2 immunoreactivity ratio	81.22 ± 1.63a	18.84 ± 0.21b	70.89 ± 1.21c	72.34 ± 1.22d	40.61 ± 0.22e	40.12 ± 2.32f	321.61	<0.0001
BAX/BCL-2 ratio	0.08 ± 0.02a	3.16 ± 0.04b	0.27 ± 0.03c	0.15 ± 0.26d	0.83 ± 0.12e	0.57 ± 0.09f	88.81	<0.0001
StAR immunoreactivity ratio	68.54	20.54	29.05	33.40	42.23	48.76	130.32	<0.0001

	± 2.08a	± 1.22b	± 1.12c	± 0.34d	± 0.44e	± 2.42f		
3β-HSD immunoreactivity ratio	44.08 ± 2.63a	19.89 ± 1.02b	21.33 ± 1.11c	33 ± 1.23d	28.28 ± 1.30e	35.00 ± 0.89f	37.51	<0.0001
LHR immunoreactivity ratio	33.44 ± 1.02a	3.04 ± 1.09b	10.33 ± 1.31c	23.40 ± 0.99d	19.70 ± 1.05e	21.89 ± 2.11f	64.79	<0.0001

Datas are presented as mean ± standard error mean (n = 5). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p < 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p > 0.05$.**AST:ALT ratio**= AST/ALT, **Blood urea nitrogen**= Serum urea × 0.467,**VLDL**= 0.2 × TAG,**LDL** = TC − (HDL + VLDL),**CI** = TC/HDL,**AI** =(total cholesterol−HDL)/HDL,**CAI**=LDL/HDL,**HOMA-IR** = [(Serum insulin level (ng/mL) × Serum glucose level (mg/dL)] / 22.5,**HOMA-β** = [20 × Insulin (ngl/mL)] / [Glucose (mg/dL) - 3.5] *100,**AI** = number of apoptotic cells/number of total cells × 100),PCNA/BCL-2/StAR/LHR/3β-HSDimmunoreactivity ratio = Positively reacted cells/total number of cells×100, **Control diet** standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g, **FPF- diet** Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g, **VLDL** Very low-density lipoprotein ,**HDL** High density lipoprotein,**LDL** Low density lipoprotein, **PCNA** Proliferating cell nuclear antigen,**BCL-2**B-cell lymphoma 2,**BAX** Bcl-2-associated X protein, **StAR** Steroidogenic acute regulatory protein,**3β-HSD** 3β-hydroxysteroid dehydrogenase ,**LHR** Luteinizing hormone receptor

5.3.5. Long-term FPF feeding repressed serum reproductive hormones and amelioration of PEITC

FPF diet induced impairment of spermatogenesis and male reproduction was reflected in the levels of male reproductive hormones namely, testosterone and serum LH and FSH **(FIG 19** A, B, C (0.21– 0.38 ,0.34 – 0.54, 0.32 – 0.64 fold) showed a significant decrease in FPF diets significantly than the control and PEITC treated groups**.**

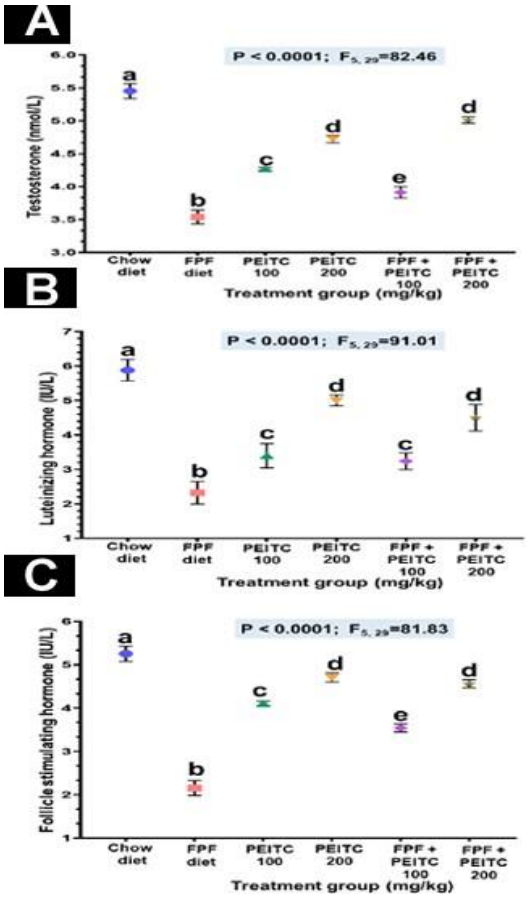

FIG 19: Serum Hormones A) Testosterone (mol/L) B) Leutenising hormone (IU/L) C) Follicle stimulating hormone (IU/L)

5.3.6.. Histological analysis and morphological changes of seminiferous tubules

Normal testis histologicalfeatures including the compactness of seminiferous tubules (SFT) with complete stages of spermatogenesis (*) and full sperm mass in lumen (®)were more evident in the control diet **(FIG 20)** while testis structural disorganization, dearth of sperm mass in lumen (+), vacuolization (µ), and loss of compactness of the SFT were observed in HFD diet fed rats (**FIG 20 Cand D**). The alterations in the SFT histo-architecture were more prominent with HFD tissue sections showing disrupted seminiferous tubules, emptiness of sperm mass in lumen (+), fragmented spermatocytes ($), apoptotic spermatocytes (#), delamination/disorientation of germinal epithelial cells (@), multinucleated germ cells (^), sloughing of germ cells (©), increased vacuolization within the tubules (µ), and in interstitial spaces start Leydig cell dystrophy. Drastic testis tissue structuralalterations were detected in HFD diet as indicated by depletion of Leydig and Sertoli cells (**), wider interstitial space (%), vacuolated spermatocyte (β) and degeneration of Sertoli cells (→), prominentvacuolization, and extensive tubular degeneration along withdisorientation of the different developing stages of spermatogenicand Sertoli cells **(FIG 20**). Long term high dietary FPF consumptionimposed maximal testis tissue damage in HFD to the magnitude of causingpartial to complete tubular atrophy **(FIG 20).**

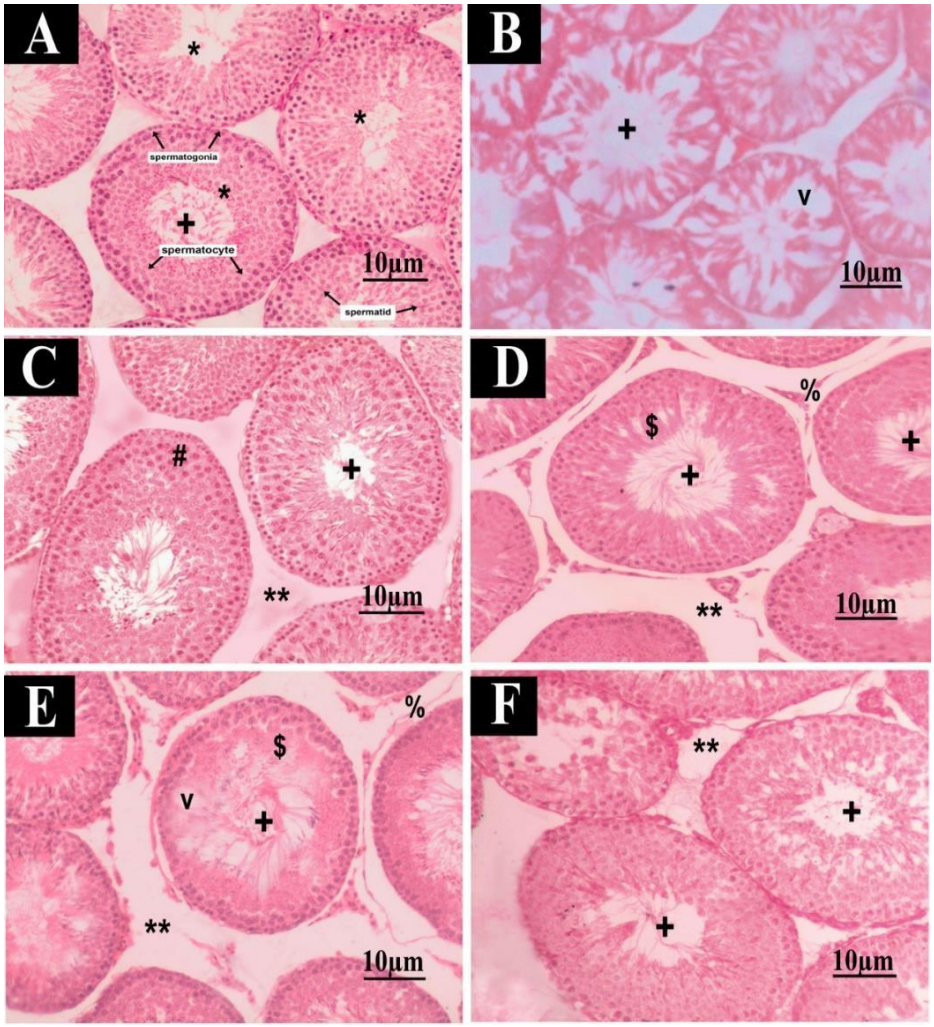

FIG 20: Histological analysis of the testis (A-F) showing effects of high fat diet in control (A), FPF-M (B), PEITC 100 (C), PEITC 200 (D), FPF M+PEITC 100 (E) and FPF-M+PEITC 200 (F) for 60 days

The high testis tissue damage score of 4.22 and 5.71 was registered in FPF-M diet group showing testicular atrophy of about 77– 100%, where seminiferous tubules lined by one layer of cells with marked cellular necrosis and loss of spermatogenesis, with interstitial edema and hyperemia while 45 – 75% testicular atrophy was noticed in control (2.34 score) diet (**Table 29).** A significant decrease in the JTBS was observed in the FPF-M (7.30) with the control diet (9.90) and PEITC (9.80), signifying that the FPF diet induced progressive deterioration of testicular histo-architecture leading to disruption of seminiferous tubules, hypo-spermatogenesis, maturation arrest and testicular damage **(Table 29).** Long-term feeding of FPF diet resulted in severe morphological changes in the seminiferous tubules, i.e., a significant reduction in the MSTD (38.00 – 46.13%), STEH (22.00 – 39.00%), TAT (20.94 – 56.73%) and an increase inIS (31.22 – 56.00%), and TL (11.45 – 52.03%) demonstrating FPF diet induced testicular tissue disintegrity and sperm loss (**Table 29)**. Further, a drastic decline in the germinal and interstitial cells was detected in the FPF supplemented diet comprising spermatogonia (0.63 – 0.96 fold), spermatocytes (0.34 – 0.99 fold), spermatids (0.34 – 0.82 fold), Sertoli cells (0.46 – 0.78fold) and Leydig cells (0.57 – 0.45 fold) resulting in hypo-spermatogenesis and testicular dysfunction (**Table 29)**

Table 29: Long-term feeding of fermented pork fat diet (Sa-um) on morphological changes of seminiferous tubules and quantification of germ and Leydig cells in testis of male Wistar albino rats and amelioration of PEITC

Parameters	**CHOW- Diet**	**FPF-M**	**P 100**	**P 200**	**FPF-M + P100**	**FPF-M +P200**	**F $_{5,29}$**	**P value**
Morphological changes of seminiferous tubules (10 random non-overlapping fields/treatment)								
Johnsen's mean testicular biopsy scc (JTBS)	9.90 ± 0.05a	7.30 ± 0.02b	9.40 ± 0.05c	9.80 ± 0.02a	8.80 ± 0.03e	9.60 ± 0.02f	810.14	<0.0001
Mean diameter of seminiferoustubule (MSTD, µm)	128.00 ± 0.12a	115.00 ± 2.23b	125.00 ± 0.11c	124.00 ± 0.11d	127.00 ± 0.13e	127.00 ± 0.09e	27.47	<0.0001
Seminiferous tubule epithelial height (STEH, µm)	83.33 ± 0.13a	69.09 ± 1.01b	80.99 ± 0.09c	82.00 ± 0.11d	79.98 ± 0.11e	81.22 ± 0.12f	149.12	<0.0001
Interstitial space (IS, µm)	9.78 ± 0.04a	7.94 ± 0.02b	9.34 ± 0.06c	9.72 ± 0.04d	8.99 ± 0.05e	9.11 ± 0.02f	267.22	<0.0001
Tunica albuginea thickness (TAT,	29.33	19.78	25.66	27.89	23.44	25.45	21.17	<0.0001

µm)	± 0.98a	± 1.11b	± 0.12c	± 0.11d	± 1.0e	± 0.11f		
Tubular lumen (TL, µm)	13.33 ± 0.06a	10.34 ± 0.07b	12.11 ± 0.06c	12.37 ± 0.03d	11.89 ± 0.02e	12.18 ± 0.08f	285.45	<0.0001
Testis tissue damage score	1.00 ± 0.01a	5.16 ± 0.12b	3.44 ± 0.13c	1.07 ± 1.11d	2.13 ± 0.03e	1.23 ± 0.14f	12.94	<0.0001
Enumeration of germ cells and Leydig cells (10 random non-overlapping fields/treatment)								
No. of Spermatogonia cells	94.06 ± 1.12a	56.30 ± 3.01b	87.06 ± 2.01c	88.92 ± 2.08d	79.21 ± 3.20e	83.42 ± 4.22f	22.79	<0.0001
No. of spermatocytes	89.10 ± 0.98a	61.55 ± 2.69b	86.54 ± 2.03c	87.07 ± 1.08d	73.14 ± 3.14e	77.98 ± 0.97f	27.84	<0.0001
No. of spermatids	155.00 ± 3.11a	111.90 ± 6.88b	143.40 ± 2.89c	146.70 ± 2.62d	133.90 ± 1.08e	141.50 ± 2.34f	16.75	<0.0001
No. of Sertoli cells	11.2 ± 0.89a	3.40 ± 0.88b	6.02 ± 0.35c	7.80 ± 0.11d	5.10 ± 2.89e	5.33 ± 0.62f	4.28	0.006
No. of Leydig cells	9.90 ± 0.08a	2.10 ± 0.08b	8.77 ± 0.21c	8.90 ± 0.21d	7.84 ± 0.67 e	8.02 ± 0.09f	83.51	<0.0001

Data are presented as mean ± standard error mean. Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons. Different letters column wise indicate that treatment groups are significantly different at $p < 0.05$ and with similar letters indicate that treatment groups are not statistically significant at $p > 0.05$.Abbreviations: **CHOW- Diet**: standard pellet diet - protein-24%, carbohydrate-65%, fat-11% and total energy-12.56 kJ/g; **FPF-M diet**: Fermented pork medium fat diet - protein-20%, carbohydrate-50%, fat-30% and total energy-17.5 kJ/g

5.3.7. Long-term feeding of FPF diets inhibited germ cell proliferation, diferentiation, apoptosis and steroidogenesis

As assessed by the TUNEL assay, occurrence of an increased number of TUNEL-positive germ cells (dark brown stained, IRDV) was detected in the testis tissue sections of the FPF-M (3.9%) and PEITC (2.3%) diet fed rats, as compared with no TUNEL-positive cells in the control group rats **(FIG 21)**. The rate of germ cell apoptosis (measured by TUNEL assay) was computed in terms of apoptotic index (AI). Increased incidence of apoptotic index (apoptotic germ cells) was observed in the FPF-M (55.65%) diet group than the control diet group (1.55%) indicating activation of apoptosis and induction of DNA damage in the germ cells.

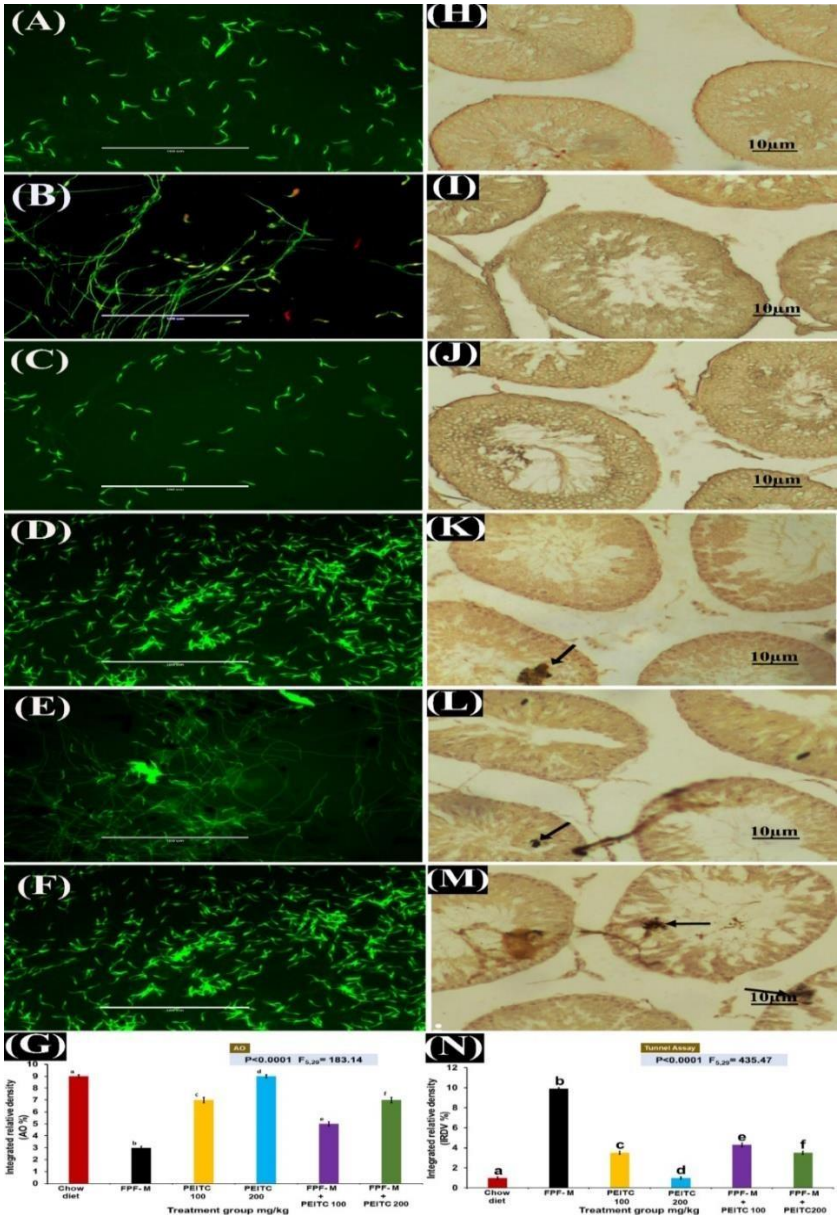

FIG 21: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet and PEITC. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with diferent letters (a, b, c, d) are statistically signifcant ($p>0.001$) if they do not share the same letters**.** Immunoreactivity quantification data (% area) of AO (G), Tunel (N),Control diet standard pellet diet; FPF-M and PEITC.

BCL-2 family genes are known for their anti-apoptosis or pro-apoptosis property and play an essential role in process of mitochondrial apoptosis pathway. This gene is also involved in regulation of spermatogenesis as dead cells or damaged spermatozoa needs to remove from seminiferous tubules. BAX ia a BCL- 2 gene family responsible as apoptotic marker. Many other factors such as environmental toxicants, many cytotoxic agents (pesticides), excessive heat and radiation from different sources may increase the process of germ cell apoptosis in mammalian testis. Bcl-2 expression may directly altered by exposure of any cytotoxic compound leading into abnormal spermatogenesis in male testis. In normal mice testis, its prime location is germ cell, spermatocytes (primary and secondary) and developing spermatozoa. HFD increases the rate of apoptosis in testicular tissue of normal healthy mice possibly by up-regulating Bcl-2 expression. Immunohistochemical analysis of Bcl-2 protein revealed a significant Bcl-2 expression in testis of all HFD treated groups when compared to control and PEITC group **(FIG 22)**. However, some Bcl-2 positive Leydig cells were also seen after FPF diet exposure. HSP 70 increased due to declining binding ability. However, HFD treatment to normal healthy rats significantly declined in LHR and increased HSP70 expression in dose dependent manner when compared to normal control group and PEITC groups. PCNA (proliferating cell nuclear antigen), a potent biomarker for proliferation in spermatogonia and spermatocytes (leptotene, zygotene and pachytene spermatocytes) has been extensively used for to immunohistochemical studies It has been used as an early biomarker for detecting chemically induced testicular toxicity due to its DNA repair nature. In our study, we observed that FPF exposure in normal healthy rats significantly affected expression of PCNA protein in germ cells and spermatocytes of seminiferous tubule **(FIG 22).** In another side, normal control group showed well expression of PCNA in nuclei of outer germinal layer (spermatogonia). Moreover, some staining in spermatocytes (prophase I) was also noticed in their nuclear content however, other cells involved in spermatogenesis like spermatids and spermatozoa were devoid of its expression. PEITC has been induced to ameliorate the effects of

HFD, improving the function of PCNA at various levels and promotes functional cell integrity.

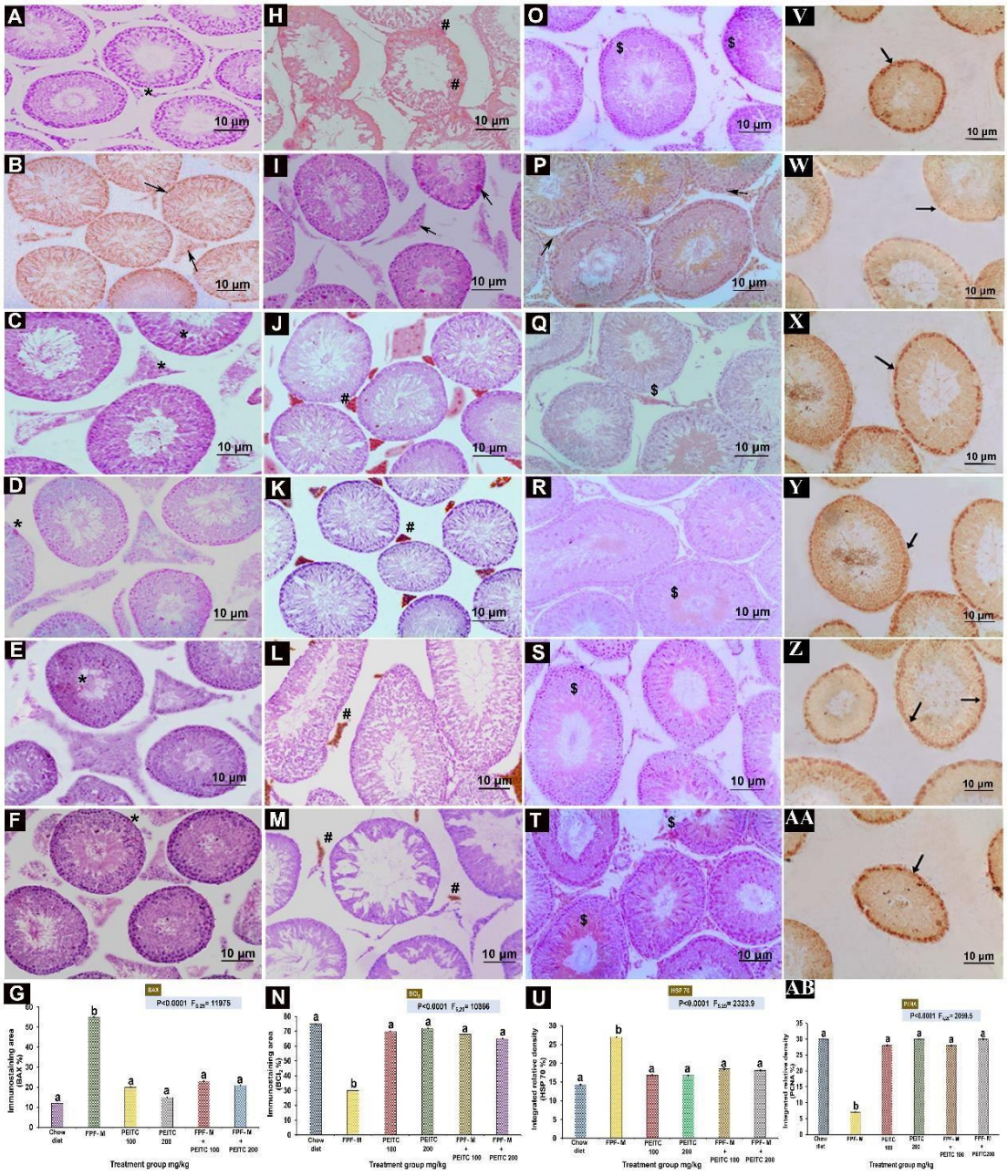

FIG 22: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet and PEITC. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc

tests for all pair-wise multiple comparisons and data marked with diferent letters (a, b, c, d) are statistically signifcant ($p>0.001$) if they do not share the same letters**.** Immunolocalization of BAX (A-F), and BCL2 (H-M) and HSP70 (O-T) in FPF diet supplemented rat testis tissue and PEITC. Immunoreactivity quantification data (% area) of BAX (G), BCL2 (N), HSP70 (U) and PCNA(AB); Control diet standard pellet diet; FPF-M and PEITC.

In rats testis, immune histochemistry study revealed its expression in Leydig cells however in many other primate studies revealed its expression in Sertoli cells. It is well documented that expression of 3β-HSD is regulated by LH receptor hence activation of LH receptor timulates more catalization of steroids into sex hormones and corticosteroids through steroidogenesis. Since LH secretion from anterior pituitary is necessary for stimulation of enzymes involved in steroid production therefore LH receptor plays a regulatory control over 3β-HSD expressions.3β-hydroxysteroid dehydrogenase (3β-HSD) is crucial enzyme in process of steroidogenesis that catalyzes the steroid into progesterone, 17αhydroxyprogesterone, androstenedione and testosterone from pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone (DHEA) and androstenediol respectively. It is generally well located in gonads (testis and ovary) and cortical part of adrenal gland therefore involved in production of sex hormone and corticosteroids (glucocorticoids and mineralocorticoids) respectively. In normal control group, the majority of 3β-HSD expression was documented in Leydig cells and rarely seen in other cells of seminiferous tubules. An interrupted steroidogenesis has been documented after HFD treatment to healthy normal mice as there was decline in expression of 3βHSD testicular tissue **(FIG 23).** LH receptors (LHR) or human chorionic gonadotropin (hCG) is well located in gonads (testis and ovary) and their accessory sex organs. Besides that, LHR expressions are also reported in brain and placental tissue. LHR plays a major role in Leydig cells development and differentiation in the early phases of testicular cells development necessary for steroidogenesis. It shows regulatory control on hypothalamic pituitary gonadal (HPG) axis through high-affinity G protein coupled receptors (GPCRs). Moreover, in

testis, cAMP independent messengers regulates activation of LHR, leading a controlled over steroidogenesis in Leydig cells. It is already reported in many study that LHR is highly susceptible to exogenous steroid present in numerous food leading into decline in altered steroidogenesis. In our study, we claimed that HFD, altered the expression of LHR either via interfering in binding ability with steroid receptors or by various unknown mechanism. We observed that normal control group consist of a massive density of LHR positive stained Leydig cells in interstitial spaces of seminiferous tubules. In testicular tissue, StAR (steroidogenic acute regulatory protein) is one of the most important enzymes in steroidogenic pathway controls cholesterol transfer to inner mitochondrial membrane through a series of event. It is primarily located in Leydig cells situated between interstitial spaces of seminiferous tubules and on head of developing spermatozoa. The expression of StAR is dependent on the concentration of Leydig cells. Like other steroidogenic enzyme involved in steroidogenesis, StAR is also affected with exogenous steroids. In this study, we revealed that the expression of StAR in testicular Leydig cells was seriously affected with FPF diet treatment when compared to control and PEITC groups**.** The immunohistochemical study of StAR in normal control group showed a positive expression in Leydig cells and in sperm masses towards lumen. In addition to that, it is concluded that higher doses of FPF diet seriously declined the StAR expression however, the lower doses were not that much effective than compared to normal control group and PEITC groups. **(FIG 23).**

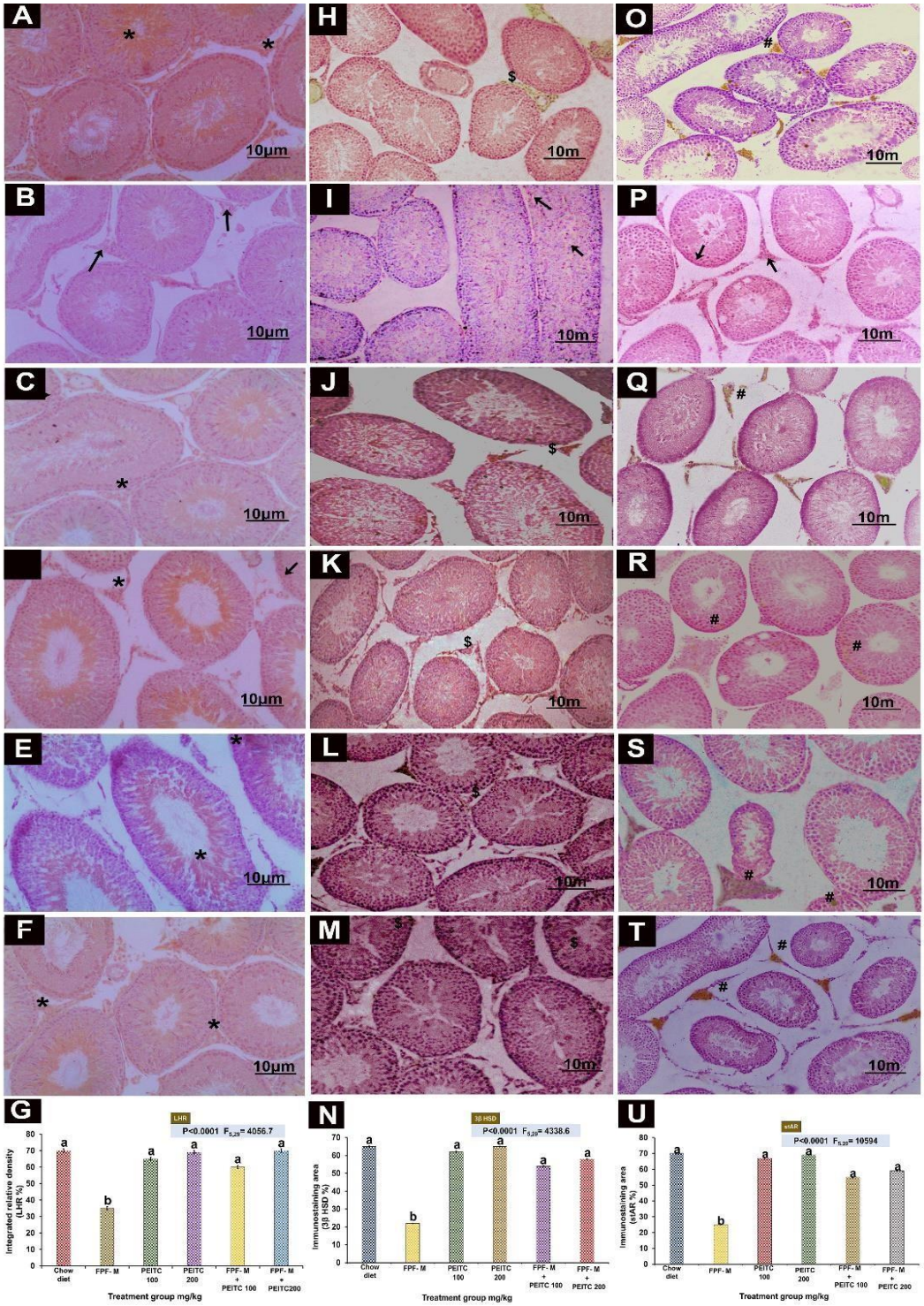
A
H
O
B
I
P
C
J
Q
K
R
E
L
S
F
M
T
10µm
10m
G
N
U
LHR
P<0.0001 F5,23= 4056.7
3β HSD
P<0.0001 F5,23= 4338.6
sbAR
P<0.0001 F5,23= 10594
Integrated relative density (LHR %)
Immunostaining area (3β HSD %)
Immunostaining area (sbAR %)
Chow diet
FPF- M
PEITC 100
PEITC 200
FPF- M + PEITC 100
FPF- M + PEITC200
Treatment group mg/kg

FIG 23: Immuno-expression pattern of steroidogenic and apoptotic regulator proteins in the testis of adult rats fed with fermented pork fat (FPF) diet and PEITC. Data are presented as mean±standard error mean (n=5 feld/diet group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post hoc tests for all pair-wise multiple comparisons and data marked with diferent letters (a, b, c, d,e,f) are statistically signifcant ($p>0.001$) if they do not share the same letters. Immunolocalization of (LHR, A-F) 3β-hydroxysteroid dehydrogenase (3β-HSD, H-M), and stAR (O-T) in FPF diet and PEITC supplemented rat testis tissue. Immunoreactiv - ity quantifcation data (% area) of LHR (G), 3β-HSD (N) and stAR (U); Control diet standard pellet diet; FPF-M and PEITC.

5.3.8. Western blotting analysis

HFD directly or indirectly interferes in the process of androgenesis and apoptosis by means of activation and suppression of several enzymes involved in testosterone synthesis and germ cell survival within testicular tissue. HSP 70 promotes epithelial germ cell proliferation in testicular compartment and its expression is sensitive with exogenous steroids. HFD significantly reduced the PCNA expression in spermatogonia of testicular tissue which is ameliorated by PEITC. In this study, it has been observed that HFD administration affect the cholesterol transfer to outer membrane of mitochondria as there was reduced expression of StAR protein in testicular tissue. Other factor that strengthens our results is reduced expression of one of the steroidogenic enzyme, 3β-HSD, leading into decline in testosterone synthesis. LH receptors widely localized in interstitial compartment were also recognized with its low expression after HFD administration suggesting interference in gonadal-pituitary pathway. A balance level of estrogen is required for healthy spermatogenesis however; excess level of estradiol promotes fertility potential in male. Our data documented an elevated level of serum LH receptors which is one of the causes of infertility. In addition to that, germ cell survival and apoptosis is modulated by several enzymes and necessary for maintenance of homeostasis between productions of germ cell ratio

with mature spermatozoa. However, some exogenous compound may increases the tendency of apoptosis in germ cells and other cells of seminiferous tubules causing sever testicular injury. In our research, we observed HFD exposure may directly or indirectly stimulated BCl-2 and caspase-3 to encounter damaged cell which is recovered by PEITC. The chronic exposure of HFD altered several biochemical and physiological process of sperm formation and level of circulatory testosterone. Overall, low circulatory testosterone due to hazardous effect of HFD affects the spermatogenesis and finally fertility.

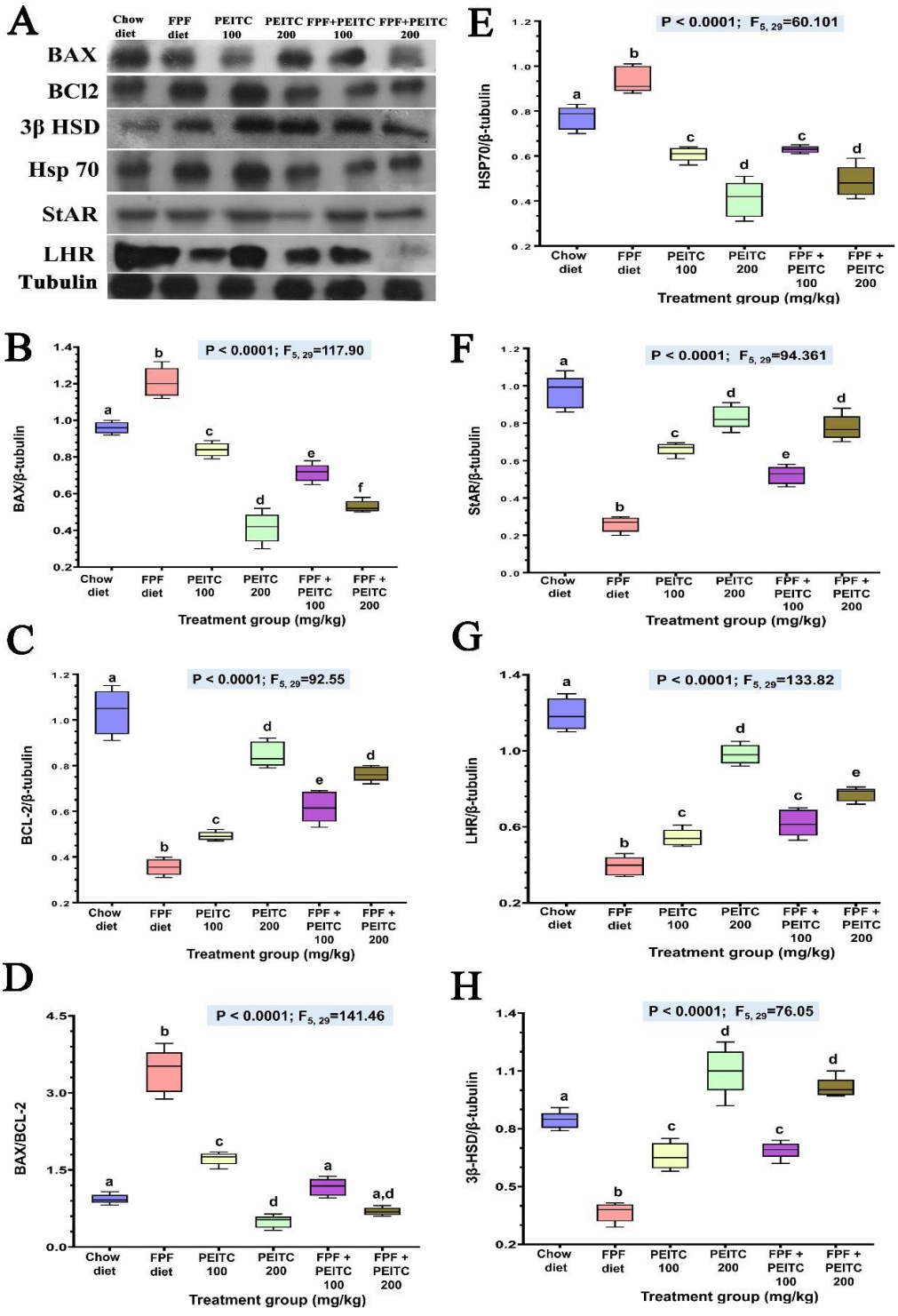

FIG 24: Representatives of Western blot analysis (A-H) showing effects of high fat diet in control (A), FPF-M (B), PEITC 100 (C), PEITC 200 (D), FPF M+PEITC 100 (E) and FPF-M+PEITC 200 (F) for 60 days

5.3.9. RT-PCR analysis

IL 6 (1.28-3.22 fold)) in FPF diet compared to control and PEITC groups, COX 1(1.33-156 fold) in FPF diet compared to control and PEITC groups, IL-1B(2.33-3.22 fold) in FPF diet compared to control, TNF a (1.67-2.65 fold)) in FPF diet compared to control and PEITC groups, iNOS (0.11-1.22 fold)) in FPF diet compared to control and PEITC groups , LT-B4 (1.23-3.23 fold)) in FPF diet compared to control and PEITC groups, PG-E2 (0.11-0.99 fold)) in FPF diet compared to control and PEITC groups , IL-10 (2.11-2.33 fold), MPO (1.89-2.45 fold)) in FPF diet compared to control and PEITC groups respectively. **(FIG 27)**

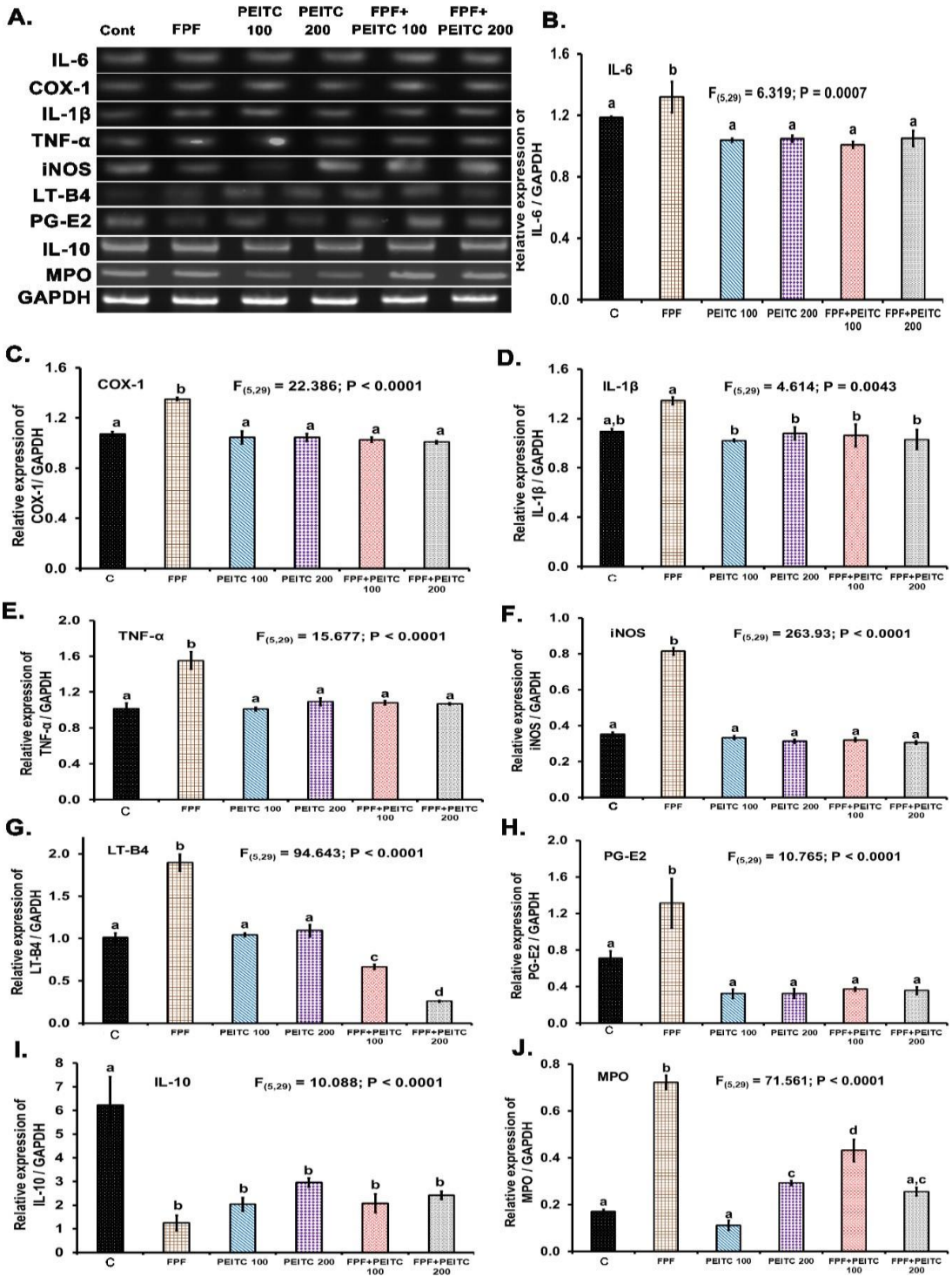

FIG 25: Representation of RT-PCR (A-J) showing effects of high fat diet in control (A), FPF-M (B), PEITC 100 (C), PEITC 200 (D), FPF M+PEITC 100 (E) and FPF-M+PEITC 200 (F) for 60 days

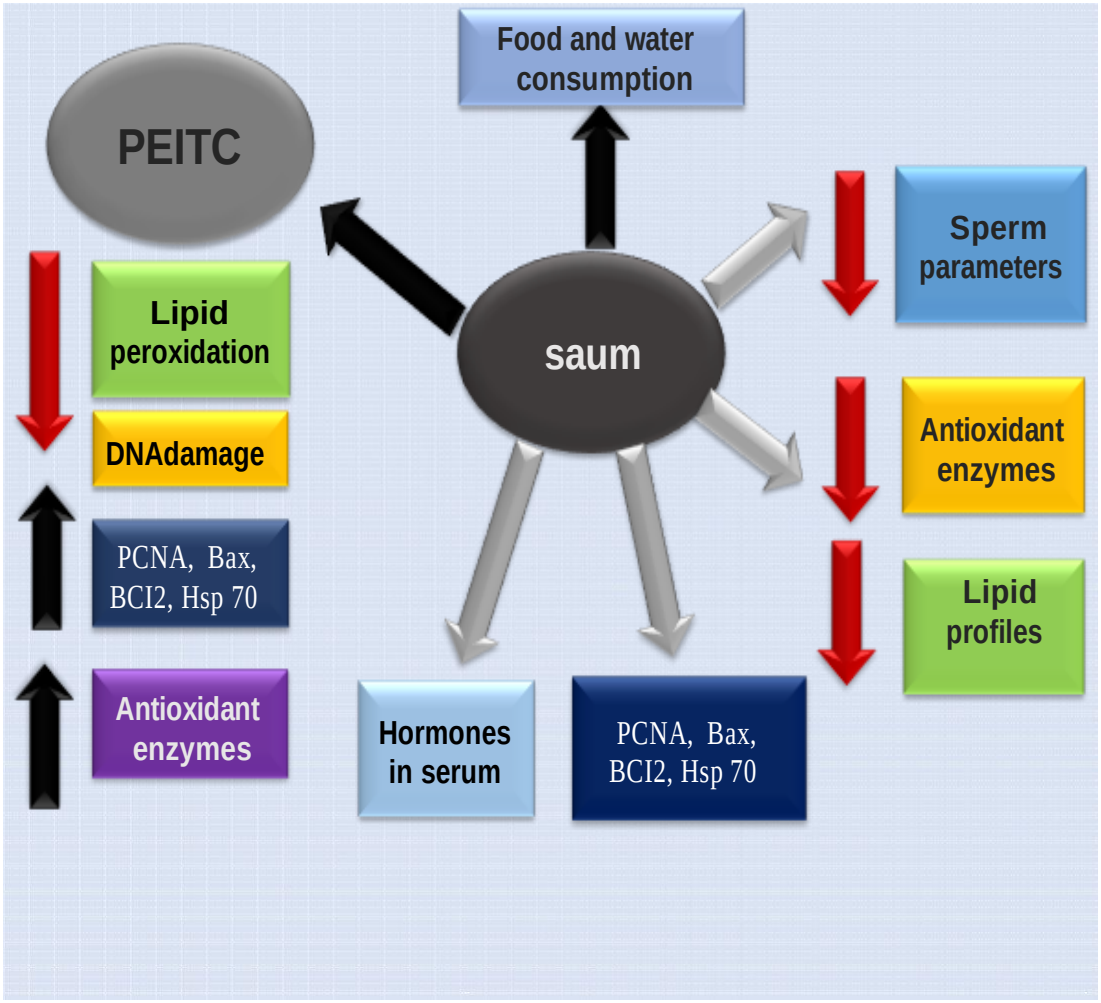

FIG 26: Diagramatic representation of the mechanism of long term feeding FPF- diet effect ameliorated by PEITC

CHAPTER 6

DISCUSSION

6.1. Discussion

To enjoy good quality of life, consumption of healthy and nutritious food is important. Eating food should also be pleasurable. Saum obtained from fermented pig fat has been consumed since Mizo traditional times. It is consumed in 90% of the family among Mizos. It is usually taken with Mizo bai (mixed vegetables). The consumption of a high-fat and high-energy diet is a major cause for the development metabolic and obesity-related health complications. FPF diet is accorded a vital status in Mizo cuisine and has peculiar sensorial attributes and can be used as a flavor enhancer in all dishes, especially in vegetable stew, soup and salads (Ajiboye *et al.*,2016; Emelyanova *et al.*,2019; Lalrohlui *et al.*,2021).

6.2. Effects on food and water consumption

Food consumption increased steadily in all groups. In the present study, the effects of short- and long term administration of HFD and PEITC on body weight and food consumption were assessed in rats. Previous studies on experimental animals suggested a possible link between HFD and obesity, where weight gain was found to be significantly greater in saum -treated animals compared to controls and that this might be due to an increase in appetite or even with consumption of similar amounts of food (Iwase *et al*., 2000), and improvement in the palatability of foods by exerting a positive influence on the appetite centre (Hermanussen and Tresguerres., 2003; Hermanussen *et al*., 2006). On the contrary, other studies reported that long term adminstration of high fat diet did not increase food intake or induce obesity (Boutry *et al*., 2011). Furthermore, other studies on rats showed an association of high fat diet with suppression of body weight gain, fat deposition, and plasma leptin levels which were probably related to the increase in energy expenditure as food intake was not altered by the ingestion (Kondoh and Torii., 2008; Kondoh *et al*., 2009). The mechanisms of action that would allow saum to promote obesity are not clear. Different studies were carried out in order to understand the relationship between HFD and obesity. These studies reported that chronic saum intake might intoxicate the arcuate nucleus and disrupt the hypothalamic signaling cascade of leptin action, causing leptin resistance related to overweight/obesity (Hermanussen and Tresguerres., 2003; Hermanussen and Tresguerres., 2005). Moreover, the observed

weight gain associated with high fat diet intake might be due to destruction of several brain regions (including the hypothalamus) involved in appetite and energy metabolism (Monno *et al*., 1995; Kondoh and Torii., 2008; Kondoh *et al*., 2009). PEITC could reduce food intake and increase energy expenditure by leptin signaling-mediated transcriptional control by inhibiting PTP1B. Leptin is mainly secreted by adipose cells in proportion to white adipose tissue mass and conveys an adiposity signal to the brain, particularly the hypothalamus, by binding to the leptin receptor (Ob-Rb). (Yagi *et al*., 2018). Administration of PEITC to mice significantly reduces food and water intake, and stimulates hypothalamic leptin signalling. Leptin is a weight reducing hormone produced by adipose tissue, which decreases food intake via hypothalamic leptin receptors (Ob-Rb) and the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway. (Yagi *et al*., 2018).

6.3. Effects on body weight, organ weight, BGL and rectal temperature

Rectal temperature increased because of high cholesterol levels and low metabolism which leads to increase in blood glucose levels in high fat diet treated rats compared to control which is ameliorated by the administration of PEITC. The body weight and the respective organ weight of the animals changed throughout the experimental period (Jorge *et al*., 2012). There was an increment in the body weight of high fat diet (saum) treated rats and the organ weight was decreased which might be due to the interference of HFD with signalling systems that regulate apetite centers, thus also upscaling food consumption.(Onaolapo *et al*., 2013). There is an increasing prevalence of obesity in children and adults, and high consumption of dietary fats are associated with alterations in male fertility and hormonal dysregulation. High BMI is associated with low total sperm count (Eisenberg, 2014) and around 65% of men with infertility have hyperlipidaemia (Ramírez-Torres., 2000). It is also known that sperm volume and motility can be affected by elevated total cholesterol and low density lipoprotein (Lu, 2016). Together, these observations suggest that abnormal lipid metabolism in the male reproductive system can affect fertility. Administration of PEITC has helped in the normal lipid metabolism which maintained normal body and organ weight. (Jorge *et al*., 2012).

6.4. Effect on Sperm parameter and motility

The number of motile sperms and the sperm count have decreased in saum treated rats as compared to control and PEITC treated rats. The sperm parameters include the sperm concentration in testis and epididymis. The percentage of motile sperms was counted in treatment groups with respect to control. (Nayanatara *et al.*, 2008) recorded high fat diet reduction in testicular weight and decrease in the sperm count in rats treated with high fat diet. Treating rats with high fat diet caused decrease in testicular weight, decrease in tubular diameter, reduction in germinal epithelium height, decrease in the spermatic count and abnormalities of sperms morphology (Nosseir *et al*., 2012). PEITC administration had increased sperm count, reformation of abnormal sperms and increased the number of motile sperms. The magnitudes of sperm DNA damage and sperm morphological anomalies (head and tail abnormalities) increased with the fat content vs high calorie in the FPF diets regimen, highlighting the damaging effects of long-term feeding of FPF-diet consumption on sperm characteristics (motility, viability, morphology, epididymal sperm concentration, DSP, and sperm transit time).Male fertility is habitually evaluated using the standard semen quality of sperm concentration, motility, and morphology (Aghazarian *et al*., 2020).

6.5. Lipid peroxidation and antioxidant status

Lipid peroxidation is one of the main process of oxidative damage, which plays a critical role in the toxicity of many xenobiotic (Ongjanovic *et al*., 2010). It was evaluated by assessment of TBARS (Qiao *et al*., 2005). Oxidative damage primarily occurs via production of reactive oxygen species such as superoxide anion, peroxides, and it can damage lipids, proteins and DNA. Therefore, it may cause to loss of enzymatic activity and structural integrity of enzymes and activate inflammatory processes (Ozyurt *et al*., 2004).There is a defense system which consists of antioxidant enzymes such as SOD and CAT (Celik *et al*., 2009; Uzun *et al*., 2010; Demir *et al*., 2011). The testis, epididymis, sperm and seminal plasma contain hydrogen peroxide; CAT converts hydrogen peroxide into water (Mansour and Mossa. 2009). SOD is the primary step of the defence mechanism in tne antioxidant system against oxidative stress by catalysing the demutation of 2 superoxide radicals(O2) into molecular oxygen (O2) and hydrogen

peroxide (H2O2) as it prevents further generation of free radicals. (Mohammed *et al*., 2014). Therefore, SOD-CAT system provides the first defense system against oxidative stress and these enzymes work together to eliminate active oxygen species (El-Demerdash., 2011; Wafa *et al*., 2011). GSH and GST are also integral antioxidants which plays a crutial role in protecting cells from oxidative stress and xenobiotics. They can react non-enzymatically with super oxide (Winterbourn *et al*., 1993), nitric oxide8, hydroxyl radicals (Bains and Shaw., 1997) and peroxynitrile (Koppal *et al*.,1999). Thus functions directly as a free radical scavenger.

High body mass index is associated with low total sperm count (Eisenberg *et al*., 2014) and around 65% of men with infertility have hyperlipidaemia (Ramírez-Torres *et al*., 2000).It is also known that sperm volume and motility can be affected by elevated total cholesterol and low density lipoprotein (Lu J-C *et al*., 2016). In combination, these observations suggest that abnormal lipid metabolism in the male reproductive system can affect fertility. PEITC helps in amelioration of lipid peroxidation caused by high fat diet, activated CAT and SOD, increased production of glutathione and glutathione S-transferase.

On a high-fat and high-cholesterol diet, the rats also developed a higher liver weight and higher serum levels of ALT and AST in addition to hepatic steatosis.(Chin-Yu Liu *et al*., 2020) The FPF-H, FPF-M, and FPF-L and 30,60,90 days of FPF diets contained significantly higher levels of SFA and PUFA-ω6 (linoleic, γ-linolenic and arachidonic acid) in control and PEITC groups while MUFA and PUFA-ω3 (α-linolenic, eicosatrienoic and eicosapentaenoic acid) levels were found to be lower. Because of excessive calories, fat content and FAME in the FPF-diets, an increased level of serum FAME was observed that is linked with an increasing oxidative stress, inflammation and apoptosis response in FPF-diet fed rats. It is proved that FPF-diet induced metabolic syndrome caused a series of functional deficits such as hyperglycemia, hyperinsulinemia, dyslipidemia, leptinemia, insulin resistance, obesity, hepato-renal and reproductive dysfunction in male rats. High amount of SFA and PUFA-ω6 present in the FPF-diet are responsible for the increase in the glucose, insulin, leptin and lipid profiles, which ultimately activate the lipid accumulation in different organs and development of

lipotoxicity. It is revealed that a FPF encourages passive overfeeding, which incites to obesity and high lipid profiles. Dietary fatty acid composition rather than dietary fat content produces insulin resistance (Ajiboye*et al*., 2016; Emelyanova *et al*., 2019; Lalrohlui *et al*., 2021). Increased FAME, especially SFAs (C16:0 and C18:0), are the main sources that cause insulin resistance in obesity and type-2 diabetes (Wang *et al*.2020). In this study, compared with the control-diet rat, rats in the three FPF-diet groups developed obesity. However, the body and organ weights, body fat mass distribution, food and calorie intake nutritional indices, Lee index, hepatic insulin resistance profiles, liver and kidney function profiles, serum lipid and FAME profiles, of rats in the FPF-H group were much higher than those of the rats in the FPF-M and FPF-L groups. Earlier studies support this finding that SFAs with longer chain lengths have slower oxidation rates. These outcomes suggest that obesity induced by the FPF-diet is associated with a high C18:0 and C16:0 diet and n-6:n-3 PUFA ratio is more likely to lead to obesity and insulin resistance and further research needs to be carried out. The FPF-diet rich in SFAs led to a higher level of palmitic, stearic, lauric, and myristic acid in the serum of the FPF-diet fed rats. This resulted in more intermediate lipid metabolites due to a relatively lower capacity of C18:0 in oxidation and incorporation into triacylglycerol. Likewise, the higher level of C18:0 may inhibit the conversion of C18:0 from C16:0 in the body, leading to the accumulation of C16:0 in the FPF-diet group, as higher serum C16:0 levels in the FPF-diet group than in the control-diet group detected that possibly together contribute to the severe insulin resistance in the FPF-diet group. This observation is in accordance with the findings of (Wang *et al*., 2020). Fat accumulation in the liver is considered non-alcoholic fatty liver disease (NAFLD) and further progresses to steatosis, which is evident from the increased liver weight, and liver dysfunction markers (ALT, AST and ALP). Moreover, the escalation of myeloperoxidase activity in FPF fed rats is an indicator of NAFLD. Lipid metabolism disorder was categorized by elevated serum lipid profiles especially TC, TAG, LDL, VLDL and FAME and declined HDL in the FPF-diet fed rats and absorbed fats are directed towards TAG synthesis and impairment of mitochondrial beta oxidation of free fatty acids, hence compromising ATP production. The higher atherogenic, cardiac and coronary artery indices of FPF-diet fed rats could persuade the rats to cardiovascular, coronary heart and ischemic diseases (Ajiboye *et al*., 2016). Consumption of FPF-diet triggers hepatic

insulin resistance because of imbalance in glucose homeostasis, provoking the risk of type 2 diabetes (Lalrohlui *et al*.,2021).The FPF-diet comprised pathogenic *Clostridium* (*C. perfringens* and *C. tetani*) and *Helicobacter* (*H. pylori*) and pro-inflammatory molecules producing bacteria (Bacillaceae, Bacteroidaceae, Clostridiaceae, Corynebacteriaceae and Enterobacteriaceae species) (Mandal *et al*., 2018). High fat content in the FPF (94.56%) causes leakiness of intestinal mucosa, which favored the entry of lipopolysaccharide rich gram-negative bacteria into the gut that produces endotoxemia and inflammation. Further bacterial fragments interact with the Toll-like receptor and activate innate and adaptive immunity, causing hyperglycemia and insulin resistance. However, pathogenic and pro-inflammatory molecule producing bacteria present in the FPF are capable of weakening the epithelial barrier and can induce LPS-mediated inflammation and hyperglycemia (De Mandal *et al*., 2018). FPF-diet supplementation impaired the glucose usage as evidenced by the HOMA-IR and HOMA-β (Ghosh and Mukherjee., 2018; Wang *et al*., 2020). Based on the current investigation, long-term FPF-diet consumption also stimulated the distribution and deposition of epididymal, retroperitoneal, peri-renal, mesenteric and dorsal sub-cutaneous fat, causing metabolic abnormalities including obesity as observed in previous studies (Lasker *et al*., 2019; Wang *et al*., 2020). Long-term feeding of a high-fat diet generated excessive ROS production that triggered oxidative stress. The elevation of oxidative stress is linked to chronic inflammation and further increases the accumulation of pro-inflammatory cytokines, which is evident in several animal experimental models. Oxidative damage in tissues also leads to hepato-renal damage in FPF-diet fed rats, as evidenced by the increased levels of the hepato-renal function markers (AST, ALT ALP, urea, creatinine, BUN, AST/ALT and B:C ratio). IL-1β, a major proinflammatory cytokine, launches various malignant processes by activating different cells to increase key molecules driving oncogenic events (Yeung *et al*.,2 013). As shown in the current study, the levels of the oxidative stress (conjugated dienes, lipid hydroperoxides, malondialdehyde, protein carbonyl and fragmented DNA) and inflammatory (IL-1, IL-6, LTB4, prostaglandin, NO, MPO, LDH, and TNF-α) marker levels were elevated while the antioxidant defense markers (CAT, SOD, GST and GSH) were declined in the FPF-diet fed rats could lead to mitochondrial dysfunction, impaired bioenergetics leading to uncontrolled production of ROS, tissue damage and cell death. Disorganization of testes

histoarchitecture, sperm defects and functional loss, and sperm DNA damage in FPF-diet fed rats are substantial effects of lipid peroxidation. Thus, the FPF-diet mediated elevation of oxidative stress markers in the testis of rats could have resulted from the peroxidation of the PUFAs by accumulated ROS (Su *et al*., 2019). Besides, these antioxidant markers are capable of removing free radicals and ROS in male reproductive organs, balancing the prooxidant:antioxidant ratio, maintaining testicular homeostasis and protecting the germ cells against oxidative damage (Ajiboye *et al*., 2016; Zhao *et al*., 2017). A significant increase in protein carbonyl and fragmented DNA levels in the FPF-diet fed rats could have resulted from oxidative degeneration of cellular proteins and oxidative assault on DNA arising from the increased generation of ROS particularly hydroxyl radical. The reduced-to-oxidized glutathione ratio (GSH:GSSG) is a means to assess the mitochondrial or cellular redox state. The testis tissue total-glutathione level is significantly lower but the GSSG level was higher in FPF-diet groups resulting in very low GSH:GSSG ratio than the control-diet, suggesting that the FPF-diet supplemented rats endured with high oxidative stress compared with the control-diet. Thus, FPF-diet supplementation elicits the oxidative stress and inflammation and imbalance the tissue antioxidants. These results are consistent with preceding studies showing that serum and testis tissue antioxidant defenses are compromised in FPF-diet fed rats (Lasker *et al*., 2019; Wang *et al*., 2020).This study identified FPF nutrition mediated elevation of SFA, PUFA-ω6 and decline in MUFA and PUFA-ω3 levels in the rat serum as one important factor amplifying oxidative stress and inflammation with increased body and liver weight, BMI, Lee index, body fat mass distribution, disturbed glucose homeostasis, insulin resistance and increased secretion of leptin and pro-inflammatory adipo-cytokines. Previous reports have described the inflammatory effects of SFA and PUFA-ω6 and anti-inflammatory properties of PUFA-ω3 (Bielawiec *et al*., 2021). Thus, several mechanisms contribute to both FPF-diet and obesity-mediated exacerbation of oxidative stress and inflammation. It is demonstrated that long-term feeding of FPF-diet fed rats resulted in an increased serum SFA and PUFA-ω6 levels a concurrent shift in the n-6/n-3 PUFA balance toward PUFA-ω6, mostly due to the high content of linoleic acid, γ-Linolenic acid, and arachidonic acid. The increase in the n-6/n-3 levels in response to FPF feeding turned out to be more pronounced in FPF-H than in the FPF-M and FPF-L, which is reliable with the metabolism and use of SFME as energy substrates.

Concurrently, lipid oversupply encouraged an elevation in the proportion of circulating PUFA-ω6 to PUFA-ω3 in the serum, which conformed to the findings of other researchers (Bielawiec *et al*., 2021). Serum linoleic acid is considered an obesity-promoting FAME since it acts as a precursor to arachidonic acid, which is a substrate in the synthesis of N-arachidonoyl ethanolamine anandamide and 2-arachidonoylglycerol. Thus, the increased synthesis of these endocannabinoids mainly elucidates the capability of linoleic acid to induce obesogenic effects. In this study, it is revealed that the arachidonic acid concentration was significantly elevated in the serum FAME of rats fed with FPF diets. Besides, this study exhibited a link between the shifts in the n-6/n-3 PUFA balance towards PUFA-ω6 and the increasing inflammatory response in fatty acid surplus situations. PUFA-ω6, particularly arachidonic acid, are regarded to be the most vital source of precursors for the synthesis of endocannabinoids and proinflammatory eicosanoids, including prostaglandins, leukotrienes, thromboxanes and hydroxyeicosatetraenoic acids, which step up the inflammatory signaling cascade (Bielawiec *et al*., 2021). Dependably, in this study, it is observed a significant elevation in the levels of IL-1, IL-6, LTB4, prostaglandin, NO, MPO, LDH, and TNF-α which are ameliorated by the administration of PEITC.

PEITC demonstrate a variety of functions in inflammation, both participating directly in a number of cellular processes and acting as a precursor for subsequent biosynthesis of lipid mediators. Polyunsaturated fatty acids (PUFAs) are important participants in many physiological processes and are associated with the regulation of inflammation, antioxidant protection, the regulation of vascular hemodynamics and other important biological functions. A growing body of evidence has made it possible to determine the leading role of impaired lipid metabolism in many processes occurring in the vascular wall in atherosclerosis (Rafieian-Kopaei *et al*., 2014). Fatty acids are essential molecules that exhibit a variety of functions. Because of their diversity, they are involved in a variety of processes. Depending on the length of the carbon chains, fatty acids are commonly classified as short-chain (have less than 6 carbon atoms), medium-chain (6–12 carbon atoms) and longchain (more than 12 carbon atoms). In addition, fatty acids are divided by the degree of saturation of the carbon chain with hydrogen atoms. This classification distinguishes saturated fatty acids (SFAs), monounsaturated fatty acids

(MUFAs) and polyunsaturated fatty acids (PUFAs). Of clinical importance is another classification that distinguishes ω-3 PUFAs with the end double bond at C3, counting from the methyl end of the hydrocarbon chain, and ω-6 PUFAs with the end double bond at C6. According to modern concepts, atherosclerosis is an inflammatory disease of arterial intima, in which the balance between the mechanisms of activation and resolution of inflammation is disturbed. PUFAs are substrates for the synthesis of both proinflammatory and specialized proresolving lipid mediators) (Kotlyarov and Kotlyarova., 2022). Arachidonic acid is ω-6 PUFA and is metabolized through the lipoxygenase (LOX) pathway, the cyclooxygenase (COX) pathway and the cytochrome P450 pathway (Kotlyarov and Kotlyarova ., 2022). In the lipoxygenase pathway, arachidonic acid is a substrate for the formation of proinflammatory leukotrienes and pro-resolving lipoxins. The 5-LOX enzyme is at the crossroads of the proinflammatory and specialized pro-resolving lipid mediators synthesis pathways (Samuelsson, 2000). PEITC has an effect on NF-κB, on interleukin (IL) -1β, IL-6, tumor necrosis factor-α (TNF-α) expression, while enhancing IL-10 expression and improving hemodynamic indices, myocardial structure and function (Chen *et al*., 2013). 15-epi-lipoxin A4, acting through endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) promotes NO production, negatively regulating leukocyte-endothelial interaction. ω-3 PUFA and is the precursor for the formation of E-series resolvins. PEITC has anti-inflammatory and pro-resolving effects through several mechanisms, including inhibition of the release of inflammatory mediators (Seki *et al*.,2010 and Kebir D *et al*., 2012) and activation of the process of efferocytosis of apoptotic neutrophils by macrophages (Schwab *et al*., 2007 and Oh *et al*., 2011). PUFAs are an important source of both proinflammatory and anti-inflammatory mediators, the balance of which is important for atherogenesis. In addition to NO, lipid mediators derived from PUFAs play an active role in the regulation of vascular hemodynamics. The crosslinks between hemodynamics and lipid mediators are due to the fact that shear stress can activate cytosolic phospholipase A2, which promotes the release of arachidonic acid from phospholipids of the plasma membrane. It is also suggested that NO can enhance COX2 activity through S-nitrosylation, resulting in increased prostaglandin production (Kim, 2011). ω-3 PUFAs exhibit endothelium-independent vasodilatory effects that are associated with the opening of large conductance calcium-activated potassium channels, KATP and members of the

Kv7 family of voltage-gated potassium channels in VSMCs. The resulting effect is hyperpolarization and relaxation of VSMCs and, consequently, a vasodilatory effect. Fatty acids that are part of plasma membrane lipids are involved in the regulation of membrane biophysical properties and physiological functions (Kotlyarov., 2021). Chain length and degree of unsaturation are important factors determining the role of ω3 PUFAs in cell membranes. The lipid bilayer of the plasma membrane has a complex structural organization in which the leading role is assigned to the spatial orientation of cholesterol molecules as well as the fatty acid tails of phospholipids, whose unsaturation can significantly influence such biophysical properties as fluidity (Marguet *et al*., 2006). At the same time, the biophysical properties of plasma membranes provide functions for many membrane proteins (Zabroski *et al*., 2021 and Grouleff *et al*., 2015). Lipid ordering is known to affect the possibility of necessary conformational changes in proteins to perform their functions. Fatty acids in membrane phospholipids are involved in maintaining the biophysical properties of plasma membranes, providing, on the one hand, optimal fluidity to allow proteins to perform the conformational changes required for their function and, on the other hand, providing sufficient viscosity, which is required for their membrane localization (Axelrod, 1983 and Phillips *et al*., 2009). Phospholipid tails of saturated fatty acids maintain lipid organization, whereas phospholipids containing polyunsaturated fatty acids are significantly more disordered. ω-3 PUFAs are incorporated into phospholipids of lipid domains, displacing cholesterol and affecting the molecular order of lipid microdomains. This is due to the fact that PUFAs chains have many rapidly changing conformations that push away the rigid steroid moiety of cholesterol molecule, affecting the lipid ordering of the membrane (Shaikh *et al*., 2014 and Wassall *et al*., 2008). Meanwhile, unordered PUFA rich domains coexist with highly ordered lipid rafts enriched in sphingolipids and cholesterol (Williams *et al*., 2012). The incorporation of ω-3 PUFAs into phospholipids of plasma membranes has enormous potential to change the organization of their molecular architecture, to remodel lipid-protein interactions and the functions of membrane proteins (Shaikh *et al*., 2014). It was shown that eicosapentaenoic acid changes the lipid composition in caveolae and induces eNOS translocation from caveolae to soluble fractions, which was accompanied by a stimulated ability to produce NO in cells (Li *et al*., 2007). In turn, docosahexaenoic acid changes the microenvironment of caveolae not only by changing the lipid composition of

the membrane but also by changing the distribution of basic structural proteins, such as caveolin-1, and also promotes eNOS displacement from caveolae. Moreover, treatment of cells with docosahexaenoic acid significantly increases eNOS activity compared to the control (Li *et al*., 2007). In addition, docosahexaenoic acid was shown to enhance eNOS and Akt activity, increase HSP90 (heat shock protein 90) expression, and increase NO bioavailability in response to Akt-kinase activation (Stebbins *et al*., 2008). Thus, ω-3 PUFAs, when incorporated into membrane phospholipids, may influence the biophysical properties of membranes and the function of membrane proteins, which, in addition to their role as lipid mediators, is a promising area for future research. The extensive data available to date demonstrate the multifaceted role of PUFAs in many physiological and pathological processes. As has been shown in many studies ω3 PUFAs demonstrate predominantly a thero protective effects, which formed the basis for evaluating their preventive and therapeutic efficacy. Dietary and circulating eicosapentaenoic acid and docosahexaenoic acid have been shown to be inversely associated with the incidence of cardiovascular disease. No significant associations with cardiovascular disease were observed for alpha-linolenic acid and ω-6 PUFAs (linoleic acid, arachidonic acid) (de Oliveira Otto *et al.*, 2013). Interestingly, the heart tissue of mice on a high-fat diet showed an increase in arachidonic, linoleic, and docosahexaenoic acids and a decrease in eicosapentaenoic acid. The incorporation of PUFAs into the cell membrane can change the biophysical properties of the membrane and affect cardiac function (Pakiet *et al*., 2020). Thus, PUFAs are involved in various physiological and pathological processes, whose complex relationships may be disturbed during atherogenesis. In addition, ω-3 PUFA supplementation significantly improved endothelial function and reduced inflammatory markers in the offspring of patients with type 2 diabetes (Rizza *et al*., 2009). Dietary supplements containing marine ω-3 PUFAs improved endothelium-dependent dilatation of large arteries in patients with hypercholesterolemia but had no effect on endothelium independent dilatation (Goodfellow *et al*., 2000) PEITC has been shown to reduce LDL cholesterol levels (Rassias *et al*.,1991). And higher intake of linolenic acid was associated with a lower risk of atherosclerotic plaques in the carotid arteries .However, ω-3 PUFA intake may marginally reduce the risk of mortality from coronary heart disease and complications of coronary heart disease (Abdelhamid *et al*., 2020). Heart failure is a complex syndrome that is the final outcome of the progression of

many cardiovascular diseases, such as coronary heart disease and arterial hypertension. Heart failure is an important cause of reduced quality of life, hospitalizations, and death. The rate of progression of heart failure depends on many factors and is an important target for therapeutic interventions, primarily regarding its causes. Because of the great medical and social significance of heart failure, new effective therapies are required. Numerous studies have investigated the possible benefits of taking ω-3 PUFAs as a means of preventing and treating heart failure. Nrf2 is a transcription factor that participates in the control of oxidative stress, thus providing myocardial protection against the occurrence of fibrosis (Kang *et al*., 2020). In addition, Nrf2 was shown to suppress TNFα-induced monocyte chemoattractant protein (MCP)-1 and VCAM-1 mRNA and protein expression in a dose-dependent manner and inhibited TNF-α-induced adhesion of human monocytic U937 cells to human aortic endothelial cells (HAEC) (Chen X-L *et al.*, 2006). PEITC treated rats showed significant decrease in the levels of IL-1, IL-6, LTB4, prostaglandin, NO, MPO, LDH, and TNF-α by lowering the levels of cholesterol, reducing the levels of oxidative stress and inflammation. PEITC is regarded as an effective phase II enzymes inducer, so it increase the levels of GSH, GSH:GSSG ratio and GST which is a defense enzyme and helped in the maintenance of cholesterol and proteins in liver and testis. Glutathione helps in sperm development, maturation and plays important role in spermatogenesis. Therefore, helps reduce the ALT, AST and AP. (Smith *et al*., 1993; Lieshout *et al*., 1996) This enzyme liberates glucose from stored glycogen as needed. This result indicates that glycogen constituted the main source of energy at short-term action. An increment of liver glycogen stores was observed after 2 weeks of PEITC oral administration with statistically significant increment of glucose concentration. In this state, glycogen stores probably had to be replenished by elevated glucose in the blood serum in order to maintain blood glucose concentration. PEITC having integrated defence strategy helps in catatlysing reactions and detoxifications by increasing superoxide dismutase. (Sies, 1999).

6.6. Testosterone, LH and FSH

Abnormal variations in the reproductive hormone levels (decline in the levels of serum and testicular testosterone and LH, and elevation in serum estradiol and FSH) during

feeding regimen that cause decreased cellularity, tubular disorganization and atrophy. Previously, numerous reports have documented low testosterone and LH levels and high FSH, and estradiol levels in the animal models of obesity and humans. LH and FSH play a major role in the synthesis of testosterone and maintenance of spermatogenesis. These gonadotrophins further stimulate Sertoli and Leydig cells to maintain sperm production and maturation. Besides the hormones of the HPG axis, leptin partakes in spermatogenesis directly by regulating testicular cells and/or indirectly by interacting with the HPG axis (Zarezadeh *et al*., 2021). Therefore, the hormonal profile can be used as a marker to assess the status of spermatogenesis. The outcome of this study clearly indicates that long-term feeding of FPF-diet significantly elicited a negative impact on testicular and serum testosterone, estradiol, FSH and LH levels. Testosterone synthesized by Leydig cells under the influence of LH, which is accountable for the induction, maintenance and regulation of spermatogenesis. Experimental studies proved that high-fat diet-induced obesity modifies the HPG axis, suppresses the serum testosterone level, which in turn impairs spermatogenesis and quality of sperm. Long-term feeding of FPF-diet markedly suppresses the LH production, impairs Leydig cells function and testosterone synthesis that causes failure in sperm maturation and sperm defects. The serum and testicular testosterone levels were drastically declined in the FPF-diet fed rats due to suppression of 3β-HSD expression in the testis. Under obese condition, the testosterone synthesis was severely inhibited in Leydig cells along with the elevation of IL-1β and estradiol levels. Interleukin-2 (IL-2) was upregulated by PEITC in this investigation. IL-2 is a cytokine produced by T cells whose main function is to stimulate the growth and cytotoxic response of activated T lymphocytes.IL-2 has been used to stimulate the immune system for the treatment of a number of different tumors, including breast cancer (Grande *et al*., 2006) (Foa *et al*., 1992)reported that the constitutive secretion of IL-2 by tumor cells led to a reduced or abrogated tumorigenicity in several different tumor models (Foa *et al*.,1992).IL-2 also induces G2 cell cycle arrest via the Akt pathway (Kibe *et al*.,2008). However, other studies have suggested that IL-2 therapy may stimulate tumor cell growth. For example, a short 2-day treatment of low-dose IL-2 resulted in a decrease in tumor load and an increase in survival (Shrikant and Mescher., 2002). Also, the addition of IL-2 to cyclophosphamide therapy reversed the growth inhibitory effects of cyclophosphamide on B16 melanoma cells and decreased survival

time, (Palomares *et al*., 1997). Therefore, IL-2 may have concentration/time-dependent effects on tumor growth and cytotoxicity. Hence, the administration of PEITC helps increase the levels of testosterone, LH and FSH levels in rats.

Our research outcomes evidenced the intensification of oxidative stress markers and depletion of antioxidant reserves in the testis tissue of FPF-diet fed rats were consistent with the earlier reports relating that over the production of free radicals leads to apoptotic death of the testicular cells consequent to long-term FPF-diet feeding (Ghosh and Mukherjee., 2018; Lasker *et al*., 2019).

6.7. Histological and Immunohistochemical analysis

According to histological analysis, chronic HFD-fed rats had markedly distorted seminiferous tubules and fewer germ cells overall, especially spermatogonial stem cells and mature spermatids, which had an impact on the meiotic index. There were fewer Sertoli cells, which are essential for maintaining germ cells. Therefore, HF diet not only affects Sertoli cell formation but also has an impact on germ cell development, and the two are probably connected. Understanding the molecular pathways impacted by the HF diet in the testis has been made possible through an examination of the proteomic data. Since proteins cannot function alone, a protein-protein interaction analysis revealed high interaction scores, indicating participation in a network of proteins with related functions. IPA pathway analysis helped to identify the key roles of these proteins and further analysis of canonical pathways revealed the top deregulated pathways related to 'cavelolar-mediated endocytosis signalling', as well as effects on 'Sertoli: germ cell signalling'. These pathways have particular relevance to the blood testis barrier (BTB), an androgen-regulated structure which is disrupted in animals exposed to a HFD diet (Fan *et al*., 2015; Morgan *et al*., 2014).

H&E stain contain haemotoxylin and eosin. Haemotoxylin is basic stain. It stained basophilic component, such as nuclei as dark blue (Kiernan, 2008). Meanwhile, eosin, which is acidic stain, will stain acidophilic substrate as pinky red in different degree (Kiernan, 2008). For instance, cytoplasm which fulfilled with granular mass is stained pink (Kiernan, 2008); collagen and muscle is stained pink (Kiernan, 2008) and erythrocyte (red blood cell) is stained intensely red. The tissue is more appropriate for

microscopical observation after the fixation, processing, embedding, and staining procedures. Fixation prevents the effects of putrefaction and autolysis while maintaining the original state of the cell. The embedding and processing steps change the tissue's hardness, provide adequate support, and enable clean cutting on tissue substrate. In order to make it easier to examine and distinguish between distinct tissue substrates, staining processes connect coloured compounds to specific tissue substrates and raise different hues on the various tissue sections dependent on their tissue affinity. It was decided to utilise a fixative agent and staining solution that would have an irreversible effect on the tissue substrate. A normal control group showed large round tubules with outer thin layer of columnar pseudo stratified epithelium and inside lined by stereocilia or microvilli. There was abundance of sperm mass in the lumen of caudal lumen. Seminal vesicle is an androgen dependant accessory sex organ of testis provides essential fluid consisting of fructose and prostaglandins to sperm (Cunha *et al*., 1987). A thin layer of columnar pseudo stratified epithelial cells with smooth muscles wall, villous mucosa and much finger-like projection along with many secretary glands towards lumen was observed in PEITC and normal control seminal tissue. (Cunha *et al*., 1987). PEITC helps repair the damaged tissues enhancing the formation of spermatogenesis and development of seminiferous tubules.

Acridine orange fluorescence of sperm nuclei helps determine which rats are fertile and could give offsprings. Acridine orange fluorescence turns from red to yellow to green as the spermatozoa mature within the epididymis. According to (Kosower *et al*., 1992), virtually of the nuclei from the cauda epididymal and ejaculated spermatozoa of hamster, mouse, and rabbit exhibited green AO fluorescence. The administration of high fat diet had promoted the damage in DNA showing orange stains which may be due to distortion of the sperm due to high cholesterol levels and elevation in hormones. However, rats almost always contains spermatozoa whose nuclei exhibit yellow or red AO fluorescence possibly associated, in part, with rapid transport of rats spermatozoa through the epididymis. Spermatozoa of infertile rats have a higher incidence of yellow-red fluorescent nuclei. (Kazuhiko Hoshi *et al*., 1996). PEITC significantly improved the physiological and metabolic processes and in turn recovered the spermatogenic process, germ cell proliferation, DNA repair, and sperm quality. The treated rats show mature

spermatozoa with green heads, provides nutrition to sperms and helps in the normal morphology and distinct physiology of the sperms. According to (Zhao et al., 2017), oxidative stress is regarded as a key promoter of apoptosis, and the BAX/BCL-2 ratio is a key indicator of how apoptosis will proceed. In the testis of the HFD-fed rats, there was a higher incidence of TUNEL-positive apoptotic cells, upregulation of BAX immunoreactivity, downregulation of BCL-2 immuno-expression, and an increased ratio of BAX/BCL-2. This resulted in oxidative stress, which led to defective spermatogenesis and reproductive damage by intensified apoptosis of primary spermatocytes causing germ cell loss. (Zhao *et al*., 2017; Ghosh and Mukherjee., 2018; Rahali *et al*., 2020). PEITC is found to decrease or inhibit the formation of apoptotic cells which gives a lesser chance for the occurrence of oxidative stress and increase the antioxidation status.

The immunuhistochemical evaluation showed great differences in the positivity of staining among the experimental groups in which PCNA (cellular proliferation marker) was used as the biomarker where the testis sections of the HFD treated groups showed slightly faint staining nuclei indicating mild cell division of the spermatogonia. (Kiernan, 2008). PCNA expression has been for detecting early testicular toxicity by identifying proliferating spermatogonia and early-phase primary spermatocytes in the seminiferous tubules. It was highly expressed in spermatogonia and early-stage spermatocytes of the control-diet group rats while its expression decreased significantly HFD fed rats due to a direct effect on germ cell proliferation, by disturbance of the hypothalamic-pituitary-thyroid axis pathway or by alterations in the Leydig and Sertoli cells function (Zhao *et al.*, 2017). PCNA expression had been detected in the nuclei of rat testis in consistent with observations previously described as (Oktay *et al* 1995; Wandji *et al*.,1996). The FPF-diet is considered as an ethnic food and a probiotic, which positively supress free radical metabolism by up-regulating the functions of antioxidant enzymes and decreasing the concentrations of oxidative stress and pro-inflammatory molecules (Lasker *et al*, 2019). The enormous pathogenic bacteria, antibiotic resistance genes and pro-inflammatory molecules in the FPF-diet may enhance the oxidative stress and inflammation and inhibit the antioxidant defenses in tissues (Mandal *et al.*, 2018). Oxidative stress is considered a significant promoter of apoptosis (Zhao *et al.*, 2017), and the BAX/BCL-2 ratio is the hallmark for determining the course of apoptosis. Higher

incidence of TUNEL-positive apoptotic cells, upregulation of BAX immunoreactivity, downregulation of BCL-2 immuno-expression and increased ratio of BAX/BCL-2 were observed in the testis of the FPF-diet fed rats resulting in oxidative stress leading to defective spermatogenesis and reproductive damage by intensified apoptosis of primary spermatocytes causing germ cell loss (Zhao *et al.,* 2017; Ghosh and Mukherjee2018; Rahali *et al.,* 2020). The selected target star had conserved expression with high expression levels in both control and treated rats. StAR activation is the first stage in steroid hormone synthesis and controls the translocation of cholesterol from the outer to the inner mitochondrial membrane in Leydig cells. LH could stimulate StAR gene expression and histone acetylation via LHR-stimulated cAMP pathway (Li *et al.,* 2021). In this study, long-term feeding of FPF-diet significantly affected serum LH levels due to downregulated LHR immuno-expression, thus decreasing StAR and 3β-HSD protein synthesis. In the inner mitochondrial membrane, P450scc metabolizes the cholesterol to pregnenolone, which is then metabolized to progesterone via 3β-HSD and then to testosterone. FPF-diet feeding induced the serum insulin levels that block the testicular steroidogenesis by limiting the StAR and 3β-HSD immuno-expression and further corroborated by Zhang *et al.,* 2017). Based on our findings of vacuolization, exfoliation of germ cells and loss of Leydig and Sertoli cells in the seminiferous tubules of testicular sections from FPF-diet fed rats, it is confirmed that long-term feeding of FPF-diet causes Leydig cell dystrophy, suppression of testosterone production, thereby further limiting steroidogenesis and spermatogenesis (Ghosh and Mukherjee., 2018). A model has been proposed that signifying the prospective relations between FPF-diet mediated oxidative stress, apoptosis, inflammation, metabolic syndrome, spermatogenesis and steroidogenesis disruption and reproductive dysfunction. PEITC and normal control group showed adequate expression of PCNA, BCl2, 3β-HSD, LH receptor and down regulation of BAX in nuclei of outer germinal layer (spermatogonia). Moreover, some staining in spermatocyte (prophase I) was also noticed in their nuclear content however, other cells involved in 120 spermatogenesis like spermatids and spermatozoa were devoid of its expression. There are many enzymes involved in steroidogenic pathway that initiate and precede steroidogenesis in gonads and adrenal tissue. Steroidogenic acute regulatory protein (StAR) transports cholesterol to inner mitochondrial membrane during steroidogenesis. Its expression in testicular tissue has been well reported in Leydig cells

(LaVoie and King., 2009). Like other steroidogenic enzyme involved in steroidogenesis, StAR is also highly expressed with PEITC exposure (Scott *et al.,* 2009). HSP70 proteins have an important role in the male testis as translocating proteins through membranes during stress conditions to protect the cellular functions. It can be used as a biomarker to oxidative stress (Biggiogera *et al.,* 1996; Wu *et al.,* 2011). Localization of HSP70 was upregulated in PEITC treated rat testis to protect the germ cell proliferation as well as to maintain the function of testis. Hence, HSP70 can be used as an oxidative stress marker for testicular toxicity. HSP70 was localized in the cytoplasm of pre-spermatogonia (germ cells), spermatogonia, spermatocytes, spermatids, and Sertoli cells within the seminiferous tubules and Leydig cells in the interstitium. The expression level of HSP70 protein in the rat testis indicated expression (up regulated) in the control and PEITC treated rats. (Maurya *et al* ., 2017).

6.8. Western blotting

Complimentary Western blot studies were conducted on selected key proteins for further validation, based on the magnitude of fold change (compared to control rats), novelty and relevance to human testicular biology. HFD exposure to rats induced oxidative stress which is shown by increased levels of HSP70, a heat shock protein which is a marker of gonado toxicity. HFD is directly changing the extent of follicular lipid peroxidation through free radicals generation, which may affect the testicular steroidogenesis of 3β HSD expression. In the present study, expression of Bax proteins tend to increase in saum treated groups according to the stage of follicular development. BCl2 is known to protect against apoptosis triggered by a wide range of agents. However the inhibitory effect of BCl2 on apoptosis is determined by the interaction with Bax proteins. BCl2 can form heterodimers with Bax and lose its protective effect. BCl2 when present excess can protect the cells from apoptosis. But, when Bax is in excess and its homodimers dominate cells are prone to programmed cell death. Therefore, it appears to be the relative relation of BCl2 and Bax that determine the fate of cell (Oltvai *et al.,* 1993). PEITC (≤10 μM), and Western blot analyses demonstrated that increased expression of p53 protein was associated with PEITC-induced apoptosis (Y.-F. Kuang and Y.-H. Chen., 2004). PEITC exposure resulted in the upregulation of Hsp27 cells in this study. The induction of

Hsp70 in response to various types of stress correlates with increased resistance to subsequent cellular damage (C. Schafer et.,1999). Hsp70 has also been reported to inhibit apoptosis in NFκB and p53 signaling pathways (J. C. H. Neo *et al.,* 2005). However, activation of Hsp70 by PEITC may result in inhibition of apoptosis, as reported for PEITC-treated human hepatoma HepG2 cells (Neo *et al.,* 2005). TNF-alpha acts as a host defense and plays double role as a proinflammatory mediator by initiating a strong inflammatory response and an immunosuppressive mediator by limiting the extent and duration of inflammatory processes and by inhibiting the development of autoimmune diseases and tumorigenesis; epithelial apoptosis. (Mubeccel *et al.,* 2016).

The nuclear factor (NF)-kB is a primary regulator of inflammatory responses and may be linked to pathology associated with obesity. HFD increased NF-kB activity in rats. A central mediator of inflammatory and stress responses is the NF-kB family of transcription factors. As a response to foreign pathogens and general stressful insults, NF-kB is activated in most cell types. In addition, NF-kB activity is linked to cancer development through its regulation of apoptosis, cell proliferation, angiogenesis, metastasis and cell survival (Baud and Karin .., 2009). Recent evidence also suggests that NF-kB activation is crucial for development of insulin resistance (Arkan *et al.,* 2005) disabled the inflammatory pathway within macrophages by creating myeloid-specific IjB kinase b (IKKb) knockout mice. These mice were more insulin sensitive and partially protected from high-fat diet (HFD)-induced glucose intolerance and hyperinsulinemia. Moreover, (Cai *et al*., 2005) reported that the activation of NF-kB in transgenic mice expressing constitutively active IKKb in hepatocytes lead to hyperglycemia and insulin resistance. The testis of males, showed enhanced NF-kB activity in response to HFD. HFD may influence and even increase NF-kB activity in the lymphoid tissues (Mito *et al.,* 2004; Hick., 2006 and Howard *et al.,* 1999). It has been suggested that the ingestion of HFD may induce low-grade inflammation in adipose tissue (Clement and Langin., 2007). Especially, the adipose tissue located in the abdominal region has been linked to the development of diseases such as type-2 diabetes and atherosclerosis (Yusuf *et al* 2004). The abdominal region would be the major site of HFD-induced NF-kB activity associated with glucose intolerance. We did observe an increase in abdominal NF-kB activity in response to HFD in which the effect was restricted to male mice. males

developed glucose intolerance and displayed elevated levels of plasma resistin, indicative of the development of diabetes type 2. Abdominal NF-kB activity may be due to immune cells invading the adipose tissue depots or an alteration of the microbial environment of the colon (Cani *et al.,* 2008 and Cani *et al.,* 2007).

Alternatively, enhanced NF-kB activity may be due to HFD-induced activation of T cells in the mesenteric lymph nodes surrounded by visceral fat. (Helenius *et al.,* 2009) detected increased NF-kB DNA binding in heart, liver, kidney and brain of older mice and rats, as compared to younger animals, whereas Spencer *et al*. found increased NF-kB activation in splenic macrophages and lymphocytes of aged mice (Spencer *et al.,* 1997). Although these previously published studies have assessed NF-kB activation measured as NF-kB DNA binding or phosphorylation of NF-kB components, none of the previous studies have analyzed NF-kB transactivation. The use of reporter constructs, such as the NF-kB luciferase transgene, we have used in the present reporter mice enables direct analysis of NF-kB transactivation. Thus, the luciferase reporter measures the integrated effects of different protein modifications regulating the NF-kB signal transduction pathway leading to DNA binding and transcriptional regulation, as well as the effects of other genetic and epigenetic factors affecting NF-kB signaling. Reporter mice are particularly useful for analyzing gene regulation over time in a physiological context as opposed to cell cultures. Our in vivo model is also ideally suited to take into account absorption efficiency, transport in blood or other extracellular fluids, and cellular uptake, metabolism and degradation. Furthermore, the present technology also provides the possibility for elucidating the full anatomical expression profile of the regulatory module of interest. The recent description and validation of reporter mice open new horizons for nutrition research and drug discovery because these novel animal models provide a global view of gene expression following acute, repeated or chronic dietary or pharmacological treatment. In summary, we are the first to report a dynamic assessment of NF-kB activity as a function of high versus low-fat feeding. The results show that NF-kB activity is more elevated in mice-fed HFD. We find that weight gain in HFD mice may be a strong predictor of NF-kB activity in the thoracic region of female mice. Moreover, male mice displayed a modest, but significant increase in abdominal NF-kB activity possibly derived from abdominal adipose tissue depots.

In the current study, we found that 4-week HFD consumption significantly increased TNF-a level in rat spleen, which was accompanied by upregulating the mRNA levels of TNF-a, TLR4, and NF-kB p65, and by downregulating the mRNA of a7nAChR in macrophages from rat spleen. This result suggests that the effect of HFD on TNF-a level in the spleen may be attributed to impaired balance of inflammation and anti-inflammation in the spleen. Additionally, we demonstrated that moderate-intensity exercise during HFD feeding abolished the detrimental effect of HFD on TNF-a level in the spleen and prevented abnormal expression of TNF-a and other relevant genes in splenic macrophages. Previous studies have verified that HFD results in increased expression or production of TNF-a in intestinal macrophages (Kawano *et al* 2016; Lee, 2014 and André *et al*., 2017) . (Kizaki *et al.*, 2011and Nakashima *et al*., 2013) In the current study, we found that 4-week HFD consumption significantly increased TNF-a expression in splenic macrophages from rats. It appears that elevated TNF-a expression in macrophages might play an important role in HFD-associated increase in splenic TNF-a level. Indeed, macrophage depletion has been shown to suppress the infiltration of macrophages in some tissues and the production of inflammatory cytokines (Suzuki, 2019). TNF-a is mainly released from activated macrophages and can trigger a deleterious signaling cascade to induce the production of other pro-inflammatory cytokine (Hadjiminas *et al*., 1994). Based on the crucial role of TNF-a in chronic low-grade inflammation, it has been used as markers to monitor the dynamic change of inflammation in responses to high-fat meals and exercise (Calder *et al*., 2013). In the current study, we also demonstrated significant increase in plasma TNF-a in HFD rats. High levels of circulating TNF-a are believed to lead to severe inflammatory response, metabolic alteration, and insulin resistance (Calder *et al*., 2011 and Rodrigues *et al*., 2017). In lipopolysaccharide induced endotoxemia, the spleen has been verified to be the major source of circulating TNF-a (Tracey., 2005). In the context of HFD feeding, elevated circulating TNF-a may come from different tissues, such as the spleen and white adipose tissue, because of a change in the inflammatory profile in multiple tissues. The white adipose tissue has been shown to release TNF-a and other inflammatory mediators into the circulation. We found that increased TNF-a level in the spleen is accompanied with upregulation of TNF-a, TLR4, NF-kB mRNAs, and downregulation ofa7nAChRmRNA. TLR4 is one of the toll-like receptors, a family of proteins playing a

role in the innate immune system, and is believed to be an important trigger of obesity associated inflammatory response (Rogero and Calder., 2018). Saturated fatty acids and lipopolysaccharides are agonists for TLR4, which can bind to and activate TLR4 signaling pathways, subsequent transcription factor NF-kB, to lead to production of pro-inflammatory cytokines, including TNF-α (Rogero and Calder., 2018). a7nAChR was identified as a major component of efferent vagus nerve-based cholinergic antiinflammatory pathway (Wang *et al*., 2003). This pathway is a predominant modulatory circuitry in neural regulation of immunity and inflammation, and it interacts with immune cells to modulate and restrain chronic inflammation (Gallowitsch-Puerta *et al*., 2007)

Activation of this pathway improves blood glucose, insulin resistance, and other obesity associated complications in mice fed with HFD (Satapathy *et al*., 2011). a7nAChR activation suppresses pro-inflammatory cytokine production and release by inhibition of NF-kB in splenic macrophages (Martelli *et al*., 2013). In vitro, antigen-stimulated spleen cells from a7nAChR-deficient mice produce more TNF-α (Fujii *et al*., 2017). In the current study, the upregulation of TLR4 and NF-kB genes and downregulation of a7nAChR expression by HFD consumption suggest it tips the balance of TNF-α regulation in splenic macrophages. It also suggests that inflammatory response increases but anti-inflammatory capacity decreases in the spleen, which is in agreement with the notion that disruption of immune homeostasis is a key aspect of low-grade inflammation development (Lori *et al*., 2017). Specifically, reduction of anti inflammatory capacity in the spleen, as demonstrated by downregulation of a7nAChR expression in macrophages, is likely more critical for HFD-induced low-grade inflammation because vagus nerve regulation of peripheral anti-inflammatory activity mainly depends on the spleen (Lori *et al*., 2017). It has been shown that HFD can result in splenic lesions, such as histological changes, atrophy, splenocyte apoptosis, and lipotoxicity (Bedhiafi *et al*., 2018). In the current study, we observed that HFD decreased spleen mass and total spleen cell number, although the decrease is not statistically significant. However, HFD did not affect splenic macrophage number. The change of spleen mass and total spleen cell number may indicate splenocyte apoptosis. Inflammatory factors are shown to trigger splenocyte apoptosis (Rashid *et al*., 2017). In our study, HFD-induced increase in splenic TNF-αlevel might be a major cause for the change of spleen mass and total spleen cell number.

In the present study, we also found that moderate-intensity running exercise for 4 weeks abolished not only the decrease in spleen mass and total spleen cell number but also the increase of TNFα expression in splenic macrophages during HFD feeding. Physical exercise, an inexpensive and side effects-free way, has been well proven to be an effective clinical intervention to reduce body weight and improve insulin resistance and type 2 diabetes mellitus (Tremblay *et al*., 1985 and Teixeira-Lemos *et al*., 2011). Exercise also decreased HFD-induced body weight gain and metabolic syndrome in experimental rats (Carbajo-Pescador *et al*., 2019). The beneficial effects of physical exercise on obesity and type 2 diabetes mellitus have been verified to be involved in the decrease in chronic low-level inflammation (Teixeira-Lemos *et al*., 2011). Indeed, physical exercise is documented to markedly restrain chronic low-grade inflammation and is acknowledged as an efficient anti-inflammatory intervention (Teixeira *et al*., 2018). However, the molecular mechanism about its beneficial effects is poorly understood. One study shows that voluntary wheel-running exercise inhibits the increase of TNF-α expression in peritoneal macrophages in mice. In another study targeting macrophages, suppression of TNF-α production has been shown to be the basis of mechanical stress anti-inflammatory effect (Saitou *et al*., 2018). A meta-analysis including 17 animal studies concludes that chronic endurance exercise leads to a marked tendency towards TLR4 downregulation in macrophages and other immune cells in rodents with obesity or metabolic syndrome (Rada *et al*., 2018). Based on the fact that physical exercise enhances cardiac parasympathetic tone, Lujan hypothesizes that a7nAChR cholinergic anti-inflammatory pathway mediates the anti-inflammatory phenotype associated with physical exercise (Lujan *et al*., 2013). Similarly, through analyzing the effect of exercise on the levels of brain-derived neurotrophic factor that increases cholinergic activity, Papathanassoglou also proposed that physical exercise likely upregulatesa7nAChR signaling in the central and peripheral nervous system and in immune cells (Papathanassoglou *et al*., 2015). Intriguingly, in the present study, running exercise downregulates the expression of TLR4 and NF-kB mRNA and meanwhile upregulates the expression of a7nAChR mRNA in splenic macrophages from HFD feeding rats, suggesting that moderate intensity physical exercise maintains the balance of TNF-a production in splenic macrophages, or the balance of inflammatory and anti-inflammatory activities in the spleen during HFD feeding. It is clear that the efficacy of

exercise depends on its duration, intensity, and modality (Fillon *et al*., 2019). A minimum of 150 min of moderate-to-vigorous intensive physical activity per week is able to promote health, as recommend by the American College of Sports Medicine (Suzuki, 2018). Previous studies show that, in humans and in animal models, 2 weeks' moderate-intensity exercise can lower blood glucose level and improve plasma lipoprotein profiles (Suk and Shin ., 2015). In the current study, it is not surprising that moderate intensity running exercise for four consecutive weeks prevents TNF-α expression in splenic macrophages during HFD feeding. Taken together, our results reveal that HFD consumption leads to increase in TNF-α level in the spleen, which is along with upregulation of TLR4 and NF-kB expression, as well as downregulation of a7nAChR expression in splenic macrophages from rats. Exercise reduced TNF-α level in the spleen and prevented abnormal expression of TNF-α and its relevant genes in macrophages in HFD-fed rats. Therefore, this research may deepen our understanding of the pathogenesis of HFD-associated diseases and shed light on the management of these diseases.

Caspase-3 belongs to cysteine proteases family and plays an essential role in process of apoptosis. It is well documented that abnormal formation of cell during spermatogenesis need to remove by a process of well-organized programmed cell death, apoptosis. Caspase-3 activation induces apoptosis by protein degradation within cell and stimulate Sertoli cell to phagocytes the cells. Moreover, abnormal activation of caspase-3 within cell may affect the process of sperm production as excessive death of germ cell affect fertility output. As a main and final perpetrator of apoptosis, caspase-3 activation may affected by environmental condition (heat, radiation and pesticides) and other cytotoxic agents stimulates the proteases to induce morphological and physiological changes leading into cell death. in our study, it is concluded that FPF exposure in normal healthy mice induces testicular germ cell apoptosis through a series of events. Caspase-3 positive cells were observed mainly in germ cells and primary spermatocytes after FPF exposure. However, some spermatids and Leydig cell were also seen positive for caspase-3 protein expression. In our study, control group showed a light to moderate staining in germinal epithelial cells (spermatogenic cells series) and Leydig cells. Higher doses of FPF were more likely to affect germ cell survival and induce apoptosis of germinal cells and other cells produced during spermatogenesis. Leydig cells localized in interstitial compartment,

responsible for production of testosterone were significantly affected by FPF diet administration leading into declined serum testosterone (Chandrasekaran *et al.*, 2006).

Poly (ADP-ribose) polymerase (PARP) is a member of the enzyme family which is involved in DNA damage and repair. PARP can be activated by DNA strand breaks, irregular DNA structures or other post-translational modifications (Cantó *et al.*, 2013). Its activation can regulate the protein function, chromatin compaction and gene expression by modifying target proteins via poly (ADP-ribosyl) ation (D'Amours *et al.*, 1999). PARP, a classic DNA repair enzyme, may participate in multiple cellular functions by modulating multiple target proteins via the modification of poly (ADP-ribosyl)ation. In this study, short-term PARP inhibition promoted the lipid accumulation and up-regulated the expression levels of lipid synthesis genes in HFD rats Specifically, PARP activation repairs damaged DNA, and affects cellular energy through NAD depletion (Virág L and Szabó C., 2002) PARP deletion probably exerts both positive and negative effects. A previous study demonstrated that PARP1 deficiency promotes the lipid accumulation in liver (Erener *et al.*, 2012) and exacerbates high-fat diet-induced obesity (Devalaraja and Padanilam., 2010). In the present study, short term PARP inhibition also enhanced the lipid synthesis in the liver tissues of rats fed with a high-fat diet, indicating that PARP is involved in the lipid metabolism via gene modulation. (Wang *et al.*, 2016). PARP inhibitor treatment up-regulated the expression level of SREBP1 both in vitro and in vivo.Some researchers have demonstrated that PARP1 activation promotes cell cycle progression by inhibiting the Sp1 signaling pathway in hepatoma cells (Yang *et al.*, 2013). Consistent with these findings, we observe that PARP is involved in lipid metabolism by modulating Sp1-mediated SREBP1 expression in hepatocytes. Interestingly, short-term PARP inhibition slightly increased insulin sensitivity, which contributed to an improvement in glucose metabolism. PARP1-deficient rats fed with a high-fat diet possessed increased insulin sensitivity than wild-type rats fed with a high-fat diet (Bai *et al.*, 2011). HFD increases the insulin sensitivity by up-regulating intracellular NAD+ levels in hepatocytes (Pang *et al.*, 2015; Pang *et al.* and Pang *et al.*, 2013).According to these observations, PARP inhibition exerts beneficial effects upon the glucose metabolism. Lipid metabolic dysfunction is always accompanied by glucose metabolic disorders in patients with metabolic syndrome. PARP can catalyze multiple

target proteins in vivo (D'Amours D *et al*.,1999). Our results suggest that different proteins involved in glucose and lipid metabolism may be modulated by PARP activity. During short-term HFD treatment, some direct reactions of PARP catalysis were observed, while after long-term observations, some complex interactive effects appeared. A molecular change in PARP inhibition in liver tissues was observed. StAR is an important regulator of steroid hormone synthesis. It was only detected in the cells located in the testicular interstitium and not observed within the seminiferous tubules. StAR was apparently decreased in the saum-treated group compared with the control group .A concentration-dependent decreasing trend of 3β-HSD, and 17β-HSD was observed following exposure in Leydig cells. Meanwhile, the expression of StAR in Leydig cells was decreased remarkably with increasing HFD concentrations and increased with PEITC concentrations. Up-regulation of 3β-HSD and 17β-HSD represents another significant effect of oral administration of PEITC, as this may contribute to increased elimination of carcinogens. (Haibo *et al*., 2022)

6.9. RT-PCR

PEITC treatment can produce significant changes in genes involved in tumor suppression and cellular proliferation/apoptosis. PEITC, a dietary agent found in cruciferous vegetables, transformed the hepatic gene expression of enzymes that are responsible for the metabolism of carcinogens in vivo. Of the modified genes, the down-regulation of these genes was confirmed by RTQ RT-PCR methods. (Schafer *et al*.,1999). RT-PCR Obesity incidence is positively correlated with excessive calorie intake. Activation of myeloperoxidase (MPO), a heme protein primarily expressed in granules of neutrophils, is associated with the development of obesity. However, whether MPO mediates high-fat diet (HFD)-induced obesity and obesity-associated insulin resistance remains to be determined.

Obesity is characterized by immune cell infiltration in adipose tissue and high levels of proinflammatory molecules in the circulation. These proinflammatory mediators may impair insulin signaling, resulting in insulin resistance. MPO is an enzyme released from neutrophils during inflammation and has been implicated in the development of obesity and insulin resistance (Olza *et al*., 2012). However, the molecular mechanisms by which

MPO promotes the development of obesity and insulin resistance have not been established. The current study demonstrates that obese mice have high MPO protein levels and activity in adipose tissues and neutrophils. Genetic deletion of MPO led to less body weight gain, inhibition of inflammation, and improvement of insulin sensitivity and glucose tolerance in HFD-fed mice. The improvement of insulin sensitivity is associated with the restoration of IR-b protein levels in HFD-fed mice. b protein levels in HFD-fed mice. Emerging evidence suggests that activation of neutrophils is involved in the development of obesity and obesity-associated insulin resistance. In obese and type 2 diabetic patients, circulating neutrophil counts are significantly increased (Victor *et al*., 2011) in association with increased oxidative stress and inflammation (Kaur *et al*., 2013). In HFD-fed mice, adipose tissue was infiltrated with neutrophils after 3 days of HFD feeding (Hadad *et al.,* 2013), and neutrophil infiltration was maintained throughout the 90-day study period (Talukdar *et al.*, 2012). Suppression of HFD-induced neutrophil infiltration by deletion of neutrophil elastase led to improved glucose tolerance and increased insulin sensitivity. Consistent with these findings, we observed neutrophil accumulation in adipose tissue of HFD-fed mice, which was accompanied by enhanced body weight gain and impaired insulin sensitivity. Many inflammatory stimuli can stimulate neutrophils to release the contents of their granules, including MPO, neutrophil elastase, and proteinases, into the surrounding tissues to induce acute inflammation. Our data indicate that the infiltrating polymorphonuclear neutrophils are the major source of MPO activity in adipose tissue of HFD-fed mice and that deletion of MPO attenuated the HFD-enhanced inflammatory response and inhibited HFD-induced insulin resistance. Thus, all evidence supports the view that neutrophil infiltration of adipose tissue contributes to insulin resistance in obesity (Rovira-Llopis *et al*., 2013). MPO may promote tyrosine nitration of proteins, leading to alteration of protein structure and function. Nitration of a tyrosine residue either prevents subsequent phosphorylation of the residue or stimulates its phosphorylation, resulting in constitutively active protein. In addition, tyrosine nitration may alter the rate of proteolytic degradation of nitrated proteins, leading to faster degradation or accumulation of the nitrated proteins in cells. Tyrosine nitration of proteins by MPO has been reported in lung (Narasaraju *et al*., 2003), blood, and vasculature (Baldus *et al*., 2001). MPO chlorinates and nitrates Tyr192 of apolipoprotein A-I, leading to impaired cholesterol efflux (Shao *et al*., 2012). In

vascular endothelial cells, MPO and HOCl increase ONOO2 production (Vaz and Augusto., 2008), resulting in endothelial NOS uncoupling and exacerbating oxidative stress. Moreover, tyrosine nitration of protein has been shown to be associated with a strong inflammatory response in human atherosclerotic plaques (He *et al*., 2010) and retina (Zou *et al*., 2011). In agreement with the previous findings that tyrosine nitration of the insulin signaling molecules, including IRS-1 and Akt, contributes to HFD-induced insulin resistance, we found that HFD feeding resulted in tyrosine nitration of IR-b, which was associated with a reduction in IR-b protein and phosphorylation levels and also with impaired insulin signaling. Deletion of MPO prevented HFD-induced tyrosine nitration of IR-b, restored IR-b protein expression, and improved insulin sensitivity. Whether tyrosine nitration of IR-b accelerates its degradation warrants further investigation. Consistent with the findings that high-fat feeding results in less body weight gain and higher body temperature and oxygen consumption in neutrophil elastase knockout mice than in control mice (Mansuy-Aubert *et al*., 2013), we observed less body weight gain in MPO2/2 mice after HFD feeding, with no effect on energy intake. Deletion of MPO also led to higher levels of UCP1 expression in BAT. UCP1 is a proton transporter of the mitochondrial inner membrane that uncouples oxidative metabolism from ATP synthesis and dissipates energy as heat. UCP1 has been reported to play important roles in energy homeostasis, and ablation of UCP1 prevented Western diet–induced obesity (Inokuma *et al*., 2006). In agreement with the upregulation of UCP1, rectal temperature and oxygen consumption measured in isolated BAT mitochondria were increased in the current study, but ATP production did not increase in HFD-fed MPO2/2 mice, suggesting the uncoupling of oxidative metabolism and ATP synthesis and increase in thermogenesis, which may account for the smaller body weight gain in HFD-fed MPO2/2 mice. Further investigations are warranted to elucidate the mechanism by which MPO regulates UCP1 expression and consequent thermogenesis and energy metabolism. Because loss of body weight and fat mass almost always improves insulin sensitivity (McAuley *et al*., 2006), which inhibits the Jun NH2-terminal kinase and inhibitor of kB kinase–nuclear factor-kB pathways and reduces inflammation (Schenk *et al*., 2009), the smaller body weight gain in HFD-fed MPO2/2 mice may also contribute to the improvement of insulin resistance in these mice. In summary, activation of MPO was a critical event in HFD-induced obesity and insulin resistance. Deletion of MPO reduced

body weight gain through the upregulation of UCP1 in BAT. In addition, MPO deficiency is associated with a restoration of IR-b protein levels and improved insulin sensitivity in HFD-fed mice. Thus, inhibition of MPO may be a potential strategy for prevention and treatment of obesity and its complications.

iNOS transcription and translation are activated by cytokines such as tumor necrosis factor- (TNF-α). We found that nNOS, eNOS, iNOS protein expression levels and eNOS, iNOS mRNA expression rose in the small intestines of high fat diet-induced obese rats. The resulting increased production of NO may have improved the absorption of nutrients and energy in the rats' small intestines. nNOS can produce NO that acts as an enteric nervous system non-adrenergic, non-cholinergic inhibitory neurotransmitter (Sanders and Ward., 1992).we concluded that the increased NO generated by iNOS had a protective effect and did not cause pathological changes in small intestine vascular permeability. iNOS protein expression levels and eNOS, iNOS mRNA expression were significantly lower than those of the obese control rats. In another study, we found that serum levels of TNF- α also decreased in octerotide intervention rats than obese control rats ($p < 0.05$) (a manuscript currently in revision). HFD can inhibit TNF α (Levite and Chowes ., 2001) which in turn decreases small intestine iNOS mRNA and protein expression. Many studies have shown that nutritional obesity causes overexpression of NF-kB in vivo (Bhatt *et al*., 2006) There are specific NF-kB binding sites on the iNOS gene promoter and enhancer regions. At the transcriptional level NF-kB regulates iNOS expression, causing rapid synthesis of iNOS mRNA (Taylor *et al*., 1998).

The increased circulating levels of LTB4 in the fat mobilization, hyperlipidemia, and changes in macrophage metabolism associated with T1D was found in this condition (Filgueiras *et al*., 2015). Also we showed that LTB4 is implicated in the activation of peritoneal macrophage lipid deposition, glycolysis, oxidative metabolism, and Ucp1 and 2 expression. PEITC induced rats is associated with a marked reduction in body weight as a consequence of fat and lean mass losses and dehydration. These complications, which are generally attributed to insulin deficiency and the absence of its anabolic and anti-catabolic actions, hinder life quality in T1D patients. (Rosenfalck *et al*., 2012) Interestingly, we found that inhibition of LTB4 receptor BLT1 attenuated the fat, but not the lean mass loss or the dehydration associated with HFD. In spite of the absence of

impact on body weight, this attenuation of fat loss induced by BLT1 inhibition was associated with an abrogation of HFD hyperlipidemia. Hyperlipidemia is a frequent metabolic complication in HFD patients, even in those with well-controlled glycemia, (Miller *et al*., 2011) that mainly results from increased white adipose tissue lipolysis found in this condition. The mechanisms promoting the activation of white adipose tissue lipolysis and therefore hyperlipidemia in HFD are complex and not completely elucidated. In addition to insulin deficiency, combined effects of systemic and local prolipolytic factors including glucocorticoids, glucagon, catecholamines, cytokines, among others are involved. Interestingly, the attenuated fat loss induced by BLT1 inhibition in HFD rats are associated with reduction in white adipose tissue expression. (Hotta *et al*., 2009) Altogether, the findings that BLT1 inhibition attenuated fat loss and hyperlipidemia in HFD mice, suggest a previously unrecognized involvement of LTB4 in the regulation of these processes. Circulating lipids seem to have a major impact in peritoneal macrophages in HFD rats. In association with hyperlipidemia, peritoneal macrophages from HFD rats displayed increased intracellular TGs content and elevated protein levels of CD36, a transmembrane glycoprotein that acts as transporter for fatty acids and oxidized phospholipids and low-density lipoproteins. (Pepino *et al*., 2014) Importantly, abrogation of hyperlipidemia in HFD rats induced by BLT1 inhibition, abolished the increase in peritoneal macrophage TGs and CD36 protein content. further supporting a role for LTB4/BLT1, perhaps by modulating lipolysis and hyperlipidemia, in the regulation of peritoneal macrophage lipid storage. On the functional level, excessive lipid uptake and accumulation in macrophages have been associated with polarization of these cells to a proinflammatory profile,(Choi *et al.*, 2009) which is indeed the case in peritoneal macrophages from HFD rats. Therefore, peritoneal macrophages from HFD rats seem to phenocopy macrophages (foam cells) residing in the atherosclerotic plaques, which displayed increased lipid accumulation and proinflammatory profile in response to hyperlipidemia. (Pourcet and Staels ., 2018). At the functional level, accumulation of lipids in peritoneal macrophages of HFD rats may also be a protective mechanism to buffer excessive amounts of fatty acids released from adipose tissue and to attenuate lipotoxicity. Noteworthy, a similar lipid buffering function was also seen in adipose tissue resident macrophages in conditions of excessive fatty acid release by adipocytes. These findings reveal the complexity and heterogeneity of

response of different macrophage populations to excessive amounts of lipids. High-fat diet (HFD) feeding of rats has been reported to increase expression of COX-1 and PG-E2. The increased glycolysis in HFD rats is in the agreement with its role as the most predominant metabolic pathway for energy production in proinflammatory macrophages, whereas the concomitant increase in oxidative metabolism, which responds for energy production in alternatively activated in anti inflammatory macrophages.(O'Neill *et al.*, 2016) was quite unexpected. In this study, however, macrophage UCP1 expression was related to IL-6 treatment and alternative anti-inflammatory polarization. (Gundra *et al*., 2014) BLT1 inhibition also abolished the increase in oxidative metabolism in peritoneal macrophage from HFD rats reinforcing the causal relationship between these variables. Another interesting finding from our study is that PEITC deficiency abolished the increase in both glycolysis and oxidative metabolism induced by T1D in mice peritoneal macrophages, suggesting that UCP1-mediated mitochondrial uncoupling is an event required and precedent to the activation of these metabolic processes LTB4 effects under BLT1 activation in T1D and other metabolic diseases. In conclusion, this study unveils previously unrecognized actions of LTB4/BLT1 in the regulation of important metabolic and immunologic responses associated to HFD rats. Indeed, we present strong evidence indicating that LTB4/BLT1 is, through unknown mechanisms, involved in the fat loss, hyperlipidemia and proinflammatory macrophage response in HFD rats, such variables that underlie some important complications found in this condition.

CHAPTER 7

Summary

- The present study was designed to examine the body and organ weight, food and water consumption, sperm parameters, oxidative stress and antioxidant effects, biochemical parameters, histological and immunohistochemical, western blotting and RT-PCR analysis of PEITC on saum induced testicular toxicity in chronic (60 days) treatment.

- The higher the dose of saum intake by the rats, the higher is the food and water consumption, increased in body weight and organ weight, decreased in motility % of sperms.

- The rectal temperature was effected with either of the treatment groups, also the blood glucose levels was significantly higher in the saum treated rats.

- Elevated results of lipid peroxidation was observed in saum treated groups and decreased in PEITC treated groups which results in the decrease of the activity of the antioxidant enzymes CAT, SOD, GSH and GST in the chronic treatment.

- AST increased with Saum treated groups and decreased with control and PEITC treated groups. ALT increased with increased treatment with saum groups and decreased with control and PEITC treated groups. AP increased with saum treated groups and decreased with control and PEITC treated groups. Their increased may be mainly due to liver damage in 60 days treatment of saum.

- The significant increase in the creatinine and urea content of the serum following the administration of saum may be attributed to compromise of renal function capacity. Cholesterol levels decreased in saum treated groups which may be due to decrease in the level of testosterone. HFD exposure elevated the hepatic and renal function and increases the disorder of lipid metabolism as there was increase in cholesterol and triglycerides level whereas serum HDL and LDH were significantly decreased ameliorated by

PEITC.

- Interrupted spermatogenesis due to saum interference significantly increases the apoptotic rate via abnormal protein expressions of BCl 2 and caspase 3 genes

- PEITC is an essential nutrient which plays a major role in cellular energy production and has been considered a promising cytoprotective agent.

- PEITC was injected intraperitonially to rats at 100 mg/kg and 200mg/kg for 60 days which greatly affected and increased the sperm motility, sperm count and daily sperm production (DSP). Daily sperm production and epididymal sperm concentration were also severely affected with HFD exposure.

- The sperm count and daily sperm production decreased with increase in the saum treated groups since it has toxic effect in the testis, which may ultimately lead to reduced fertility but increased with the PEITC treated groups.

- PEITC is a very important antioxidant which helps in DNA repair, germ cell recovery and sertoli cell metabolism. HFD significantly increases the morphological abnormalities and sperm DNA damage possibly via increased oxidative stress or hormonal imbalance

- The sperm abnormalities in saum group was significantly higher as compared to PEITC groups.

- Oxidative stress was reduced by PEITC and antioxidant enzymes were elevated in chronic treatment groups.

- The changes caused by saum with respect to biochemical parameters was attenuated by PEITC exposure.

- PEITC treatment greatly improved the damaged caused by saum induced histopathological changes in rat testis in treatment groups.

- Immunohistochemical changes were observed in HFD treatment resulting in the degeneration of proliferating cells in saum treated groups and regeneration of positive cells in PEITC treated groups.

- Our work provides a foundation for understanding how obesity may affect male fertility and future functional studies should be directed towards providing support for these potential players.

- An imbalance in serum LH, FSH, testosterone and estradiol significantly affected the proliferation and maturation of germ cell as well spermatozoa.

- HFD induces oxidative stress mediated toxicity that influence abnormal physiological changes due to elevated MDA content has been observed in this study.

- Due to excess generation of ROS in testicular tissue, there is imbalance in ROS and antioxidant defense system leading into disrupted testicular function in HFD treated groups.

- HFD increases the interstitial spaces between seminiferous tubule leading into uneven distribution of Leydig cells that adversely affect testosterone biosynthesis due to poor connectivity with blood vessels.

- HFD exposure enhances the shrinkage of seminiferous tubule leading into a decreased in Johnson‘s score (assessment of spermatogenesis).

- Increase of weight as well pathological changes in ultra structure of cauda epididymis and seminal vesicle due to oxidative stress or reduced testosterone could be the one of reasons behind the abnormal sperms.

- HFD not only disrupted testicular steroidogenesis, it also altered the adrenal cortiosteroidogenesis as there was decline in volume density of cortical area.

- HFD administration has shown to suppress the cholesterol transport to inner mitochondria membrane as there was reduced expression of StAR protein in testicular tissue.

- HFD, an exogenous steroid/drug interferes in endocrine function as well as steroidogenic enzymes that leading into suppression of 3β-HSD and LHR expression. Interrupted spermatogenesis due to HFD interference significantly increases the apoptotic rate via abnormal protein expressions of Bcl-2 and caspase-3 genes.

- HFD exposure induces the testicular atrophy leading into degeneration of testicular tissue and poor blood supply leading excessive cell death in tubule.

- Use of HFD for a longer duration can make sever fertility issue as herein we observed a decline in percentage of fertility potential as well decrease in litter numbers

CHAPTER 8

Conclusion

The FPF-diets consisted of high amounts of SFA and PUFA-ω6 with lower quantity of MUFA and PUFA-ω3 than the control-diet. Excessive calories, fat content along with FAME, especially SFA and PUFA-ω6 are associated with increasing oxidative stress, inflammation and apoptosis response, which results in excessive accumulation of serum FAME in rats, including SFA and PUFA ω6.The novelty of the study is the finding that the long-term consumption of the FPF-based diets different in calorie, fat content and FAME levels for 30,60,90 days significantly induces dysregulation in lipid and carbohydrate metabolism, induction of inflammatory and oxidative stress markers, disturbance in male sex hormonal levels, elevation of serum FAME and lipid levels, suppression of steroidogenesis and spermatogenesis, damaging the testicular histoarchitecture with testicular atrophy, sperm defects, poor sperm quality, sperm DNA damage, depletion of antioxidant enzymes reserve and apoptotic germ cell death in the testis, which is critical in male reproductive impairment under conditions of FPF-diet mediated metabolic syndrome and obesity which is ameliorated by PEITC.

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PUBLICATIONS

Lalrinzuali Sailo1, Meesala Krishna Murthy2, Khandayataray Pratima3, Vikas Kumar Roy4 and Guruswami Gurusubramanian5*(2019). **Monosodium Glutamate Toxicity and the Possible Protective Role of L–Carnitine.**Science and Technology Journal. Vol. 6 Issue: I January 2018 ISSN: 2321-3388

Sailo Lalrinzuali, Maurya Khushboo, Roy Dinata, Baishya Bhanushree, Nisekhoto Nisa,Rema Momin Bidanchi,Saeed-Ahmed Laskar, Bose Manikandan, Giri Abinash, Buragohain Pori, Vikas Kumar Roy, Guruswami Gurusubramania.(2023). **Long-term consumption of fermented pork fat-based diets difering in calorie, fat content, and fatty acid levels mediates oxidative stress, infammation, redox imbalance, germ cell apoptosis, disruption of steroidogenesis, and testicular dysfunctionin Wistar rats.** Environmental Science and Pollution Research. https://doi.org/10.1007/s11356-023-26018-0

Maurya Khushboo, Sanasam Sanjeev, Meesala Krishna Murthy, Maibam Sunitadevi, Roy Dinata, Baishya Bhanushree, Rema Momin Bidanchi, Nisekhoto Nisa, **Sailo Lalrinzuali**, Bose Manikandan, Laskar Saeed-Ahmed, Giri Abinash, Buragohain Pori, Chettri Arati, Vikas Kumar Roy, Guruswami Gurusubramanian.(2023).**Dietary phytoestrogen diosgenin interrupts metabolism, physiology, and reproduction of Swiss albino mice: Possible mode of action as an emerging environmental containment, endocrine disruptor and reproductive toxicant.** Food and Chemical Toxicology. 176 (113798).

Rema Momin Bidanchi, Lalrinsanga, Lalrindika, Maurya Khushboo, Baishya Bhanushree, Roy Dinata, Milirani Das, Nisekhoto Nisa, **Sailo Lalrinzuali**, Bose Manikandan, Laskar Saeed-Ahmed, Sanasam Sanjeev, Meesala Krishna Murthy, Vikas Kumar Roy, Guruswami Gurusubramanian (2022). **Antioxidative, anti-inflammatory and anti-apoptotic action of ellagic acid against lead acetate induced testicular and hepato-renal oxidative damages and pathophysiological changes in male Long Evans rats**☆ Environmental Pollution 302 (119048)

CONFERENCE/ WORKSHOP/SEMINARS/WEBMINARS

- **Oral presentation** in **International conference** on **Recent Advances in Animal Sciences** (ICRAAS) , "Ameliorative effect of phenethyl isothiocyanate on high fat diet (*Saum*) induced spermatogenesis dysfunction in Wistar albino rats,"6-8 th November,2019 organised by Department of Zoology, Pachhunga university college, Aizawl, Mizoram.

- **Poster presentation** , " High fat diet (Saum) and Health risks in the 12 Annual Convention of Association of Biotechnology and Pharmacy (ABAP) and **International Conference** on Biodiversity, Environment and Human Health: Innovations and Emerging Trends(BEHIET 2018) organised at the school of Life Sciences, Mizoram University, Aizawl, Mizoram 796004 during November 12-14 th, 2018

- **Poster presentation** on **National seminar** on '**Conservation and sustainable use of Medicinal and Aromatic Plants**' held on 13-14 th September, 2018, in the Department of Forestry, Mizoram University, Aizawl.

- Participant in the National Worshop on '**National seminar on Animals Handling, Maintenance and Care'** held during 26 th March,2018 organised by Advanced State Biotech –Hub Facility, Department of Biotechnology, Mizoram University sponsored by Department of Biotechnology, New Delhi.

- Participated in Two-day training programme on "**Understamding the Impact of Forest fire on the Faunal Resources of North-Eastern**

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- Attended webinar in "**IPR Management in Universities**" on 16 th September, 2020 organised by Mizoram University, Aizawl, Mizoram

- Attended webinar presentation on **'Publish and Flourish'** on 2 th july, 2020 organised by Department of Chemical Engineering, University of South Carolina, USA, CEO, SAARP INTERNATIONAL, LLC, South Carolina, USA

- Attended webinar presentation on **'Science Communication'** held on 29th May, 2020, organised by Department of Zoology, Mizoram University, Aizawl, Mizoram

- Attended webinar presentation on **'Analysis of Scientific Publications for enhanced visibility'** held on 29 th May, 202,0 organised by Department of Zoology, Mizoram University, Aizawl, Mizoram

- Participated in webinar presentation on **'Al Research, Applications on Computer Image and Biomedical Instrumentation Research'** held on 13 th February, 2022 organised by EducareTaiwan with Yuan Ze University and National Chung Hsing University

- Participated in UGC STRIDE workshop on **'Basic and Advanced Molecular Techniques'** held from 7 June – 11th June, 2021, organised by Mizoram University, Aizawl, Mizoram

- Attended webinar presentation on **'Principles in Cell Signalling'** held on 4, 11, 18 and 27th May 2020, organised by Department of Zoology, Mizoram University, Aizawl, Mizoram

- Participated in National Workshop on **"The value and interconnections of human, animal, plant and microorganisms: Metataxonomics and Metagenomics Approach"** held from 18 March to25 March, 2023 at Mizoram University, Aizawl, Mizoram

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DEPARTMENT	Zoology
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AMELIORATIVE EFFECT OF PHENETHYL ISOTHIOCYANATE ON HIGH FAT DIET (SAUM) INDUCED SPERMATOGENESIS DYSFUNCTION IN WISTAR ALBINO RATS

AN ABSTRACT SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Introduction

Diets can have a significant impact on normal physiology, with high calorific foods and sedentary lifestyles which contributes to the development of obesity, an epidemic in adults and children of all ages all over the world (NCD-RisC., 2017). The type, source and composition of an FPF-diet strongly impact bioenergetics, modifying metabolic function through changes in gene and protein expression, metabolism, fatty acid composition and cholesterol content of cell membranes (Augustin *et al.*, 2018), resulting in mitochondrial dysfunction and the development of metabolic diseases. SFAs, particularly palmitate and stearate, are harmful and toxic to normal cellular processes and can induce abnormal plasma lipid profles, insulin resistance, infammation, activation of stress-associated protein kinases, protein oxidative stress, disruption of spermatogenesis and steroidogenesis, apoptosis, male reproductive disorders and infertility (Ly *et al.,* 2017; Dallak 2019; Li *et al.,* 2020). Increases in the ratio of n- 6:n-3 PUFA, distinct of the FPF-diet, could potentiate oxidative and inflammatory processes and increase in SFA and PUFA-ω6consumption alter the production of inflammatory mediators and regulators and immune responses on the way to a pro inflammatory process (Schulze *et al*., 2020). An increase in the ratio of n-6:n-3 PUFAs, distinct from the FPF-diet, could potentiate oxidative and infammatory processes, and an increase in SFA and PUFA-ω6 consumption could alter the production of infammatory mediators and regulators, as well as immune responses, leading to a pro-infammatory process (Schulze *et al.,* 2020). We hypothesized that exposure to a FPF-diet with excessive calories, a high fat content, and high FAME levels alters testicular physiology and metabolism, leading to permanent damage to the testicular system and its function. Therefore, the purpose of this study was to compare the effects of long term consumption of FPF-based diets (FPFH, FPF-M, and FPF-L), FPF-M for 30 days, 60 days and 90 days and PEITC on anthropometrical indices (body and organ weights, body fat mass distribution, food and calorie intake nutritional parameters),metabolic syndrome (liver and kidney function profles, hyperinsulinemia, hyperglycemia, insulin resistance, dyslipidaemia, leptinemia), infammatory (IL-1, IL-6,

LTB4, prostaglandin, NO, MPO, LDH, and TNF-α) and pro-oxidant activities (conjugated dienes, lipid hydroperoxides, malondialdehyde, protein carbonyl and fragmented DNA), compromised testicular antioxidant defenses (CAT, SOD, GST and GSH), variations in reproductive sex hormones (testicular and serum testosterone, estradiol, FSH and LH), sperm quality and functional indices, sperm DNA fragmentation and damage, progressive deterioration of spermatogenesis (histologyand histomorphometrics), germ cell proliferation (PCNA expression) and testicular function, steroidogenic impairment (StAR, 3β-HSD and LHR expression), and abnormally enhanced testicular germ cell apoptosis (TUNEL assay, BAX and BCL-2 expression) in Wistar albino rats compared to the control diet.

High fat diet- a mulifactorial disease: Dietary pork-fat comprises an expansive amount of saturated (SFA), monounsaturated(MUFA) and polyunsaturated (PUFA) fatty acids, high in calorie and fat content,which is a potential risk factor for hypertension, hyperlipidemia, hypercholesterolemia, imbalance in glucose and lipid metabolism, inflammation, gut dysbiosis and promotes the production of body obesity in rats, gastric cancer and ulcer, and type-2 diabetes (Emelyanova *et al*., 2019; Chakraborty *et al*., 2021; Lalrohlui *et al*., 2021; Zhu *et al*., 2021).Obesity is considered a serious disease affecting a large population worldwide characterized by excess adipose tissue that contributes to numerous chronic diseases and early mortality. (Bessesen, 2008; Kushner, 1993; Simopoulous, 1984). It is a complex metabolic disease that is a serious detriment to children and adult health, which induces a variety of diseases, such as cardiovascular disease, type II diabetes, hypertension and cancer. The integrity of the blood-testis barrier can be severely interrupted consistent with declines in the tight junction related proteins, occludin, ZO-1 and androgen receptor, but instead endocytic vesicle-associated protein, clathrin rose. Obesity can impair male fertility through declines in the sperm function parameters, sex hormone level, whereas during spermatogenesis damage to the blood-testis barrier (BTB) integrity may be one of the crucial underlying factors accounting for this change. Thus, efforts are continuously underway to prevent obesity in the population. (Nordmann *et al*., 2006; Yancy *et al*., 2004; Volek *et al*., 2008). Chronic ingestion of a low-carbohydrate diet is that it usually contains a high percentage of fat to compensate for carbohydrate-

calorie reduction, and most in the form of saturated fat which is one potential problem. In rats a high-saturated fat diet is used as a diabetogenic factor increasing insulin and lipid levels and it has been shown to induce severe insulin resistance in skeletal muscles (Surwit *et al*., 1988; Chun *et al*., 2010). It is certain that with the increasing prevalence of unhealthy dietary behaviours and sedentary life styles, obesity is emerging as an important risk factor for non-insulin-dependent diabetes, hypertension, cardiovascular disease, cancer, and relevant metabolic and reproductive disorders (Mayes *et al*., 2004).

PHENETHYL ISOTHIOCYANATE

Phenethyl isothiocyanate (PEITC) is a naturally occurring isothiocyanate whose precursor, gluconasturtiin is found in some cruciferous vegetables especially in watercress and cabbages (Tusskorn *et al*., 2013) . Watercress is a rich source of gluconasturtiin, the precursor of phenethyl isothiocyanate (PEITC), while garden cress is rich in glucotropaeolin, the precursor of benzyl isothiocyanate (BITC). PEITC has been studied for its potential for chemoprevention of cancers (Stan *et al*., 2013) such as prostate cancer.(Thomson *et al*., 2013).In terms of biosynthesis, PEITCis produced from gluconasturtiin by the action of the enzyme myrosinase. PEITC is found in plants of the Brassicaceae family, including broccoli, cabbage, and radish.

Ameliorative role of Phenethyl isothiocyanate Phenethyl isothiocyanate has been demonstrated to have a series of pharmacological actions, including antioxidant, antimutative, anti-inflammatory and anti-tumorigenic actions. PEITC which is a naturally occurring cruciferous vegetable-derived compound that inhibits cell growth and induces apoptosis in oral cancer cells (Yeh *et al*., 2014). It induces DNA damage-associated G2/M arrest and subsequent apoptosis in oral cancer cells with varying p53 mutations (Yeh *et al*., 2014). PEITC is one of the most studied members of the ITC family of compounds because of its ability to chemically inhibit induced cancers, including oral cancer, in animal models (Solt *et al*., 2003; Shabany *et al*., 2002). In addition, PEITC displays minimal or no toxicity toward normal cells, thus making this class of compounds ideal chemopreventive agents against various malignancies (Trachootham *et al*., 2008; Musk *et al*., 1995). The chemopreventive and therapeutic effects of PEITC on lung cancer in smokers are

currently being explored in clinical studies. Isothiocyanates are best known for their antioxidative, anticancer chemotherapeutic, chemopreventive, anti-angiogenic, and antibiotic properties. In vitro, PEITC increases caspase 3 activity and cleavage of poly (ADP)-ribose polymerase (PARP), inducing caspase-mediated apoptosis in Jurkat T cells and other cellular models. PEITC increases activation of JNK1, one potential mechanism behind its regulation of phase II detoxifying enzyme gene expression. The LD50 of PEITC for female rats was 1.47 g/kg. The maternal body weight gain and the number of implanted and live fetuses were decreased with the increase of PEITC dosage given during pre-implantation period. There was also a dose-dependent effect of PEITC given during post-implantation period on fetal weight/growth and placental weight. No toxicity on the organ weight of pregnant rats was observed. Given at different duration of gestation PEITC exhibited some embryo-toxicity on pregnant rats, and the no observable adverse effect level was 15 mg/kg (Liu *et al*., 2011).Over the last decade, the therapeutic effects of Phenethyl isothiocyanate on various cancer types and Alzheimer's disease have been confirmed by clinical trials (Yang *et al*., 2016). Additionally, PEITC decreases levels of Bcl-2 and increases levels of Bax, also decreasing the mitochondrial membrane potential and inducing intracellular influx of free Ca2+, resulting in cell death. This compound decreases oxidation of carcinogen NNK and increases activity of NADPH: quinone oxidoreductase and glutathione S-transferase in vitro and in vivo. In glioma cells, PEITC alters PI3K/MAPK signaling to inhibit accumulation of HIF-1α and secretion of VEGF during hypoxia. Initially, phenethyl isothiocyanate was shown to be cytotoxic to the Jurkat T lymphoma cell line with an LD50 of 7.4 μM. Bcl-2 expression had little protective effect, and even greater than 50-fold overexpression only increased the LD50 to 15.1 μM. Morphological and biochemical assays indicated that death still occurred by apoptosis despite overexpression of Bcl-2. (Annis *et al*.,2004). PEITC inhibited the migration of tumor cells to the brain after injection into the heart of mice, limiting the growth of metastatic brain tumors (Gupta *et al*., 2013). To fuel their rapid growth, invasive tumors must also develop new capillaries from pre existing blood vessels by a process known as angiogenesis. Isothiocyanates have been shown to prevent the formation of capillary-like structures from human umbilical endothelial cells. Isothiocyanates likely inhibit the expression and function of hypoxia

inducible factors (HIFs) that control angiogenesis, as reported in endothelial cells and malignant cell lines (Cavell *et al*., 2011) PEITC inhibited androgen receptor (AR) transcriptional activity in prostate cancer cells by repressing miR-141 expression and miR-141-mediated downregulation of small heterodimer partner (shp), a repressor of AR (Xiao *et al*., 2012). In this study, we initially established a high fat diet (HFD) induced obese rats in order to determine whether obesity affects declines in male fertility as well as serum reproductive hormone levels and disrupts testicular morphology. Furthermore, during spermatogenesis in obese rats testicular changes in relevant biomarkers of blood-testis barrier (BTB) function was studied

Objectives

- **Assessment of fermented pork high fat diet (saum) and its implications on biochemical parameters, sperm quality, spermatogenesis and steroidogenesis, oxidative stress and apoptosis.**

- **Ameliorative effects of phenethyl isothiocyanate on fermented pork high fat diet (saum) induced metabolic alterations and reproductive toxicity.**

Materials and methods

Animal ethics

Male Wistar albino rats (150–200 g, 13 weeks old) were inbred in the Animal Care Facility at the Department of Zoology, Mizoram University, Aizawl, Mizoram, India. Pathogen-free polypropylene cages (421×290×190 mm) and bedding materials were used to house experimental rats in an environmentally controlled chamber (23±1 °C; relative humidity, 50±10%; photoperiod, 12L:12D). The study was approved by the Institutional Animal Care Committee of Mizoram University (Approval Number: MZUIAEC/2018/15 dt. 26/03/2018) and conducted in accordance with the ARRIVE and NIH guidelines for the care and use of laboratory animals. All the experiments were conducted with minimal suffering and distress.

Experimental design

After seven days of acclimation, rats were randomly divided into 4 groups (n=20):Group 1: Control rats fed with standard pellet diet (Carbohydrate-65%, Protein-24%, Fat-11% and Total energy-12.56 kJ/g), Group 2: FPF-M, a low-calorie and low-fat diet (Carbohydrate-65%, Protein 20%, Fat 15%, and Total energy 14.21 kJ/g).), Group 3: FPF-M, moderate calorie and moderate-fat diet (Carbohydrates 50%, Protein 20%, Fat 30%, and Total energy 17.5 kJ/g), and Group 4: FPF-M, highcalorie and high-fat diet (Carbohydrate-20%, Protein-20%, Fat-60% and Total energy-23 kJ/g) for 30, 60 and 90 days respectively. Based on the energy contentanother experiment was done in which rats were randomly divided into 4 groups(n=20): Group 1: Control rats fed with standard pellet diet (Carbohydrate- 65%,Protein-24%, Fat-11% and Total energy-12.56 kJ/g), Group 2: FPF-L, a low- calorie and low-fat diet (Carbohydrate-65%, Protein 20%, Fat 15%, and Total energy
14.21 kJ/g).), Group 3: FPF-M, moderatecalorie and moderate-fat diet (Carbohydrates 50%, Protein 20%, Fat 30%, and Total energy 17.5 kJ/g), and Group4: FPF-H, high-calorie and high-fat diet (Carbohydrate-20%, Protein-20%, Fat-60%and Total energy-23 kJ/g). Rats were fed the diets and water ad libitum twice daily at 9:00 AM and 4:00 PM for 90 days. Another experiment was performed in which rats were divided into 6 groups fed with FPF diet and PEITC. Group 1: Control rats fed with standard pellet diet (Carbohydrate-65%, Protein-24%, Fat-11% and Total
35 energy-12.56 kJ/g), Group 2: FPF-M diet (fermented pork fat), Group 3: PEITC 100mg/kg, Group 4: PEITC 200mg/kg, Group5: Fermented pork fat +PEITC 100mg/kg, Group 6: Fermented pork fat +PEITC 200mg/kg. Routine analyses (body weight, food and water consumption, and rectal temperature) were performed at regular intervals. After the experiment, the rats were fasted overnight, anesthetized (ketamine, 60 mg/ kg), euthanatized, and serum was collected and stored at -20 °C for hormonal and biochemical analyses. Livers, kidneys, testes, and accessory sex organs, as well as fat contents (epididymal, retroperitoneal, peri-renal, mesenteric, and dorsal subcutaneous) were excised, weighed, and stored at -80 °C for further use. The left testis from each group was fixed in Bouin's fixative for 24 h for histological and immunohistochemical evaluation, while the right testis was preserved for biochemical assay

FPF, diet composition, FAME and TAG analysis The production and consumption of fermented pork fat (FPF) has expanded throughout the world as well as Northeast India as a source of daily food, which leads to the progress of multifactorial diseases, chronic non-communicable diseases, a global socio-economic and health problem, which affects human health (Emelyanova *et al*., 2019; Lebret and Candek-Potokar., 2022). Sa-um, a high fat diet which is obtained from pork fat, is used as flavour enhancer in Mizo cuisine. The high fat diet, an indigenous fermented pork fat is a product in Northeast India, especially in Mizoram, is prepared using methods based on cultural traditions under cottage industrial scale and as a result of variation in the organoleptic, nutritional and product quality (De Mandal *et al*., 2018; Deka *et al*., 2021). Fats of pigs are mainly collected from the inner abdominal portion and sometimes fats from other parts of the body, it is then torn/chopped into pieces and cooked and are placed in a special containercalled sa-um bur, which is prepared from the dried fruit of the plant ûm bottle gourd (*Lagenaria siceraria)*. The container is then placed over the fireplace andapproximately after three days or even longer which is called Sa-um, (fermented) and is ready for use in the preparation of other foods such as bawl, bai, etc. (Lalthanpuii *etal*.,2015). FPF is rich in fat content (90 – 95%) with high calorific value (830 Kcals/100 g) and comprises of pathogenic organisms such as *Clostridium* (7.61%),*Bacteroides*(4.57%), *Oscillospira* (4.15%), *Corynebacterium* (1.80%), *Megamonas*(1.52%), *Faecalibacterium* (1.38%),*Proteus* (1.38%), *Ruminococcus*(1.24%), and *Prevotella*, which can have adverse health effects in human (De Mandal *et al*.2018). The nutrients and mineral composition of FPF were determined according to AOAC methods (Association of Official Analytical Chemists, 2019). FPF-diet composition and analysis of FAME and TAG composition Three FPF diets were developed, and their respective caloric composition, percentage contribution of calories from proteins, carbohydrates, and fat are presented. Fatty acid methyl esters (FAME) composition of the diets was determined gas chromatography-mass spectrometry (a TRACE GC–MS with a Polaris Q mass spectrometer, Thermo Finnigan, USA)(Aldai et al., 2006; Liu et al. 2010; Li *et al.*, 2021). A capillary column (J&W DBWAX, 30 m×0.25 mm I.D., 0.25 mm flm thickness), helium carrier gas (fow rate 1.0 mL/min), oven temperature (60— 250 °C at a rate of 4 °C/min) and 1 µL sample(1:9 with hexane) were used for GC analysis (1:10 split ratio, 24.79 psi,

inlettemperature 230 ℃, solvent delay time 5 min, and scanning at 30–450 m/z with electron energy at 70 eV and 0.58 s/scan velocity). FAMEs were separated at a constant flow with an oven program of initially 50 ℃ for 2 min, followed by an increase in temperature of 10 ℃ per minute up to 200 ℃ and maintained at 200 ℃ for 10 min, and finally an increase in temperature of 10 ℃ per minute up to 220 ℃ and maintained at 220 ℃ for 15 min. The transfer line was maintained at 230 ℃. The ion trap mass spectrometer was operated under electron ionization (EI) mode (Liu *et al.*, 2010). Solutions of 16 individual triacylglycerol (10 mg TAG:10 mL dichloromethane w/v) standards (Table 1) were prepared and 1 µL was used for GC analysis [Thermo Scientifc Trace 1300 gas chromatograph, capillary column (RTX-65TG, 30 m×0.25 mm i.d. 0.1 µm), split ratio of 1:30, oven temperature (250—360 ℃ at 4 ℃/min), FID detector, carrier gas (hydrogen, 99.9%, fow rate 1.5 mL/min)]. Trinonadecanoyl-glycerol (NNN) was used as an internal reference.The validation parameters (ftness of analytical curves, linearity, recovery, limit of detection, limit of quantitation, precision, relative standard deviation and repeatability) were computed using the peak area. Peak identifcation was based onthe elution profle of known FAME chromatographic standards (fatty acid methyl esters, C4eC24, Nuchek Prep, CDDE-GLC-617-50MG, USA) and previous reports (Aldai *et al.,* 2006; Liu *et al.*, 2010; Li *et al.,* 2021). Relative quantifcation was normalized with the sum of the detected species and is shown as mg/g of total species.

Anthropometrical and nutritional measures

Body length (nose-to-anus), absolute and relative organ weights, gonadosomatic index, body mass index, Lee index, food and water consumption, body fat mass distribution, specifc rate of body mass gain, energy intake, feed effciency, and rectal temperature were measured. (Bernardis, 1970; Novelli *et al.,* 2007).

Sperm analysis and acridine orange staining

The daily sperm production (DSP) was calculated as the number of homogenizationresistant spermatids. Sperm motility, viability, morphology, epididymal sperm concentration, sperm DNA damage (acridine orange staining, AO), sperm head and tail abnormalities, and sperm transit time were evaluated (World Health

Organization1999). The dsDNA/ssDNA ratio was calculated as the number of normal dsDNA (green fuorescent) divided by the number of damaged ssDNA (yellowish orange/red fuorescent)

Insulin resistance, hepato-renal function and lipid profle

Serum insulin, glucose, aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), creatinine, urea, total cholesterol (TC), HDL, TAG, and leptin levels were estimated using the procedure outlined in commercial diagnostic kits (Coral Clinical systems, Goa, India). The Homeostasis Model Assessment of Insulin Resistance Index (HOMA-IR), Pancreatic β-Cell Function (HOMA-β), AST:ALT Ratio, Blood Urea Nitrogen, BUN:Creatinine Ratio, VLDL, LDL, Cardiac Index (CI), Atherogenic Index (AtI), and Coronary Artery Index (CAI) were computed (Ajiboye *et al.*, 2016)

Serum and testis reproductive hormone assay

Serum and testicular testosterone (Labor Diagnostika Nord, Germany), serum estradiol (DiaMetra kit, Italy), serum luteinizing hormone (LH, DiaMetra kit, Italy),and serum follicle stimulating hormone (FSH, MyBiosource, USA) hormones were estimated using ELISA kit according to the manufacturer's instructions. Serum and testis infammatory markers Serum interleukins (IL-1, IL-6, and IL-10), leukotriene B4 (LTB4), and prostaglandin levels were assessed using ELISA kits (Bioassay Technology Laboratory, China) and the testicular levels of nitric oxide (NO), myeloperoxidase (MPO), lactate dehydrogenase (LDH), tumour necrosis factor-alpha (TNF-α) were estimated using ELISA kits (R&DSystems, Thermo Fisher Scientifc) based on the manufacturer's instructions. Testis oxidative stress markers The levels of conjugated dienes, lipid hydroperoxides and malondialdehyde (Reilly and Aust., 2001), protein carbonyl (Levine *et al.*, 1990) and the fragmented DNA (Burton, 1956) were determined according to the protocols described earlier. The enzymatic activity of antioxidant enzymes (CAT: catalase, SOD: superoxide dismutase, GST: glutathione S transferase and GSH: reduced glutathione and GSSG: glutathione disulfde) was estimated in the testicular extract using commercial kits (ELBA Science, USA and Cayman Chemical Company, USA) according to the

standards and protocols provided by the manufacturer. GSH:GSSG ratio was calculated to measure the mitochondrial or cellular redox state.

Serum FAME profiles

Serum FAME levels in rats fed diferent FPF-diets, along with control-diet, were analyzed by GC–MS as described previously in the section ‑FPF-diet composition and analysis of FAME and TAG composition‖ (Aldai *et al.*, 2006; Liu *et al.*, 2010; Li *et al.*, 2021).

Testis histology and histo-morphometrics

The left testes were harvested, fxed in Bouin's fxative overnight, followed by paraffin embedding, sectioned at 5 µm thicknesses, and counterstained with hematoxylin and eosin (H&E) for histopathological analysis. Testis tissue damage was assessed using the blindfold method and scored in all tissue Sects. (10 random non-overlapping felds/diet group) at 10×and 40×magnifcations, respectively. A lesion scoring system was employed to measure the testis damage (Abdelhamid *et al.*, 2020). Spermatogenesis impairment was evaluated in terms of histomorphological changes in the seminiferous tubules (JTBS: Johnsen’s mean testicular biopsy score, MSTD: mean seminiferous tubule diameter, TAT: tunica albuginea thickness, STEH: seminiferous tubule epithelial height, IS: interstitial space and TL: tubular lumen) and the quantifcation of germ cells (spermatogonia, spermatocytes, spermatids, Sertoli cells) and Leydig cells in the testis was performed (10 random nonoverlapping felds per diet group), respectively. The Tubule Differentiation Index (TDI, %) was calculated as the percentage of tubules presenting Type A or Type B diferentiation in 600 seminiferous tubules for each treatment group (5 tissue sections per group) (Johnsen 1970; Russell *et al.*, 1990).

Immunohistochemistry

Immunohistochemical staining was performed on the testis tissue using PCNA (1:100, Santa Cruz Biotechnology Inc., USA), StAR (1:2000, St. John’s laboratory, London, UK), 3β-HSD (1:200, Santa Cruz Biotechnology, Dallas, USA), LHR (1:500, Santa Cruz Biotechnology, Dallas, USA), BAX (1:50, Elabscience, China)

and BCL-2 (1:500, Elabscience, China) polyclonal rabbit primary antibodies anda secondary antibody HRP-conjugated goat-anti-rabbit (1:500, Merck Specialties Pvt.Ltd, Mumbai, India), along with 0.05% diaminobenzidine solution, to confrm the effect of FPF-diet on testicular steroidogenesis, cell proliferation and apoptosis. The area immunostained for the antibodies was obtained using ImageJ, and the percentage area of staining at 10×and 40×magnifcations was calculated. The number of germ cells and Leydig cells that had positive reactions to PCNA, BAX, BCL-2, StAR, 3β-HSD, and LHR were manually counted and computed as the ratio of positively reacted cells to the total number of cells multiplied by 100 (Jeremy *et al.,* 2019).

TUNEL assay

Testis germ cell apoptosis was evaluated by TUNEL assay using the Apo-Brdu-IHC in situ DNA fragmentation assay kit (BioVision Inc., USA) following the manufacturer's instructions. The TUNEL-positive apoptotic cells were identifed as dark brown cells. The apoptotic index (AI) was calculated as AI (%)=(number of apoptotic cells/number of total cells)×100)

Univariate and multivariate analyses

The Kolmogorov and Smirnof test, Levene test, one-way analysis of variance (ANOVA) with Tukey's multiple range post hoc test, and correlation and regression analyses were performed to detect signifcant diferences ($p<0.05$) between the treatment groups. A multivariate analysis of variance (MANOVA) comprising principal component analysis (PCA), hierarchical cluster analysis (using an Euclidean distance measure), and detrended correspondence analysis (DCA) was conducted using PAST (PAleontological Statistics, version 4.03) software (Hammer *et al.,* 2001).

Westernblotting

Western blot analysis for StAR, **3 beta Hsd, BCl2, HSP 70** protein was undertaken as previously described by (Clark *et al.,,* 1994), except that 250 mg aliquots of mitochondrial protein was analyzed in each gel lane and a Protean II system wasused

for electrophoresis.5 samples were selected randomly from each groups. β tubulin was used as loading control. Protein estimation was done by the protocol of (Lowry *et al.,,* 1951). 50µ of protein was loaded in each lane followed by separation using SDS-PAGE which is then electroblotted in PVDF membrane (Millipore, Massachusetts, USA). The membrane was blocked for 1hour at 37°C with 5% bovine serum albumin (BSA) solution. The membrane was incubated with anti-rabbit andanti-mouse polyclonal antibody (1:1000) for overnight at 4°C, which is then followed by incubation with alkaline conjugated anti-rabbit and anti-mouse for 4 hours at a ratio of (1:1000). After washing with PBS, the membrane was developed usingECL (BIO-RAD, Cat.# 170-5060,USA). All blottings were done under samecondition. The result is the representative of one western blot data. The densiometry analysis was done using Image J software and Graph pad prism

Analysis of gene expression

In a total volume of 25 µl [1 µl synthesized cDNA, 1 µl each primer (10 pM), 12.5 µl PCR master mix and 9.5 µl sterilized deionized water], PCR was conducted. The PCR cycling conditions was set as follows: Initial denaturation for 1 cycle at 95˚C for 4 min, followed by 27 cycles (each consisting of denaturation at 94˚C for 1 min, annealing as stated in Table I for each gene, and extension at 72˚C for 1 min) with a final extension step at 72˚C for 7 min. G3PDH were used as an internal control. PCR products were separated by 1.5% agarose gel electrophoresis for 30 min and stained with ethidium bromide in Tris-borate-EDTA buffer. The gels were visualized under ultraviolet light and subsequently photographed using gel documentation system. The band intensities were densitometrically quantified and calculated. (Alkhedaide *et al.,* 2016).

Summary

- The present study was designed to examine the body and organ weight, food and water consumption, sperm parameters, oxidative stress and antioxidant effects, biochemical parameters, histological and immunohistochemical, western blotting and RT-PCR analysis of PEITC on saum induced testicular toxicity in chronic (60 days) treatment
- The higher the dose of saum intake by the rats, the higher is the food and water consumption, increased in body weight and organ weight, decreased in motility % of sperms.
- The rectal temperature was effected with either of the treatment groups, also the blood glucose levels was significantly higher in the saum treated rats
- Elevated results of lipid peroxidation was observed in saum treated groups and decreased in PEITC treated groups which results in the decrease of the activity of the antioxidant enzymes CAT, SOD, GSH and GST in the chronic treatment.
- AST increased with Saum treated groups and decreased with control and PEITC treated groups. ALT increased with increased treatment with saum groups and decreased with control and PEITC treated groups. AP increased with saum treated groups and decreased with control and PEITC treated groups. Their increased may be mainly due to liver damage in 60 days treatment of saum.
- The significant increase in the creatinine and urea content of the serum following the administration of saum may be attributed to compromise of renal function capacity. Cholesterol levels decreased in saum treated groups which may be due to decrease in the level of testosterone.
- Interrupted spermatogenesis due to saum interference significantly increases the apoptotic rate via abnormal protein expressions of BCl 2 and caspase 3 genes
- PEITC is an essential nutrient which plays a major role in cellular energy production and has been considered a promising cytoprotective agent.
- PEITC was injected intraperitonially to rats at 100 mg/kg and 200mg/kg for 60 days which greatly affected and increased the sperm motility, sperm count and daily sperm production (DSP).
- The sperm count and daily sperm production decreased with increase in the saum treated groups since it has toxic effect in the testis, which may ultimately lead to

reduced fertility but increased with the PEITC treated groups.

- PEITC is a very important antioxidant which helps in DNA repair, germ cell recovery and sertoli cell metabolism.
- The sperm abnormalities in saum group was significantly higher as compared to PEITC groups.
- Oxidative stress was reduced by PEITC and antioxidant enzymes were elevated in chronic treatment groups.
- The changes caused by saum with respect to biochemical parameters was attenuated by PEITC exposure.
- PEITC treatment greatly improved the damaged caused by saum induced histopathological changes in rat testis in treatment groups.
- Immunohistochemical changes were observed in HFD treatment resulting in the degeneration of proliferating cells in saum treated groups and regeneration of positive cells in PEITC treated groups.
- Our work provides a foundation for understanding how obesity may affect male fertility and future functional studies should be directed towards providing support for these potential players.