INFLUENCE OF DIETS HAVING DIFFERENT TYPES OF FAT ON ANTIOXIDATIVE ENZYMES AND DIET INDUCED ENDOPLASMIC RETICULUM STRESS

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILLMENT OF THE REQUIREMENT
FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE
IN
ANIMAL BIOCHEMISTRY

By

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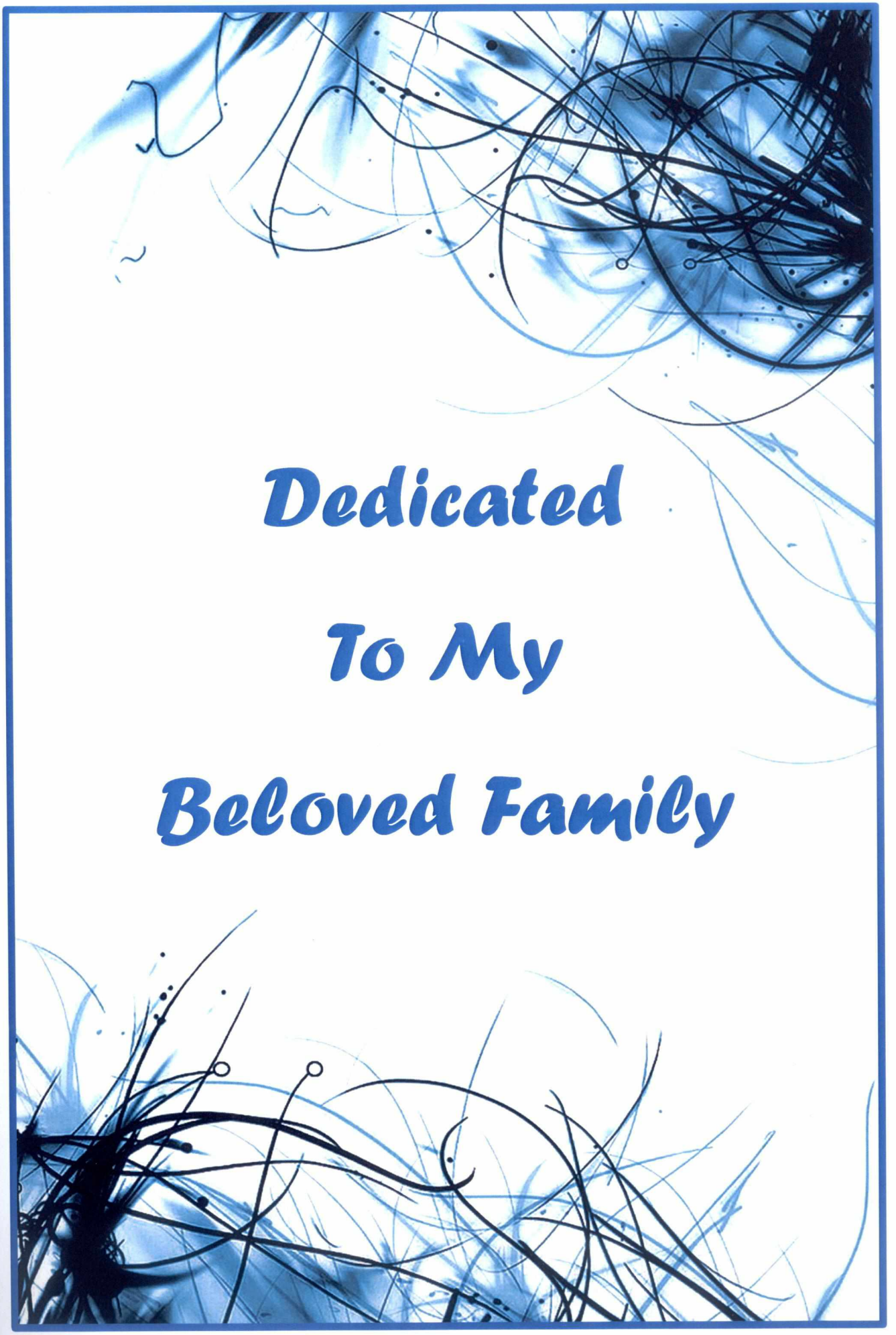
Dedicated
To My
Beloved Family

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This is to certify that the thesis entitled "**INFLUENCE OF DIETS HAVING DIFFERENT TYPES OF FATS ON AN ANTIOXIDATIVE ENZYMES AND DIET INDUCED ENDOPLASMIC RETICULUM STRESS**" submitted by **YOGESH NAGAR** in partial fulfillment of the requirement for award of the degree of **MASTER'S OF SCIENCE** in **ANIMAL BIOCHEMISTRY** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana) is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Date: 30.6.17

(Dr. GAUTAM KAUL)
MAJOR ADVISOR

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Date: *(Yogesh)*

Abbreviations

°C	degree Celsius
@	at the rate of
ANOVA	Analysis of Variance
ALA	α-linoleic acid
ATF4	Activating Transcription Factor 4
ATF6	Activating Transcription Factor6
BMI	Body Mass Index
bp	base pair
CAT	Catalase
CHOP	CCAT\Enhancer-Binding Protein Homologous Protein
cDNA	complementary DNA
DEPC	Diethyl pyrocarbonate
DHA	Docosahexaenoic acid
DIO	Diet Induced Obesity
DAGs	Diacylglyerols
ER stress	Endoplasmic Reticulum stress
ER	Endoplasmic Reticulum
FFA	Free Fatty Acids
GPx	Glutathione Peroxidase
Grp78	Glucose regulated protein 78kD
IRE-1	Inositol requiring Enzyme-1
h	hour
HDL	High Density Lipoprotein
HFD	High Fat Diet
H_2O_2	Hydrogen Peroxide
kg	Kilogram
LDL	Low Density Lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
mg/dl	milligram/decilitre
min	Minute
ml	milliliter
mM	Millimolar
MDA	Malonaldehyde

NAFLD	Non-Alcholic Fatty Liver Disease
NO	Nitric Oxide
ng	Nanogram
Nm	Nanometer
PERK	PKR like ER Kinase
qRT-PCR	quantitative Real Time PCR
ROS	Reactive Oxygen Species
SEM	Standard Error of Mean
SFA	Saturated Fatty Acid
SOD	Superoxide Dismutase
T2DM	Type 2 diabetes mellitus
Taq	*Thermus aquaticus*
TC	Total cholesterol
TGs	Triglycerides
TAGs	Triacylglycerols
TNF	Tumor Necrosis Factor
TBARS	Thiobarbituric Acid Reactive Substance
UPR	Unfolded Protein Response
VLDLs	Very Low Density Lipoprotein
WHO	World Health Organization
wk	Week

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ABSTRACT

In accordance with World health organization (WHO), obesity or overweight is defined as accumulation of abnormal or excessive fat that may impair health. Body Mass Index (BMI) is a common way for measuring the obesity in individual according to their age, gender and size. The fundamental cause of obesity is imbalance between energy intake and energy dissipation. Worldwide, there has been increase in consumption of food that contains high fat content and increase in sedentary way of lifestyle are being recognized as causative factors of obesity. A study conducted in 2015, revealed that India ranked second afterwards China in highest number of obese children (Afshin *et al.,* 2017). Obesity is major risk factor for metabolic syndrome (Furukawa *et al.*), insulin resistance, type-II diabetes, Non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases, asthma and even cancer also (Legris *et al.,* 2017). Obesity is also a contributing factor in progression of oxidative stress and impairment of antioxidative enzyme activities (Vincent *et al.,* 2006). This diet induced oxidative stress leads to the mitochondrial uncoupling, decrease in anti-oxidative metabolism and induction of Endoplasmic Reticulum stress(ER stress).Oxidative stress induced by high fat diet (HFD) causes change in endoplasmic reticulum redox homeostasis which leads to the reduction in ER chaperone activity. This leads to the imbalance between protein folding capacity of ER and client protein load, causing accumulation of unfolded or misfolded protein in ER, this phenomenon is known as ER stress. The accumulation of unfolded protein in ER leads to activation of response known as ER stress response or Unfolded Protein Response (UPR) which is an evolutionary conserved mechanism. But if ER stress level is high it even guides the cells towards apoptosis. In our study we used C57BL/6 male mice for *in-vivo* study of 16 weeks. The different dietary fat based diet was given in two concentration of fats i.e. low fat diet (7% fat by weight) high fat diet(35% by weight). Fasting blood glucose level were seen significantly higher in all high fat diet groups in corresponding to their low fat diet groups. In case of mustard oil based high fat diet (HFD-M) showed significantly lower liver weight than corresponding low fat diet while in kidney weight, HFD-M had significantly lower kidney weight than cow ghee based high fat diet (HFD-C) and lard based high fat diet(HFD-L).The adipose tissue weight after 16 week of feeding is higher in all high fat diet groups than their corresponding low fat diet groups except in case of mustard based high fat diet which have lower adipose tissue weight than its corresponding low fat diet group. Serum Total cholesterol levels is significantly higher in high fat diet groups of cow ghee, buffalo ghee and lard than their corresponding low fat diet groups and mustard oil and soyabean oil based high fat diet groups. Serum triglycerides levels is significantly higher in high fat diet groups of cow ghee, buffalo ghee and lard than their corresponding low fat diet groups and mustard oil and soyabean oil based high fat diet groups. Similar pattern was observed in LDL, VLDL,HDL levels and atherogenic index. Superoxide dismutase activity in liver was assessed lower in all high fat diet groups corresponding to their low fat diet groups. Catalase activity was significantly lower in HFD-L corresponding to its low fat diet group, while in other high fat diet groups catalase activity was lower than their corresponding low fat diet groups. Glutathione peroxidase activity was lowered in all high fat diet groups than corresponding low fat diet groups. Further the mRNA expression of endoplasmic reticulum stress marker genes, Grp78, CHOP, ATF4 and ATF6 expression were found positive in mustard oil and soyabean oil based high fat diet in comparison with other high fat diets.

□□त-आ□सीकारक एंजाइम□और आहार □□रत एंडो□ला□मक रे□टकुलम तनाव पर □व□भ□न □कार के वसा वाले आहार का □भाव

सार

□व□व □वा□□य संगठन (ड□□यूएचओ) के अनुसार, मोटापा या अ□धक वजन असामा□य या अ□यधिक वसा के संचय के □प म□पर□भाषित □कया जाता है जो □वा□□य को खराब कर सकता है। शर□र □□यमान सूची (बीएमआई) □य□तय□क□आयु, □लंग और आकार के अनुसार मोटापे को मापने का एक सामा□य तर□का है। मोटापे का मूल कारण ऊजा□का सेवन और ऊजा□अप□यय के बीच असंतुलन है। दु□नयाभर म□भोजन क□खपत म□वृ□ध हुई है जिसम□उ□च वसा वाले पदाथ□और जीवनशैल□के गत□हा□न तर□के से वृ□ध शा□मल है जो मोटापे के □रक कारक□के □प म□पहचाने जा रहे ह□। 2015 म□□कए गए एक अ□ययन म□पता चला है □क सबसे □यादा मोटे ब□च□क□सं□या म□भारत का चीन के बाद दूसरे □थान पर है। मोटापा मेटाबो□लक □सं□ोम, इंसु□लन □□तरोध, टाइप -2 मधुमेह, गैर अ□कोहल वसायु□त जिगर क□बीमार□ (एनएएफ़एडीडी), □दय रोग, अ□थमा और यहां तक □क क□सर के □लए □मुख संकट कारक है। ऑ□सीडे□टव तनाव और एंट□ऑ□सीडे□टव एंजाइम गत□वधिय□क□हा□न क□ □गत□म□मोटापा भी एक योगदानकार□ कारक है। यह आहार □□रत ऑ□सीडे□टव तनाव माइटोकां□□यल □व□छेदन क□ओर जाता है, एंट□ऑ□सीडे□टव उपापचय म□कमी और ए□डो□ला□मक रे□टकुलम तनाव (ईआर तनाव) का □रण होता है। उ□च वसायु□त आहार (एचएफडी) □वारा □□रत ऑ□सीडे□टव तनाव ए□डो□ला□मक रे□टकुलम रेडॉ□स होमो□टे□सस मा□पर□वतन□ का कारण बनता है जो ईआर संर□□ का गत□वधि म□कमी का कारण बनता है। इससे ईआर और गाहक □ोटा□न भार क□□ोटा□न तह □मता के बीच असंतुलन क□ओर बढ़ जाता है, जिससे ईआर म□खुला या मै□सफ□ड □ोटा□न का संचय होता है, इस घटना को ईआर तनाव के □प म□जाना जाता है। ईआर से उ□प□न □ोटा□न का संचय ईआर तनाव □□त□□ या या अनफ़ो□ड □ोटा□न □र□पांस (यूपीआर) के □प म□जाना जाने वाले □□त□□ या के स□□ यण क□ओर जाता है जो एक □वकासवाद□ संर□□ त तं□ है ले□कन अगर ईआर तनाव का □तर उ□च होता है तो यह को□शकाओं को को□शका □छय क□तरफ □नदा□शत करता है। हमारे अ□ययन म□हमने 16 स□ताह के इन-□ववो अ□ययन के □लए सी 57 बीएल / 6 नर चूह□का इ□तेमाल □कया। □व□भ□न वसा आधा□रत आहार को दो एका□ ता म□□दया गया था, अथा□□ कम वसा वाले आहार (वजन से 7% वसा) उ□च वसा वाले आहार (वजन का 35%)। □बना भोजन श□क□रा □तर, कम वसा वाले आहार समूह□के मुकाबले अ□धक वसा आहार वाले सभी समूह□म□काफ□अ□धक था। सरस□के तेल आधा□रत उ□च वसा वाले आहार (एचएफडी-एम) के मामले म□ कम वसा वाले भोजन क□ तुलना मे जिगर का वजन काफ□ कम जब□क गुद□ का वजन एचएफडी-एम गाय घी आधा□रत उ□च वसायु□त आहार (एचएफडी-सी) और चरबी वाले उ□च वसा आहार (एचएफडी-एल) क□तुलना म□कम था । 16 स□ताह के भोजन के बाद वसा ऊतक वजन सभी उ□च वसा वाले आहार समूह□म□उनके संबं□धत कम वसा वाले आहार समूह□क□तुलना म□अ□धक है, म□□त□क आधा□रत उ□च वसा वाले आहार के मामले म□ जो कम वसा ऊतक वजन इसके संबं□धत कम वसा वाले आहार समूह क□तुलना म□सीरम कुल कोले□□ॉल का □तर गाय घी, भ□□ा घी और चरबी के उनके वज़न कम वसा वाले आहार समूह□और सरस□के तेल और सोयाबीन तेल आधा□रत उ□च वसा वाले आहार समूह□क□तुलना म□उ□च वसा वाले आहार समूह□म□काफ□अ□धक है। सीरम □इ□लसराइ□स का □तर गाय घी, भैंसो घी और चरबी के उनके वज़न कम वसायु□त आहार समूह□ और

सरस□के तेल और सोयाबीन तेल आधा□रत उ□च वसा वाले आहार समूह□क□तुलना मा□उ□च वसा वाले आहार समूह□मा□काफ□आ□धक है। एलडीएल, वीएलडीएल, एचडीएल □तर और एथेरोजे□नक इंडे□स मा□इसी तरह क□ि□थ□त देखी गई थी। जिगर मा□सुपरऑ□साइड □ह□स□यूटेज गा□त□व□ध को कम वसा वाले आहार समूह□से संबं□धत सभी उ□च वसायु□त आहार समूह□मा□कम मू□यांकन □कया गया था। कम वसा वाले आहार समूह के मुता□बक एचएफ़डी-एल मा□क्टालेस गा□त□व□ध काफ□कम थी, जब□क अ□य उ□च वसा वाले आहार समूह□म□ उनके कम वसा वाले आहार समूह□क□तुलना मा□कमटाल गा□त□व□ध कम थी। □लूटा□थयोन पेरो□साइड क□ गा□त□व□ध कम वसा वाले आहार समूह□के मुकाबले सभी उ□च वसा वाले आहार समूह□म□कम थी। इसके अलावा एंडो□ला□मक रे□टकुलम तनाव मा□कर जीन क□ एमआरएनए अ□भ□य□ित, □ुप 78, सीओओपी, एटा□एफ 4 और एटा□एफ 6 क□ अ□भ□य□ित सरस□के तेल और सोयाबीन तेल पर आधा□रत उ□च वसा वाले आहार मा□सकारा□मक □प से पाई गई थी।

CHAPTER –1

Introduction

INTRODUCTION

Diet is the sum of food consumed by the organism having specific intake of nutrients. Diet contains various constituent like proteins, carbohydrates and fats as a major and vitamins, minerals, etc. as minor constituent. The intake of fats in diet is essential because of their high calorific value, natural antioxidant component but the excess of fat used in diet leads to obesity and related metabolic disorders, type 2 diabetes, oxidative stress etc. The different types of fats have different fatty acid component i.e. saturated fatty acid (SFA), unsaturated fatty acids, and their effect on organism is different when consumed in diet. Lard fat which is an animal fat and extracted from the pig adipose tissue. Lard is used around the world as cooking fat because of its easy availability and low cost, but recent study has showed that excess of lard fat in diet is associated with obesity and oxidative stress due to increase in reactive oxygen species(ROS) in adipose tissue and liver. This diet induced oxidative stress leads to the mitochondrial uncoupling, decrease in anti-oxidative metabolism and induction of Endoplasmic Reticulum stress(ER stress). Endoplasmic reticulum is the essential organelle of cell where the protein folding and its attainment of function occurs. Any sort of disruption in endoplasmic reticulum microenvironment leads to the improper folding or unfolding of protein. Oxidative stress induced by high fat diet (HFD) causes change in endoplasmic reticulum redox homeostasis which leads to the reduction in ER chaperone activity. This leads to the imbalance between protein folding capacity of ER and client protein load, causing accumulation of unfolded or misfolded protein in ER, this phenomenon is known as ER stress. The accumulation of unfolded protein in ER leads to activation of response known as ER stress response or Unfolded Protein Response (UPR) which is an evolutionary conserved mechanism. The UPR response initially an adaptive response which tries to restore the ER microenvironment by enhancing the production of chaperone proteins and by degrading the unfolded protein in endoplasmic reticulum. But if ER stress level is high it even guides the cells towards apoptosis.

Mustard oil , Cow ghee and Buffalo ghee which are extensively used in the Indian subcontinent as cooking oil contain different lipid composition than lard. Their effects on

the endoplasmic reticulum stress associated study are not reported. So it would be interesting to study the comparative effect of diets having different fat on antioxidative metabolism, lipid metabolism and diet induced Endoplasmic reticulum stress. Keeping this in view the following objectives have been designed

1. To study the comparative effects of different types of fat rich diet on anti-oxidative enzymes and lipid profile in mice.
2. Analysis of expression pattern of selected genes in liver.

CHAPTER –2

Review of Literature

REVIEW OF LITERATURE

In accordance with World health organization (WHO), the obesity or overweight is defined as accumulation of abnormal or excessive fat that may impair health. Body Mass Index (BMI) is a common way for measuring the obesity in individual according to their age, gender and size. The fundamental cause of obesity is imbalance between energy intake and energy dissipation. Worldwide, there has been increase in consumption of food that contains high fat content and increase in sedentary way of lifestyle are being recognised as causative factors of obesity. A study conducted in 2015, revealed that India ranked second afterwards China in highest number of obese children (Gregg *et al.,* 2017). Obesity is major risk factor for metabolic syndrome (Furukawa S. *et al.,* 2004), insulin resistance, type-II diabetes, Non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases, asthma and even cancer also (Guillemot-Legris O. *et al.,* 2017). Obesity is also a contributing factor in progression of oxidative stress and impairment of antioxidative enzyme activities (Vincent H.K. *et al.,* 2006).

2.1 Dietary fats and their constituents

Fats and oils are essential part of our diet. They are source of robust energy, contains essential fatty acids which are precursors of important enzymes like prostaglandins and transporter of fat soluble vitamins. The major source of fats in diet is vegetable oils, dairy products, fish and meats. Fats and oils are mostly comprises of fatty acids. Saturated fatty acids are hydrocarbon chains having single bonds between carbon atoms, chiefly found in product derived from animal sources. Unsaturated fatty acids characterized by one (monounsaturated) or more (polyunsaturated) double bonds in the carbon chains, mostly found in plant and sea food. *Cis* and *trans* are the another terms included in classification of fatty acids. They are the arrangement patterns of carbon atoms chains across the double bonds. In *cis* form the carbon chains are on the same side of the double bond and in *trans* form chains are arranged on opposite sides of the double bonds. Commonly, vegetable oils like mustard, soybean, sunflower and olive are low in saturated fats and unsaturation found mostly in *cis* configuration while *trans* configuration and highly saturated fats are found in dairy products, meat.

2.1.1 Lard

Lard is derived from adipose tissue of pigs. It is commonly found in diet routine of most western countries like USA, Canada, Mexico and European Countries. Lard major constituents are Triacylglycerol (TAGs), Diacylglycerol (DAGs), free fatty acids (FFAs) and minor constituents like phospholipids, sterols, carotenoids, and fat soluble vitamins (Che Man Y. *et al.,* 2010). Lard is associated with diet induced obesity related studies. Thus, it is heavily endorsed as a research diet constituent. Data available suggest that there is 45.1% monounsaturated fat, 11.2% polyunsaturated fat and 39.2% of saturated fats in 100g of total lipids. No *trans*-fat presence has been found in lard.

2.1.2 Mustard oil

Mustard oil extracted from *Brassica juncea* seeds and leafs. *Brassica juncea* belongs to the *Brassicacaea* plant family. It is also known as Indian Mustard and heavily included in daily diet routine of South Asian peoples like in India, Myanmar, China etc. Mustard oil mainly consists of glycerides like erucic, eicosanoic, arachidic, arachidonic, Ŭ-linolenic, behenic, palmitic and oleic acids and essential oil found in mustard oil belongs to isothiocyanates. It includes allyl isothiocyanate (54.8 - 68.8%), 3-butenyl isothiocyanate (4.8 -5.9%) and phenethyl isothiocyanate (2.4 - 3.4%). They represent more than 62.9% of the total essential oil (Kumar V. *et al.,* 2011).

2.1.3 Milk fats

In India, peoples have incorporated the habit of eating Milk fats like buffalo ghee and cow ghee in their daily diet routine. The lipids or fat present in milk are unique for the species, in terms of lipid biosynthesis and composition of lipids every species differ from each other. Milk carry fat soluble vitamins like A, D, E, K, essential fatty acids like arachidonic and linoleic acids. In terms of milk lipids composition 97-98% was triacylglycerol (TGs) of the total lipid, afterwards comes sterols and like cholesterols and phospholipids.

Table 2.1: Composition of milk lipids

Lipids	Weight (%)
Triacylglycerols	97-98
Diacylglycerols	0.28-0.59
Monoacylglycerols	0.016-0.038
Free sterols	0.22-0.41
Free fatty acids	0.10-0.44
Phospholipids	0.2-1.0
Hydrocarbons	Traces
Sterols esters	Traces

(*Source*: Patton and Jensen, Biomedical aspects of lactation, 1976)

This milk lipids composition varies from species to species like in Bovines especially in India where buffaloes like Murrah, Jaffarabadi, Surti have fat % 6.8, 7.3 and 8.4, while in case of cows like Sindhi, Gir, Tharparker and Sahiwal fat % is found to be 4.9, 4.73, 4.55, and 4.55 respectively.

2.1.4 Soybean oil

Soybean oil is extracted from the seeds of Glycine Max (soybean). Worldwide soybean oil is used as an cooking oil. The lipid composition of soybean oil is given in table below:

Table 2.2: Lipid composition of soybean oil

Lipid composition	Weight (%)
Total Saturated fatty acids	15.65
Total monounsaturated fatty acids	22.78
Total polyunsaturated fatty acids	57.74
Trans fatty acids	0.53

(*Source*: National Nutrient Database for Standard Reference release 28, USDA)

2.2 Excess dietary fat consumption and risk of obesity

Dietary fat have both positive as well as negative impact on individualôs health depending upon the consumption rate. Excess consumption of fat or lipids in daily routine has been linked with several disorders. Major risk is onset of obesity associated with excess consumption of fats regularly. Excess fat consumption and sedentary lifestyle leads to the increase in adipose tissue mass in relation with Body Mass Index (BMI) ratio above normal levels which marks the onset of obesity. The risk of obesity not only depends upon the excess consumption of dietary fats but also on the composition of dietary fats i.e. saturated, monounsaturated or polyunsaturated fatty acids (Yaqoob P. *et al.,* 1995). Excess of saturated fatty acids in diets is associated with increase in body weight, triglyceride and cholesterol levels in serum and triglycerides levels in liver and decrease in adiponectin levels, plasma glucagon levels, insulin sensitivity .While diet high in docosahexaenoic acid (DHA) or Ŭ-linoleic acid (ALA) upregulate the adiponectin levels and downregulate the inflammatory cytokines levels.

2.3 Obesity and associated disorders

Obesity is now recognized as major causative factor in several diseases like atherosclerosis, cardiovascular diseases, type-II diabetes, cancer (Guillemot-Legris O. *et al.,* 2016), and many more. Obesity is associated with increase in adipose tissue mass which leads to hyperlipidemia i.e. increase in total cholesterol (TC) and triglycerides (TGs) levels in serum of rats (Yang R. *et al.,* 2006). Obesity is also linked with increase in free radicals and reactive oxygen species (ROS) which leads to the oxidative stress in humans (Vincent H.K. *et al.,* 2006). Free fatty acids (FFA) elevate the blood glucose levels in obesity which leads to insulin resistance and later on towards diabetes.

2.3.1 Obesity and blood lipid metabolism

Obesity which leads to the hyperlipidemia and progress towards atherosclerosis is initiated by aggregation of excess lipids containing cells in arterial vessels or blood vessels. The activity of enzyme lipoprotein lipase (LPL) is associated with lipid metabolism. LPL production site in body is muscle, adipose tissue, heart and to some

proportion by macrophages also (Yang R. *et al.,* 2006). LPL hydrolyze the crux triglycerides from chylomicron and very low density lipoproteins (VLDLs) (Beisiegel U. *et al.,* 1996) Thus, keep the triglyceride levels low in blood. But in obese condition the activity of LPL decreases leading to increased triglycerides level and decreased level of high density lipoprotein cholesterol (HDL-C).(Yang R. *et al.,* 2006). Adiposity (abdominal or visceral) is linked with elevated plasma free fatty acids (FFA).

2.3.2 Obesity and oxidative stress

Expansion of adipose tissue associated with obesity is been linked to increase in free radicals and Reactive Oxygen Species (ROS) generation in humans and rodent models (Furukawa S. *et al.,* 2006). Obesity causes increase in lipid content (FFA, TGs and TC) in blood which hydrolyzed by the peroxisomes and converted to lipid peroxides which are the source of free radicals and ROS generation thus leading towards oxidative stress.

2.3.3 Oxidative stress

Oxidative stress is the imbalance between tissue oxidants and antioxidant levels (Vincent H.K. *et al.,* 2006). Oxidants include free radicals and ROS. Free radicals are molecules with unpaired electrons that makes them highly reactive to surrounding molecules while ROS are oxygen containing molecule that either may or may not have unpaired electrons, but extensively reactive in tissues. ROS includes hydroxyl radicals, nitric oxide, hydrogen peroxide, superoxide, peroxynitrile and hypochlorite. These effects the functionality of antioxidative enzymes like Superoxide Dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) in tissues and in blood increase the levels of Thiobarbituric Acid reactive substances (TBARS) leads to further increase in production of Malonaldehyde (MDA).

2.3.4 Oxidative stress and antioxidative enzymes activity

During Normal state the activity of antioxidative enzymes like SOD, GPx remains elevated and catalase activity remain comparatively low. But during persistent oxidative stress due to obesity, there is increase in free radicals and ROS which leads to

decrease in activity of SOD, GPx while in case of Catalase, the activity first elevated due increase in substrate i.e. hydrogen peroxide (H_2O_2) but persistent higher levels of H_2O_2 due to oxidative stress leads to exhaustion of catalase activity. Olusi *et al*. (2002) also reported that erythrocyte GPx and SOD activity was lowered in obese persons than non-obese persons.

Figure 2.1: Obesity causes increase in ROS production and decrease in antioxidative enzymes in tissue and White Adipose Tissue (WAT) leading to upregulation of inflammation marker genes PAI-1,TNF-Ŭ,MCP-1 which leads to metabolic syndrome (Furukawa *et al.,* 2006)

Apart from FFAs there are enzymes in body which activity induces increase in oxidant and ROS level and decrease in antioxidative enzymes activity. NADPH oxidase is the major source of endothelial ROS production (Vincent *et al.,* 2006) and minor contribution is given by xanthine oxidoreductase and Nitric Oxide (NO).

The persistent high level of ROS leads to inhibition of antioxidative enzymes activity which further affects the cell organelles like Endoplasmic reticulum, Golgi body etc. In Endoplasmic Reticulum (ER) due to oxidative stress there is a disturbance in normal

activity of ER which leads to accumulation of unfolded proteins in ER, this condition is also known as Endoplasmic Reticulum Stress (ER Stress).

2.4 Endoplasmic Reticulum stress(ER stress)

Endoplasmic reticulum is the site of protein, glucose and lipid metabolism. Endoplasmic reticulum is the organelle where proteins folding and maturation occurs, later on which defines the proteins function. Various insults including obesity which leads to the accumulation of unfolded protein in endoplasmic reticulum lumen, causing ER stress. It is also demonstrated by studies that ER stress play central role in lipogenesis, peripheral insulin resistance type 2 diabetes and obesity (Zhao *et al.,* 2013). But, eukaryotic cells have response system which deals with ER stress known as Unfolded Protein Response (UPR) (Kawasaki *et al.,* 2012) The UPR have three pathways involving three type I transmembrane proteins: double-stranded RNA-activated protein Kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme-I (IRE-1) and activating transcription factor-6 (ATF6).

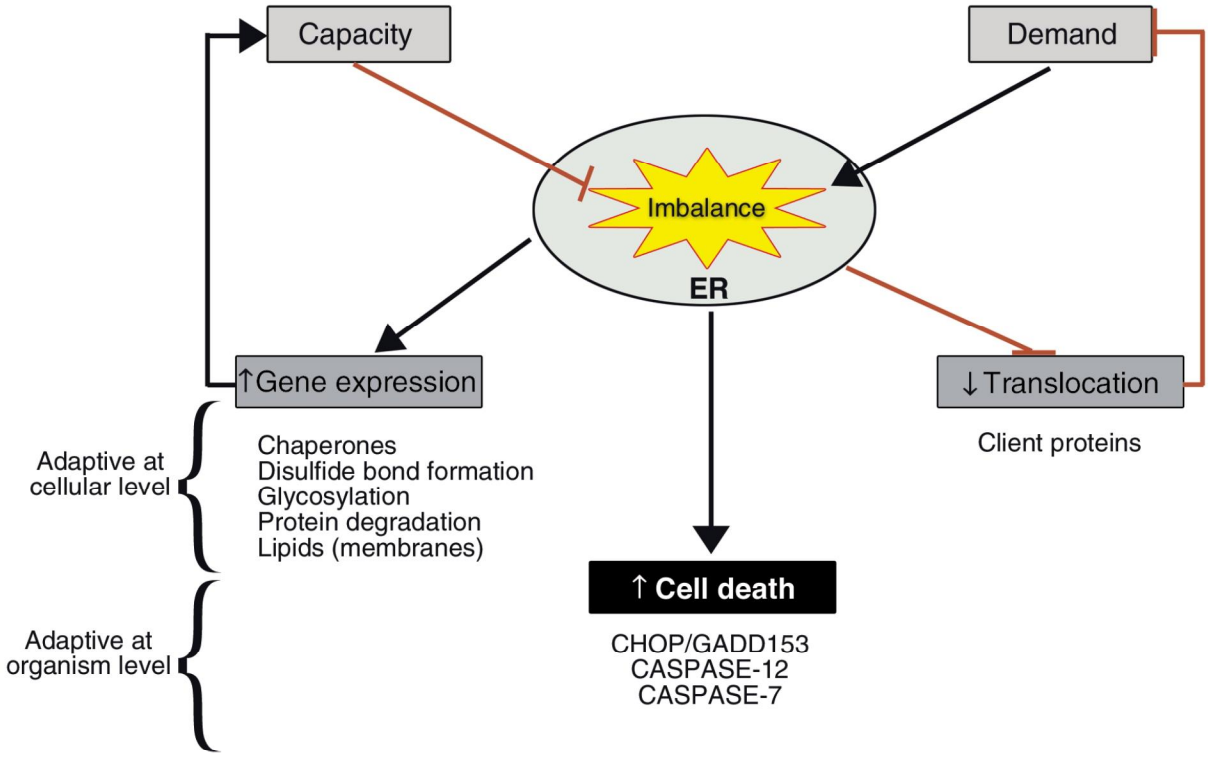


Figure 2.2: Different phases in during Endoplasmic reticulum Stress (Ron D. 2002)

2.4.1 Inositol-requiring enzyme-1(IRE-1)

It cleaves the mRNA encoding for transcription factor X-box binding protein-1(XBP1) (Yoshida *et al.,* 2001). XBP1 up regulates the expression of ER homeostasis regulating genes and itself involved in protein folding, disulfide bond formation and ER associated degradation (ERAD).

2.4.2 Protein kinase RNA (PKR)-like ER kinase (PERK)

It carries out the phosphorylation of the Ŭ-subunit of eukaryotic protein synthesis initiation factor 2 (eIF2Ŭ) inhibiting the hoard of newly synthesized proteins and initiating the expression of the activating transcription factor4 (ATF4). Later on ATF4 targets the C\EBP homologous protein (CHOP/Ddit3) and growth arrest and DNA-damage inducible protein 34(GADD34) (Claudio *et al.,* 2013)

2.4.3 Activating transcription factor 6 (ATF6)

Under ER stress condition ATF6 is transported to Golgi body and converted to active transcription factor (Shen *et al.,* 2002). After activation it is localized to nucleus where it induces the transcription of genes like Bip (Binding immunoglobulin proteins).

2.4.4 Glucose regulated protein 78kD (Grp78)

Grp78 commonly known as Bip, is a immunoglobulin heavy chain binding protein. Under non stressed condition Grp78 remain bound to the three main transducer of UPR response i.e. PERK, IRE-1 and ATF6. (Lee *et al.,* 2005). When cell goes under ER stressed condition Grp78 release all three transducer and initiate their activation. Thus, Grp78 is the key regulator of UPR.

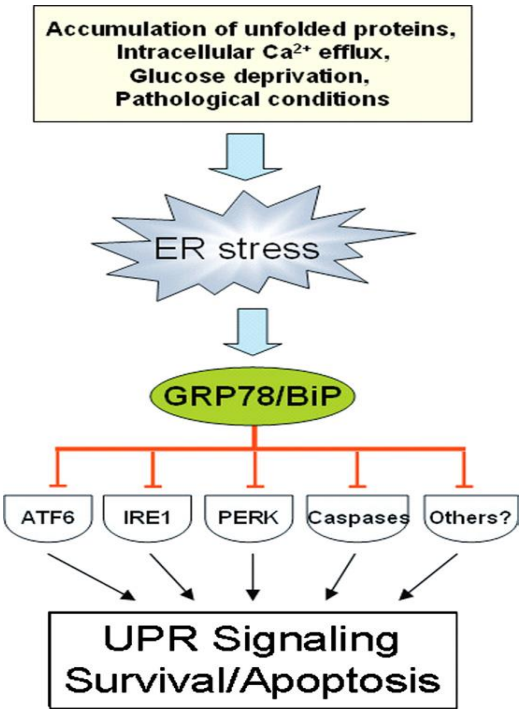
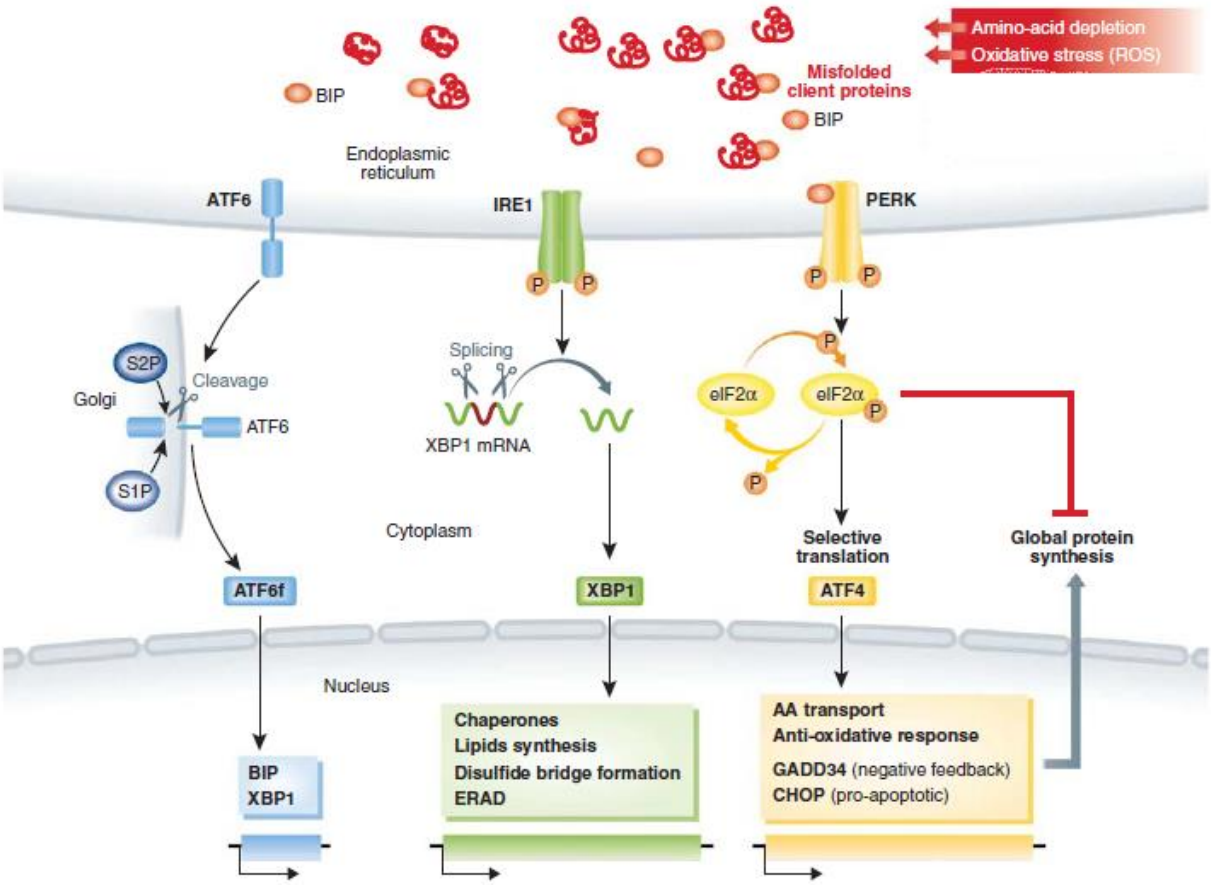


Figure 2.3: Grp78 major role in ER stress and pathways that are regulated by it. (Lee A. 2005)

2.4.5 CCAT\Enhancer-Binding Protein Homologous Protein (CHOP)

When the cell is under extreme ER stress and not able to recover, it is guided towards apoptosis. CHOP is the molecule involved in ER stress regulated apoptotic response. It guides the cell toward apoptosis by down-regulation the expression of Bcl-2 and up regulation of Bim.



(Adopted from Claudio *et al.,* 2013)

Figure 2.4: Overview of three main UPR pathways and their mechanism under ER stress.

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

3.1 Chemicals

Lipid profile analysis through kit from Recombigen laboratories Pvt. Ltd. New Delhi, India. RNA*later*®, diethyl pyrocarbonate (DEPC), RNaseZAP^{TM}, TRI Reagent®, ethidium bromide, agarose, PCR primers, *Taq* DNA polymerase were procured from M/s Sigma-Aldrich. Nuclease free water, GeneRuler (100 bp) DNA ladder, 6X DNA loading dye, dNTPs (10 mM each), RevertAid first strand cDNA synthesis kit and Maxima SYBR Green qPCR Master Mix (2X) (products of Thermo scientific) were procured from Genetix Asia Pvt. Ltd, New Delhi. Casein was a product of M/s Modern Dairies, Karnal (India). Cow milk fat incorporated in diets was obtained from experimental dairy of Institute. Glucose strips used for blood glucose analysis (Accu-Chek® active) were product of Roche diagnostics. Vitamins and salts for mineral mixture were purchased from CDH, New Delhi. Other components like corn starch, mustard oil, refined soybean oil and sucrose were purchased from local supplier. All other chemicals used in the study were of analytical grade (AR) or equivalent.

3.2 Animals

C57BL/6 male mice were procured from Indian Institute of Integrative Medicine, Jammu (CSIR-IIIM).

3.3 To study the influence of different fat rich diet on antioxidative enzymes and diet induced endoplasmic reticulum stress

All animal procedures are conducted at Small Animal House, National Dairy Research Institute, Karnal, Haryana, India, in accordance with Institutional Animal Ethics Committee (IAEC). Animals are housed in plastic cages under 12 hour light/dark conditions at 25±2ęC. All animals are fed normal chow diet and acclimatized for 10 days. Seventy male C57BL/6 mice weighing approximately 29±2 g, were taken for study. They were then randomized according to their body weight. They were fed powdered Low fat diet (LFD) in

small bowl and balls of High fat diet (HFD) placed on net of cage. The composition of low fat diet and high fat diet is given in table below

3.3.1 Preparation of buffalo ghee

120 liters of raw buffalo milk was procured from experimental dairy plant, National Dairy Research Institute (NDRI), Karnal. Buffalo milk was first processed through cream separator (P.G. Lab, Dairy technology department, NDRI) which separates the cream from raw milk. Cream weighing about 15 kg extracted from 120 liters of buffalo milk, then stored in -20ęC for 48 hours for proper separation. Afterwards, cream was heated while stirring it, and buffalo ghee was separated from cream residues and stored for utilization in diet preparation.

Table.3.1: Composition of Low fat diet

Component	**g/Kg**
Starch	530
Casein	200
Sucrose	100
Fat	7
Cellulose	5
Mineral mix(AIN-76)	35
Vitamin mix(TEKLAD 40060)	10
Choline chloride	2
Methionine	3

Table.3.2: Composition of High fat diet

Component	g/Kg
Casein	265
Maltodextrin	172.9
Sucrose	94
Cellulose	65.5
Fat	318
L-Methionine	4
Calcium carbonate	4
Mineral mix(AIN-76)	35
Vitamin mix(TEKLAD 40060)	10
Ethoxyquin	0.004

Table.3.3: Composition of Mineral mixture (AIN-76)

Component	g/Kg
Calcium phosphate dibasic	500
Potassium citrate monohydrate	220
Sodium chloride	74
Sucrose powder	118.03
Potassium Sulfate(K_2SO_4)	52
Magnesium oxide(MgO)	24
Manganese carbonate($MnCO_3$)	3.5
Ferric citrate($C_6H_5FeO_7.3H_2O$)	6.0
Zinc carbonate($ZnCo_3.Zno.3H_2O$)	1.6
Potassium iodate(KIO_3)	0.01
Sodium selenite($Na_2SeO_3.5H_2O$)	0.01
Cupric Carbonate($CuCO_3$)	0.3
Chromium potassium sulfate ($CrK(SO_4)_2.(2H_2O)$)	0.55

Table.3.4: Composition of Vitamin Mixture (TEKLAD 40060)

Component	mg/500gm
Vitamin A	500,000 IU
Vitamin D_3	0.002753 IU
Vitamin E	6.0572 IU
Vitamin K_3 Menadione	2.4779
p-aminobenzoic acid	5.5066
Vitamin C	50.8302
Vitamin B_{12}	1.468
Calcium Pantothenate	3.30395
Choline chloride	82.6711
Folic acid	0.0991
Inositol	5.5066
Niacin	4.9559
Riboflavin	1.103
Pyridoxine hydrochloride	1.103
Thiamine hydrochloride	1.103
Corn starch	333.4798
Biotin	0.02205

3.3.2 Grouping of animals

The animals were randomized according to their body weights and were grouped into 8 groups according to the diet they were fed for 16 weeks as follows

Table.3.5: Grouping of animals

S.No.	Group	Description
1.	LFD-M	Mustard oil based low fat diet
2.	LFD-C	Cow ghee based low fat diet
3.	LFD-B	Buffalo ghee based low fat diet
4.	LFD-L	Lard based low fat diet

5.	HFD-M	Mustard oil based high fat diet
6.	HFD-C	Cow ghee based high fat diet
7.	HFD-B	Buffalo ghee based high fat diet
8.	HFD-L	Lard based high fat diet

3.3.3 Feeding schedule

Different fat based low fat diet and high fat diet are provided in light phase followed by 24 hour refilling time. Water was given *ad libitum*. The feeding schedule was followed for 16 weeks.

3.4. Parameters studied

3.4.1 Body weight and feed intake

Body weight was measured weekly and feed intake was measured daily.

3.4.2 Blood glucose measurement

First, the mice were kept on fasting for 10-12 hours and blood was drawn from tail vein with needle. Blood glucose level were measured by taking one drop of sample on Accu-check glucometer strip.

3.4.3 Collection of tissue samples and blood processing

All the animals were sacrificed after 16 weeks feeding study. Animals were first exposed with anesthetic agent (i.e. Diethyl ether) than blood was collected through cardiac puncture, liver and adipose tissue were excised. Liver and adipose tissue was weighed and washed in Phosphate buffer saline 50mM (PBS pH 7.0) and stored at -20ęC in PBS (pH 7.0) or RNA Later. As far as blood is concerned it was kept at room temperature for 40 minutes so that

blood can coagulate and then centrifuged at 4000xg for 20 minutes. The upper yellowish supernatant (i.e. serum) was collected and stored at -20ęC.

3.4.4 Analysis of lipids in serum

The triglycerides, total cholesterol and high density lipoprotein (HDL)-cholesterol in plasma was estimated using kits (Recombigen laboratories Pvt. Ltd. New Delhi, India). The lipid samples were analyzed as per the instruction of manufacturer. For, triglycerides and total cholesterol 10µl of serum sample was mixed with 1ml of enzyme reagent provided with the kit and incubated for 10 minutes at 37ęC temperature then absorbance was measured at 505nm.For HDL-cholesterol, first 20µl of sample was dissolved in 20µl of precipitating reagent provided with kit then centrifuged at 4000 rpm for 10 minutes. Supernatant was collected and from that supernatant 10µl was mixed with enzyme reagent provided with the kit and incubated for 10 minutes at 37ęC. Absorbance was measured at 505nm against blank containing enzyme reagent mixed with distilled water.

Low density lipoprotein (LDL)-cholesterol, very low density lipoprotein (VLDL)-cholesterol and atherogenic index (AI) were calculated using Friedewaldęs equation (Friedewald *et al.* 1972) as given below

(a). VLDL-cholesterol:

VLDL-cholesterol (mg /dl) = Triglycerides **/** 5

(b). LDL-cholesterol:

LDL-cholesterol (mg/dl) = (Total cholesterol ï VLDL) ï HDLC

(c). Atherogenic index (AI):

AI **=** LDL-cholesterol **/** HDL-cholesterol

3.4.5 Analysis of oxidative stress in liver

The oxidative stress was determined in liver sample collected from experimental animals. The tissue homogenate was prepared and oxidative stress marker was assessed i.e. activity of catalase, superoxide dismutase

(SOD) and glutathione peroxidase (GPx). But first total protein was estimated in tissue homogenate as
follows:

3.4.5.1 Estimation of total protein in liver

Protein was estimated according to the method of Lowry *et al.* (1951).

Reagents:

Solution A: 2% sodium carbonate (Na_2CO_3) dissolved in 0.1N sodium hydroxide (NaOH).

Solution B: 1% sodium potassium tartarate dissolved in distilled water.

Solution C: 0.5 % copper sulphate ($CuSO_4.5H_2O$) in distilled water.

Solution D/Lowryøs reagent: 48 ml of solution A was mixed with 1 ml of solution B and solution C for 50 ml solution.

Solution E/Folin-Ciocalteu reagent: The reagent was obtained as stock solution (2N). This was diluted with distilled water to 1N before use.

Procedure:

In 50µl of sample, 50µl of distilled water and 5ml of Lowryøs reagent was added then incubated for 10 minutes. Afterwards 0.5ml of Folin-Ciocalteu reagent was added and whole reaction mixture was mixed vigorously then incubated for 30 minutes in dark at 37ęC temperature. Absorbance was measured at 660 nm and total protein concentration was estimated by plotting standard curve using bovine serum albumin as standard.

3.4.5.2 Assay of catalase activity in liver

Catalase activity was determined by spectrophotometer (Aebi ,1984).

Reagents:

Potassium phosphate buffer (50 mM, pH 7.0)*:*

Solution A: Dissolved 3.54g Na_2HPO_4 in 500 ml of distilled water.

Solution B: Dissolved 3.40 g KH_2PO_4 in 500 ml of distilled water.

Mixed solution A and B and pH was adjusted to 7.0.

Hydrogen Peroxide (H_2O_2) (30 mM)

Procedure:

The reaction mixture contained 50 µl tissue homogenate(10x diluted), 11-12.5µl of H_2O_2 and volume made to 3 ml with phosphate buffer. The reaction was initiated with the addition of H_2O_2 and decrease in the absorbance at 240 nm due to the decomposition of H_2O_2 was observed. Initial absorbance was approximately A = 0.500 and the decrease in the absorbance was followed for 1 min. The enzyme activity was determined by using an extinction coefficient of $43.9M^{-1}$ cm^{-1} and expressed as units/mg of protein, where unit enzyme activity is one µmole of H_2O_2 consumed/min and calculated as:

$$\frac{\text{ΔA/min x volume of assay mixture (3 ml) x dilution factor x 1000}}{\text{Volume of sample (ml) x 0.0439 x protein concentration (mg/ml)}}$$

3.4.5.3 Assay of Superoxide dismutase (SOD) in liver

The SOD activity was measured by Marklund and Marklund method (1974).

Reagents:

Diethylenetriaminepentacetic acid (DTPA)-Tris-HCL buffer: Dissolve 605mg of tris and 39.33mg of DTPA in 80 ml of distilled water. Adjust the pH to 8.2 with 1N HCL and total volume made upto 100 ml.

Pyrogallol: Dissolve 25.2 mg of pyrogallol in 100 ml of 10mM HCL.

Procedure:

Initially, the rate of pyrogallol auto-oxidation was measured from the increase in absorbance at 420 nm against reference containing 3ml of DTPA-Tris-HCL buffer (1mM DTPA dissolved in 50mM Tris, pH 8.2) using Specord 200 double beam UV/Visible spectrophotometer (Analytikjena, Germany). The volume of pyrogallol in the absence of superoxide dismutase at which the increase in absorbance to 0.02 min^{-1} was selected for the reaction. The inhibition of pyrogallol auto-oxidation was brought about by SOD, which was employed for the determination of enzyme activity. A unit of enzyme was defined as the amount of enzyme that inhibits the reaction by 50%.

$$\% \text{ inhibition of pyrogallol autoxidation} = \left[1 - \frac{\Delta A}{\Delta A_{max}}\right] \times 100$$

Where,

ΔA= Absorbance change due to pyrogallol auto-oxidation in the sample reaction system

ΔA_{max} = Absorbance change due to pyrogallol auto-oxidation in the control (without cell lysate)

The amount of enzyme that inhibits auto-oxidation of pyrogallol by 50% was defined as one unit of enzyme. The activity of SOD was expressed as units/mg protein.

1/a x dilution factor / protein concentration in the sample (mg/ml)

Where,

a= Volume of the sample (ml) that inhibits auto-oxidation of pyragallol by 50% in one min.

3.4.5.4 Assay of glutathione peroxidase (GPx) in liver

GPx activity was determined spectrophotometrically using the method of Paglia and Valentine (1967). GPx was assessed by utilizing excess of glutathione reductase that couples the rate of oxidation of NADPH to reaction of the peroxidase reaction with cumene hydroperoxidase and glutathione (reduced).

Reagents

Potassium phosphate buffer (50 mM, pH 7.0)

Solution A: Dissolve 3.54 g Na_2HPO_4 in 500 ml distilled water

Solution B: Dissolve 3.40 g KH_2PO_4 dissolved in 500 ml distilled water

Mix solution A and B and adjust pH to 7.0

EDTA (10 mM): Dissolved 37.2 mg of EDTA in 10 ml of potassium phosphate buffer

NaN_3 (10 mM): Dissolved 6.5 mg of NaN_3 in 10 ml of potassium phosphate buffer

NADPH (2 mM): Dissolved 16.6 mg of NADPH in 10 ml of potassium phosphate buffer

Glutathione reductase: 100 U/ml in potassium phosphate buffer

Glutathione (reduced) (10 mM): Dissolved 30.73 mg of reduced glutathione in 10 ml of potassium phosphate buffer

Cumene hydroperoxide (1.5 mM): Dissolve 3 µl in 10 ml of potassium phosphate buffer.

Procedure

The reaction mixture (1 ml) containing 440 µl of potassium phosphate buffer, 100 µl of EDTA, 100 µl of NaN_3, 100 µl of NADPH, 10 µl of glutathione reductase, 100 µl of glutathione (reduced) was mixed with 50 µl of tissue homogenate(10x diluted) and incubated at room temperature for 5 min. Then, added 100 µl of cumene hydroperoxide to the reaction mixture to initiate the reaction. Absorbance was taken against blank containing phosphate buffer (50 mM, pH 7.0). Oxidation of NADPH was monitored for 5 min by change in absorbance at 340 nm. The enzyme activity was calculated using extinction coefficient of NADPH (6.22×10^3 $M^{-1}cm^{-1}$), where unit enzyme activity is one µmole of NADPH oxidized per minute per mg of protein and calculated as:

$$\frac{\text{ΔA/min x volume of assay mixture (1ml) x dilution factor}}{\text{Volume of sample (0.1ml) x 6.22 x protein concentration in sample (mg/ml)}} \text{ X } 1000$$

3.5 Analysis of gene expression in liver

3.5.1 Isolation of total RNA from liver

First all the plastic wares and glass wares essential in RNA isolation were made RNase free by 0.1% diethyl pyrocarbonate (DEPC) treatment for 24 hours at room temperature. Then, they were dried off in incubator and stored in RNase free working area. Also, the gloves, micropipettes and working area used during the whole process are first cleansed with RNaseZAP (sigma-aldrich).

Total RNA from tissues were isolated using TRIzol reagent (ambion). First, 100-200mg of tissue sample was dipped in liquid nitrogen and homogenized in mortar, than 1ml of TRIzol reagent was added into this homogenized tissue. With the help of 2ml syringe homogenized mixture was sucked in needle and forcefully exerted out on the wall of mortar, process was repeated 3 times. Transfer the mixture in centrifuge tube and incubated for 40 minutes at room temperature. Centrifuge the homogenized mixture at 6000 rpm for 15 minutes at 4ęC. Supernatant was collected in another centrifuge tube and 200µl chloroform was added. Vortex it and incubate for 5 minutes. Centrifuge at 12000 rpm for 15 minutes at 4ęC. Supernatant was collected in another centrifuge tube and 500µl isopropanol was added followed by gentle mixing and incubated for 10 minutes at -20ęC. Centrifugation at 12000 rpm for 15 minutes at 4ęC, afterwards supernatant was discarded and 1ml 70% ethanol was added. Centrifugation at 6000 rpm for 5 minutes at 4ęC, supernatant was discarded and after air drying of RNA pellet in centrifuge tube, 50µl nuclease free water was added and stored at -20ęC.

3.5.2 RNA quantification and purity determination

Total RNA quantity was measured using Nanodrop spectrophotometer (ND1000). To determine the purity of RNA A_{260}/A_{280} ratio was measured which standard value range for RNA is between 1.9-2.0. A_{230}/A_{260} ratio was also measured to check that there should be no contamination of organic materials, standard range for RNA 1.9-2.0.

3.5.3 Integrity of RNA:

Integrity of RNA was assessed through agarose gel electrophoresis. 1.5% agarose gel was prepared in Tris-Acetic acid-EDTA(TAE) buffer and stained with ethidium bromide. RNA sample was loaded in wells of agarose gel and again TAE buffer was used as electrophoresis buffer and band of 28s rRNA and 18s rRNA was assessed for integrity of RNA.

3.5.4 Synthesis of first strand cDNA

cDNA synthesis was carried out by using RevertAid first strand cDNA synthesis kit (Thermo Scientific). For each reverse transcription reaction, following reagents were added into sterile, nuclease free PCR tubes on ice:

Total RNA	:	approx. 1000 ng
Primer	:	1 µl Oligo dT
Nuclease-free water	:	x µl
Total volume	:	12 µl

Contents in PCR tubes were mixed gently, centrifuged briefly and incubated in PCR machine as follows:

65 ^{o}C	:	10 min
4 ^{o}C	:	--

After completion of the above steps, the tubes were removed from PCR machine and kept quickly on ice. The following remaining components were added into the tubes for completion of first strand cDNA synthesis:

5X reaction buffer	:	4 εl
RiboLock RNase inhibitor (20 U/εl)	:	1 εl
10 mM dNTP mix	:	2 εl
Reverse transcriptase (200 U/ εl)	:	1 εl
Total volume	:	8 εl

The contents in PCR tubes were mixed gently, centrifuged briefly and incubated in PCR machine as follows:

25 ^{o}C	:	10 min
42 ^{o}C	:	30 min
95 ^{o}C	:	3 min
4 ^{o}C	:	ī pauseī

3.5.5 Real Time polymerase chain reaction (qRT-PCR)

The first strand cDNA synthesized was used directly as a template for qRT-PCR. Gapdh was used as the reference gene. The primers were designed from inter-exonic regions of respective genes by using primer3 software, and got custom synthesized from Sigma-Aldrich. The sequences of primers used in the study are given in Table,

3.5.6 List of Primers for qRT-PCR analysis of gene expression

Table.3.6: List of primers

Genes	Sequence (5'-3' direction)
Grp78	Forward- ATTCCTGCGTCGGTGTGT
	Reverse- GCATCGAAGACCGTGTTCTC
Ddit3	Forward- AGGAAACGAAGAGGAAGAATCA
	Reverse- GGGCACTGACCACTCTGTTT
Atf6	Forward- GAACTTCGAGGCTGGGTTCA
	Reverse- ACTCCCAGAATTCCTACTGATGC
Atf4	Forward- ATGGCGCTCTTCACGAAAT
	Reverse- TTGGCCACCTCCAGATAGTC

The real time PCR was performed in a total volume of 15 εl. The reaction mixture consisted of the following reagents:

Primers (both forward and reverse)	:	1 μl each
SYBR Green mix (2x)	:	7.5 μl
Nuclease free water	:	4.5 μl
cDNA	:	2 μl
Total volume	:	15 μl

3.5.7 Data analysis

The quantitative PCR data were analyzed by $2^{-\Delta\Delta CP}$ method (Schmittgen and Livak, 2001) and represented as fold change.

æC$_{P\ test}$ = C$_{P\ (target,\ test)}$ ï C$_{P\ (reference\ gene,\ test)}$

æC$_{P\ calibrator}$ = C$_{P\ (target,\ calibrator)}$ ï C$_{P\ (reference\ gene,\ calibrator)}$

ææC$_P$ = æC$_{P\ test}$ ï æC$_{P\ calibrator}$

Fold difference in expression = $2^{-\Delta\Delta CP}$

3.6 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by *post hoc* Tukeyôs multiple comparison test, using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). Data with different superscript letters are significantly different at $p<0.05$,$p<0.001$, $p<0.0001$ according to *post hoc* ANOVA statistical analysis. Data are expressed as mean ± SEM. The results were considered statistically significant when $p<0.0001$

CHAPTER –4

Results

RESULTS

The present study was undertaken with the aim to investigate the influence of diets having different types of fat on antioxidative enzymes and diet induced endoplasmic reticulum stress on obesity prone C57BL/6 male mice. The results are presented below in the following sections-

4.1 TO STUDY THE COMPARATIVE EFFECTS OF DIFFERENT TYPES OF FAT RICH DIET ON ANTIOXIDATIVE ENZYMES AND LIPID PROFILE OF MICE

Under this objective C57BL/6 male mice was fed different experimental diets for period of 16 weeks to study the effect of different dietary fat when fed as high fat diet (35% fat by weight) and low fat diet(7% fat by weight). The different groups made is already mentioned in Table 3.5.The effects on different parameters viz., body weight, feed intake, fasting blood glucose level, serum lipids, organ weights, activity of enzymes like superoxide dismutase, catalase and glutathione peroxidase were studied-

4.1.1 Body weight and feed intake

The body weights were recorded at different time periods for all experimental groups viz., LFD-L, HFD-L, LFD-M, HFD-M, LFD-C, HFD-C, LFD-B, HFD-B, LFD-S and HFD-S. The mean body weights of different groups recorded at 0 and 16 weeks are depicted in Fig.4.1. The initial body weights at the start of experiment (zero day) were measured to be 29.8±1.2, 29.8±1.3, 29.8±1.0, 29.8±0.95, 29.9±1.0, 29.8±1.0, 29.8±1.2, 29.8±0.97, 29.8±1.3, 29.8±1.3 g (mean ± SEM) for LFD-M, HFD-M, LFD-S, HFD-S, LFD-C, HFD-C,LFD-B,HFD-B,LFD-L, HFD-L groups respectively (Table 4.1). Supplementation of HFD-M and HFD-S showed the less increase in body weight 31.1±1.95 g and 35.8±2.5 g in comparison with other HFD like HFD-C 38.1±5.7, HFD-B 37.2±2.2, HFD-L 37.9±0.8 g (Table 4.2).

The cumulative feed intake of different groups was calculated for the 16 weeks of feeding period, and the data are presented in Fig. 4.2. The cumulative feed intake in HFD-M, HFD-C, HFD-B, HFD-L groups were calculated to be 272.9, 281.6, 264.3,

280.7 g, respectively which were much lower than LFD-M, LFD-C, LFD-B, LFD-L groups 412.9, 416.4, 414.1, 378.7 g (Table 4.3).

4.1.2 Organ weight

The adipose tissue mass accumulation was measured in different feed groups after 16 weeks of feeding and results are depicted in Fig. 4.3. The adipose tissue mass at 16 week was observed to be more in HFD-L, HFD-B, HFD-C as compared to their corresponding LFDs however in case of HFD-M there is less adipose tissue mass was observed in comparison with its corresponding LFD but statistically it was not significantly different. The liver weight in HFD-M (1.35g) significantly lower in comparison with LFD-M (1.9g) while in case of kidney weight HFD-M (0.4g) showed significant decrease in comparison with HFD-C (0.73g) and HFD-L (0.69g) but in spleen weight case there is no significant difference in feed groups after 16 weeks of feeding Figure 4.4 (Table 4.4).

4.1.3 Fasting blood glucose

The results of fasting blood glucose levels at 16 weeks of experimental period is presented in Fig. 4.5. The blood glucose level after 16 week of study was measured to be significantly higher in HFD-M, HFD-C, HFD-B, HFD-L 163.7±5.7, 168.2±3.4, 167±3.3, 185.5±1.5 mg/dL (mean ± SEM) comparison with LFD-M, LFD-C, LFD-B, LFD-L (137.5±2.5, 131.2±9.1,140.2±3.1, 135.2±2.4 mg/dL). In case of HFD-S the blood level was higher than LFD-S but not significantly different.

4.1.4 Serum lipid profile

Serum lipid profile was evaluated to determine the effect of different fat rich diet on triglyceride, total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL) and very low density lipoproteins (VLDL). The data is presented in Fig. 4.6. The triglycerides level at 16 week is found significantly increased in 251.9±2.6, 255±3.4, 278.2±1.8 mg/dL (mean ± SEM) HFD-C, HFD-B, HFD-L in comparison with 194.8±2.4, 208±4.5, 209.2±1.6 mg/dL LFD-C, LFD-B, LFD-L. In HFD-S (222.8±2.1 mg/dL) and HFD-M (194.4±10.4 mg/dL) the triglycerides level were not significantly different to their

corresponding LFD groups. Interesting fact have been pointed out that level of triglycerides in HFD-S and HFD-M was significantly lower than the other HFD groups (Table 4.5)

The total cholesterol level at 16 week is found significantly increased in HFD-C, HFD-B, HFD-L 260.1±2.8, 262.9±3.1, 275.6±2.2 mg/dL (mean ± SEM) in comparison LFD-C, LFD-B, LFD-L groups 199.4±1.7, 175.3±1.8, 213.4±2.7 mg/dL. The total cholesterol levels in HFD-S and HFD-M (170.8±5.6 and 195.9±7.6 mg/dL) was significantly lower than other HFD groups (Table 4.6).

HDL level at 16 week study was found to be significantly decreased in HFD-S, HFD-M, HDS-C, HFD-B, HFD-L 46.8±3.6,39.4±1.0, 39.7±2.7, 39.8±2.5,43±2.0 mg/dL (mean ± SEM) in comparison with LFD-S,LFD-M,LFD-C,LFD-B,LFD-L 59.5±1.3, 55.1±2.6, 54.0±2.3, 54.7±0.4, 56.4±2.3 mg/dL (Table 4.7).

LDL level at 16 week study was found significantly higher in HFD-C, HFD-B, HFD-L 169±0.8, 172±2.8, 176.9±2.2 mg/dL (mean ± SEM) than in LFD-C, LFD-B,LFD-L 106.4±2.9, 78.9±2.0, 115.0±4.1 mg/dL. Significantly lower level of LDL in HFD-S and HFD-M (79.4±5.4 and 117.5±10.1 mg/dL) in comparison with other HFD groups (Table 4.8).

VLDL level at 16 week study showed significantly higher in HFD-C, HFD-B, HFD-L 50.3±0.5, 51±0.6, 55.6±0.3 mg/dL in comparison with LFD-C, LFD-B, LFD-L 38±0.4, 41.6±0.9, 41.8±0.3 mg/dL while significantly lower VLDL level in HFD-S and HFD-M (44.5±0.4 and 38.8±2.0 mg/dL) than other HFD groups (Table 4.9).

Atherogenic Index (A.I.) is also significantly higher in all HFD groups in comparison with LFD groups.

4.1.5 Hepatic superoxide dismutase activity (SOD)

SOD activity in liver at 16 week study was found to be decreased in HFD-M, HFD-C, HFD-B, HFD-L 84.4±18.5, 70.6±15.8, 66.1±9.1,49.5±4.7 µmol/min/mg of protein (mean ± SEM) in comparison with LFD-M, LFD-C,LFD-B,LFD-L 98.4±9.8, 83.8±33.6, 62.6±21.3, 50.8±6.8 µmol/min/mg of protein (Table 4.10). Though the SOD activity was

decreased but no significant difference between HFD and LFD groups was identified. The data is presented in Fig. 4.7.

4.1.6 Hepatic catalase activity (CAT)

CAT activity in liver at 16 week of study was found to be significantly decreased in HFD-L 1238.4±161.2 µmol/min/mg in comparison with LFD-L 2497.1±115.8 µmol/min/mg (mean ± SEM) (Table 4.11). While in other HFD groups the activity of CAT was lowered than their corresponding LFD groups but not found significantly different. The data is presented in Fig. 4.8.

4.1.7 Hepatic glutathione peroxidase activity (GPx)

GPx activity at 16 week study in liver was decreased in all HFD groups in comparison with their corresponding LFD groups but not found any significantly difference (Table 4.12). The data is presented in Fig. 4.9.

4.2 ANALYSIS OF EXPRESSION OF GENES RELATED TO ENDOPLASMIC RETICULUM STRESS IN LIVER

Quantitative mRNA analysis of different genes related to Endoplasmic reticulum stress like Grp78, Ddit3, ATF4, ATF6 affected by different fat rich diet.

4.2.1 Gene expression in liver

4.2.1.1 Glucose regulated protein 78kD (Grp78kD)

The mRNA expression of Grp78 in liver sample was analyzed and data is presented in Fig. 4.10. There is significant increase in fold change expression of Grp78 gene in HFD-M groups as compared with other HFD as well as LFD-groups.

4.2.1.2 C\EBP homologous Protein (CHOP)/Ddit3

The mRNA expression of DdiT4 gene in liver sample was analyzed to study the effect of different fat rich diet on endoplasmic reticulum stress. There is significant increase in fold change expression of DdiT3 gene HFD-B in comparison with all other HFD as well as LFD groups. Data is presented in Fig. 4.11.

4.2.1.3 Activating transcription factor 4 (ATF4)

The mRNA expression of ATF4 gene in liver sample was analyzed to study the effect of different fat rich diet on endoplasmic reticulum stress. There is significant increase in fold change expression of ATF4 gene HFD-C and HFD-m with their corresponding LFDs while in case of HFD-C expression was found significantly higher in comparison with HFD-B and HFD-L. Data is presented in Fig. 4.12.

4.2.1.4 Activating transcription factor 6 (ATF6)

The mRNA expression of ATF6 gene in liver sample was analyzed to study the effect of different fat rich diet on endoplasmic reticulum stress. There is significant increase in fold change expression of ATF6 gene HFD-B in comparison with all other HFD as well as LFD groups. Data is presented in Fig. 4.13.

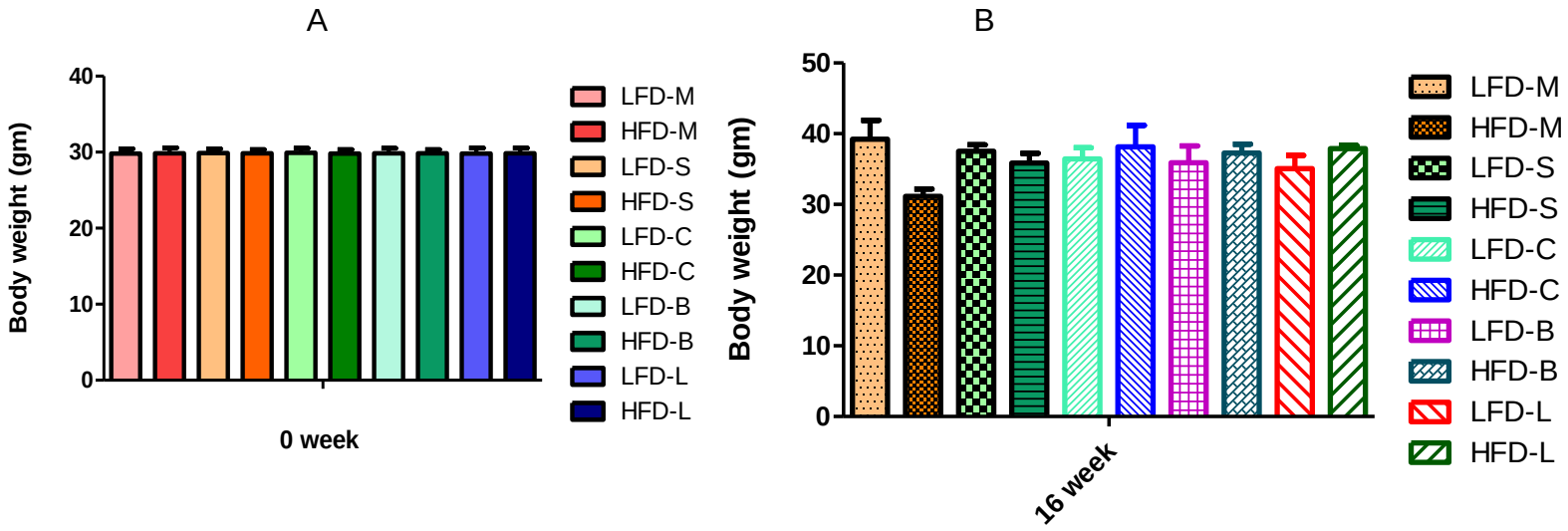


Figure 4.1: Body weight of C57BL/6 male mice under different dietary groups at different time period of 0 week (A) and 16 weeks(B). All value represent the mean ± SEM of experiment (n=4-7).

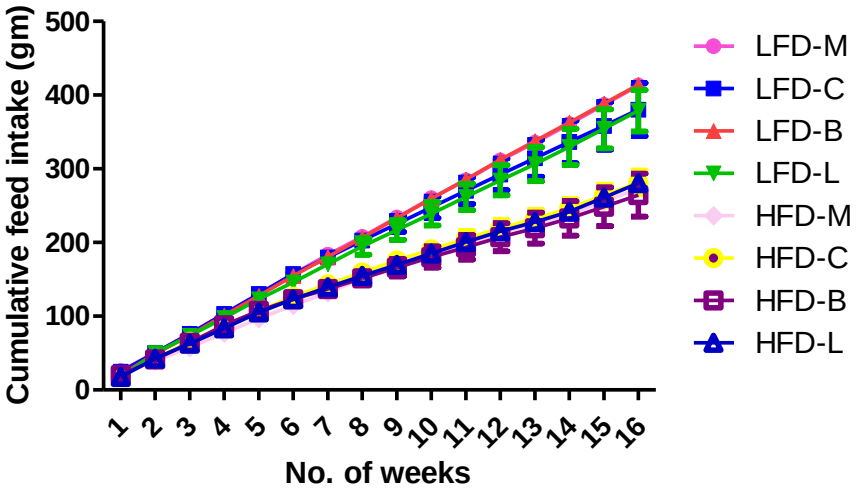


Figure 4.2: Cumulative feed intake in C57BL6 male mice under different feed groups in 16 week of study period. All value represent the mean ± SEM. of experiment (n=4-7)

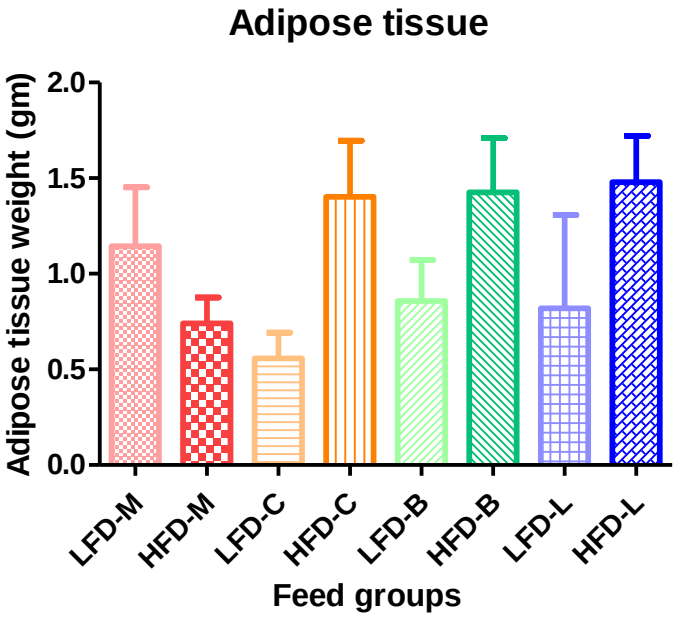


Figure 4.3: Adipose tissue weight accumulated in different feed groups after 16 week study. All value represent the mean ± SEM. of experiment (n=4-7)

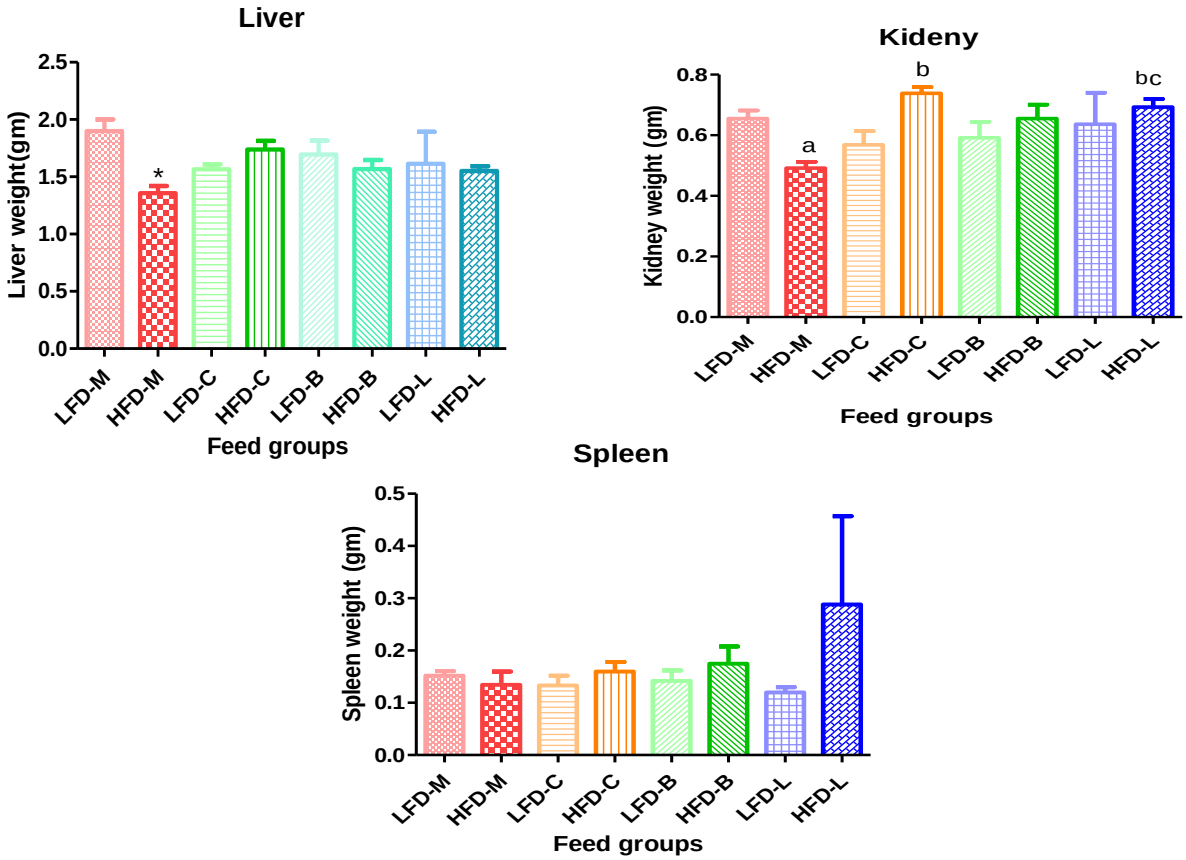


Figure 4.4: Liver and kidney weight of different feed groups at 16 week of feeding study. All values are expressed as mean ± SEM. of experiment (n=4-7). Significance level of $p<0.0055$. * represents the significant difference from corresponding LFD, Alphabets represents the significant difference from other HFD groups.

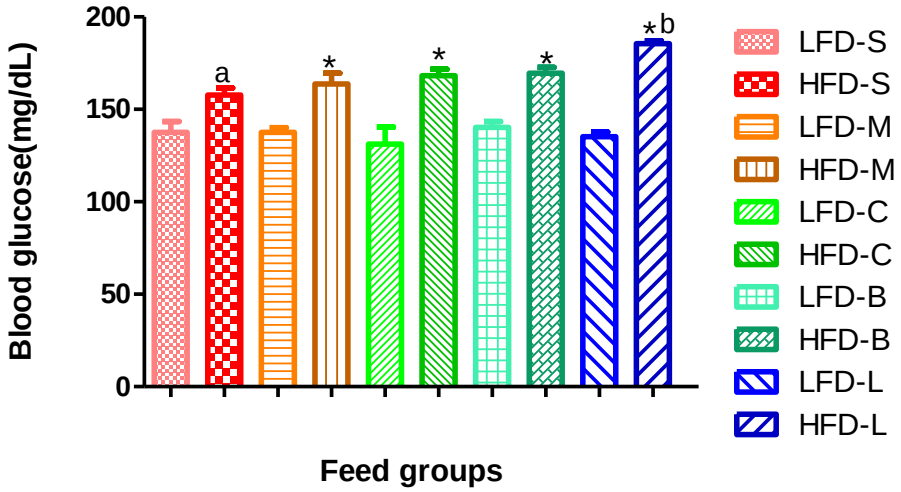
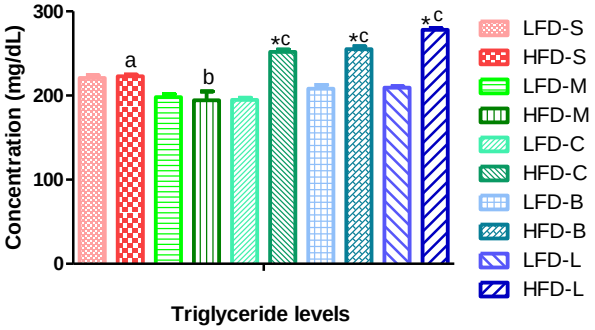
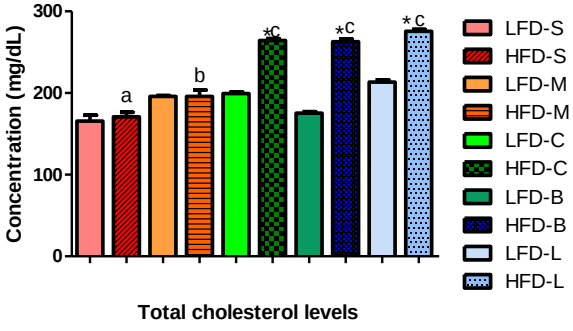
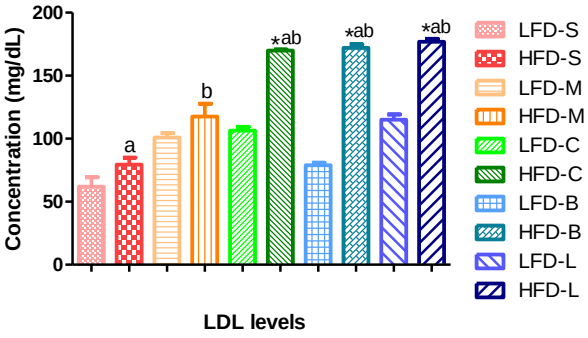
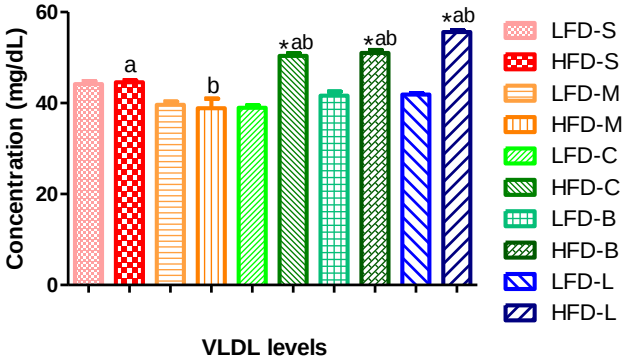


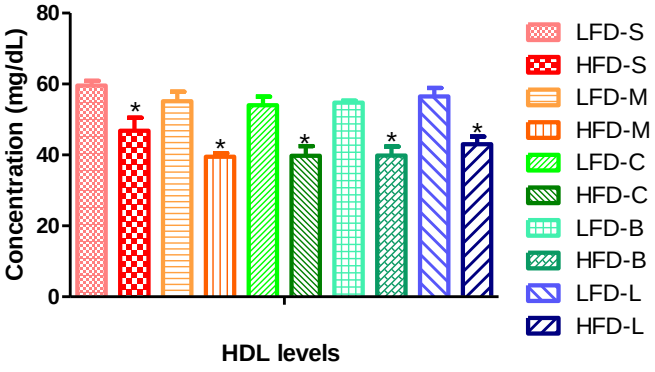
Figure 4.5: Blood glucose levels of different feed groups at 16 week of feeding study. All values are expressed as mean ± SEM. of experiment (n=4-7). Significance level of $p<0.0001$. * represents the significant difference from corresponding LFD, Alphabets represents the significant difference from other HFD groups

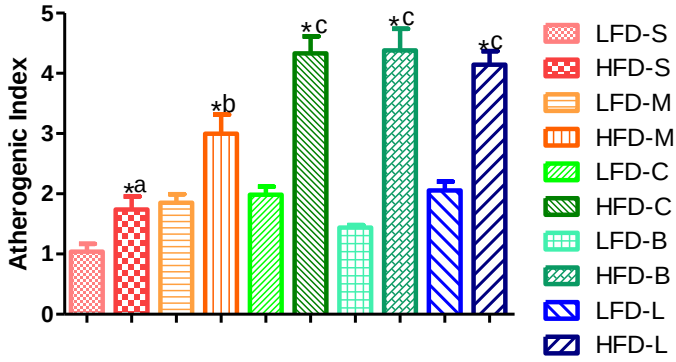












Figures 4.6:Lipid profile in serum of different feeding groups. All value represent the mean ± SEM of 3 individual experiment (n=4).Significance level of p<0.0001.*represent the significant difference from corresponding LFD. Alphabets represent significant difference from other HFD groups.

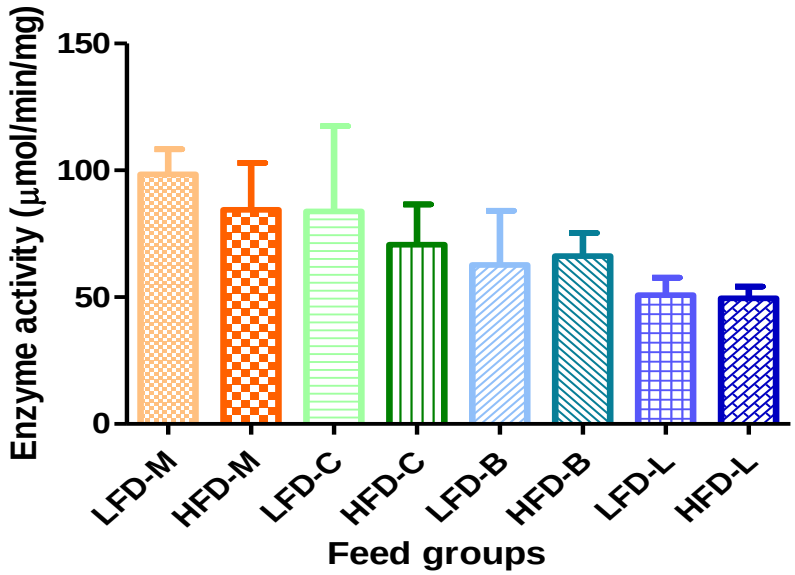


Figure 4.7: Activity profile of superoxide dismutase enzyme in C57BL/6 male mice under different feeding groups. All value represent the mean ± SEM. of 3 individual experiment (n=4).

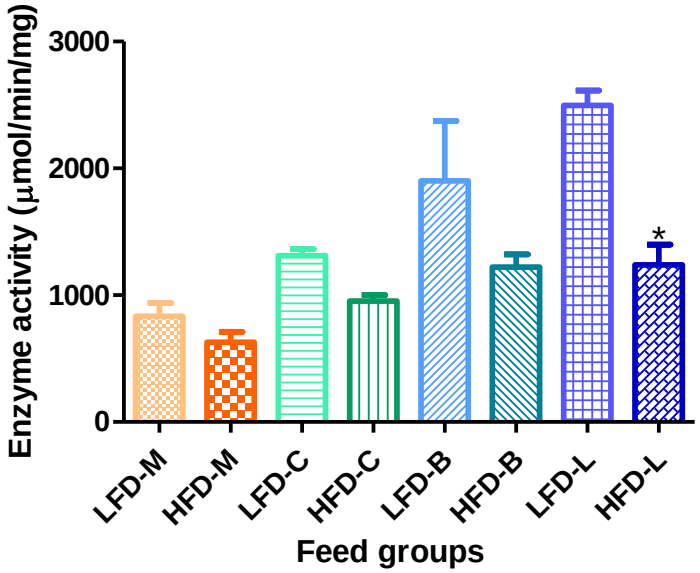


Figure 4.8: Activity profile of catalase enzyme under different feeding groups. All value represent the mean ± SEM of 3 individual experiment (n=4).Significance level of $p<0.0001$.* represent the significant difference with corresponding LFD.

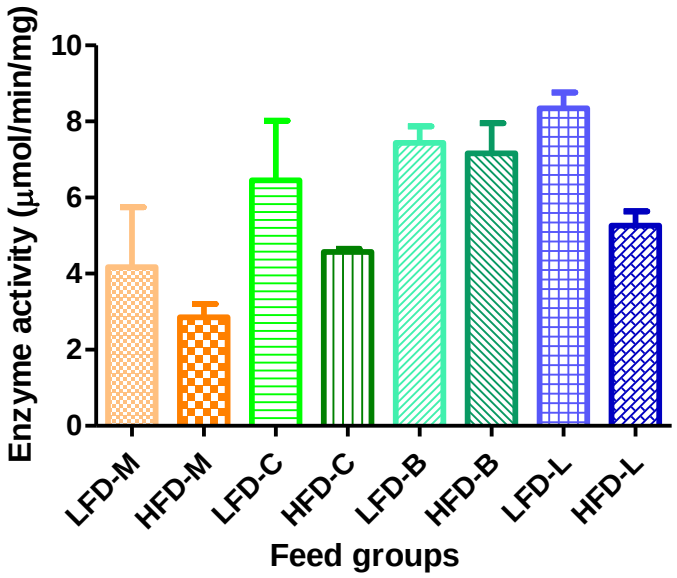


Figure 4.9: Activity profile of glutathione peroxidase enzyme in C57BL/6 male mice under different feeding groups. All value represent the mean ± SEM of 3 individual experiment (n=4). Alphabets represent the significant difference from other HFD groups.

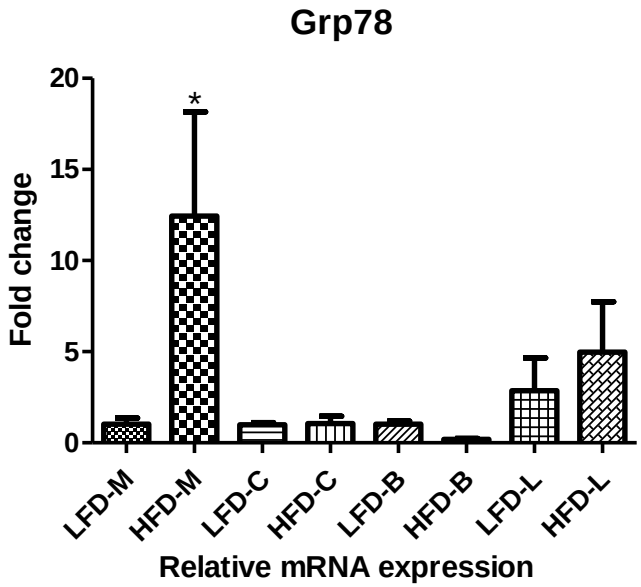


Figure 4.10: Relative expression of Grp78 gene in liver of C57BL/6 male mice of different feed groups. All value represent the relative gene expression mean± SEM of three individual experiment (n=2).* represents the significant difference from corresponding LFD.

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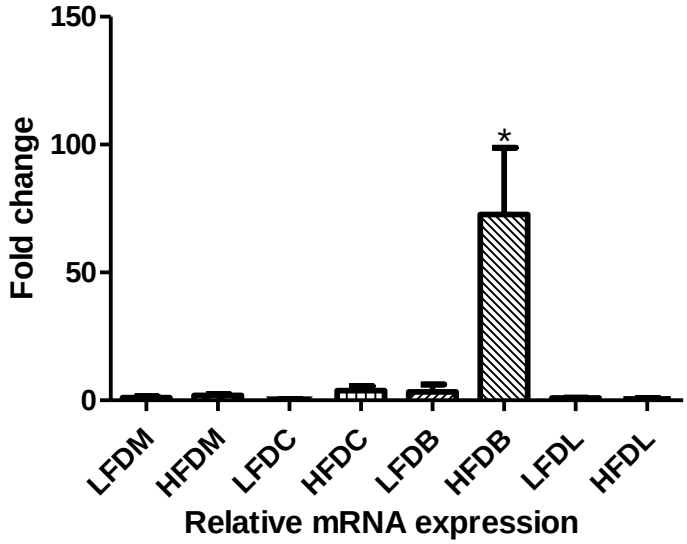

Figure 4.11: Relative expression of DdiT3 gene in liver of C57BL/6 male mice of different feed groups. All value represent the relative gene expression mean± SEM of three individual experiment (n=2).* represent the significant difference from corresponding LFD

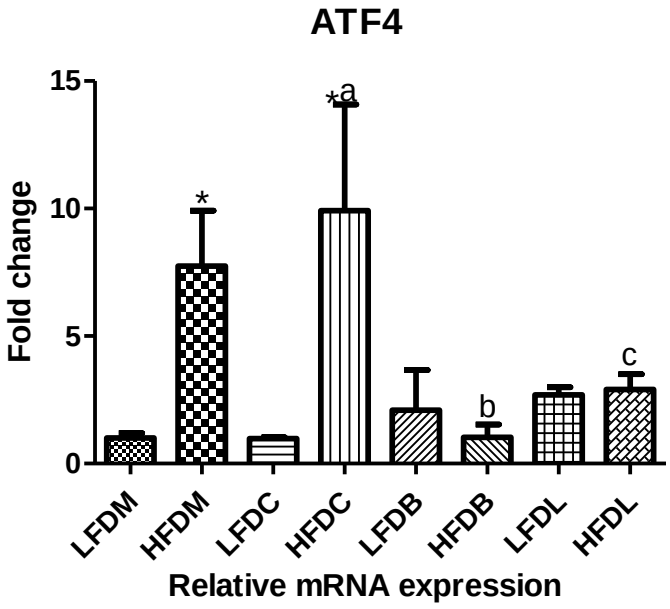

Figure 4.12: Relative expression of ATF4 gene in liver of C57BL/6 male mice of different feed groups. All value represent the relative gene expression mean± SEM of three individual experiment (n=2).*represent the significant difference from corresponding LFD. Alphabets represent the significant difference with other HFD groups.

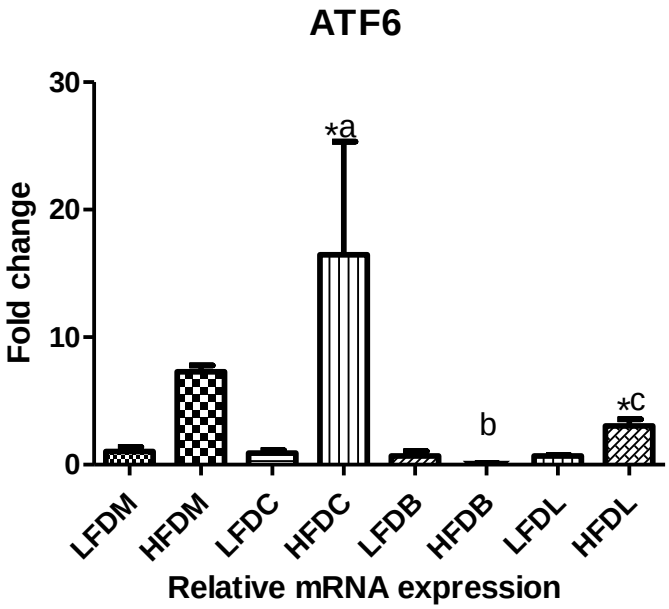

Figure 4.13: Relative expression of ATF6 gene in liver of C57BL/6 male mice of different feed groups. All value represent the relative gene expression mean± SEM of three individual experiment (n=2).*represent the significant difference from corresponding LFD. Alphabets represent the significantly different with other HFD groups.

Table 4.1: Body weight at 0 week

Feed Groups	Body Weight(g)
LFD-M	29.8±1.2
HFD-M	29.8±1.3
LFD-S	29.8±1.0
HFD-S	29.8±0.95
LFD-C	29.9±1.0
HFD-C	29.8±1.0
LFD-B	29.8±1.2
HFD-B	29.8±0.97
LFD-L	29.8±1.3
HFD-L	29.8±1.3

Table 4.2: Body weight at 16 week

Feed Groups	Body Weight(g)
LFD-M	39.2±2.7
HFD-M	31.1±1
LFD-S	37.5±0.9
HFD-S	35.8±1.3
LFD-C	36.4±1.5
HFD-C	38.1±3
LFD-B	35.8±2.3
HFD-B	37.2±1.2
LFD-L	35±1.9
HFD-L	37.9±0.5

Table 4.3: Cumulative feed intake at 16 week

Feed Groups	Cumulative Feed Intake(g)
LFD-M	412.9
HFD-M	272.9
LFD-C	416.4
HFD-C	281.6
LFD-B	414.1
HFD-B	264.3
LFD-L	378.7
HFD-L	280.7

Table 4.4: Organ weight

Parameter	LFD-M	HFD-M	LFD-C	HFD-C	LFD-B	HFD-B	LFD-L	HFD-L
Adipose	1.14	0.74	0.55	1.40	0.85	1.42	0.8	1.4
Liver	1.9	1.3	1.56	1.73	1.69	1.56	1.61	1.55
Kidney	0.65	0.49	0.56	0.73	0.59	0.65	0.63	0.69
Spleen	0.15	0.13	0.13	0.16	0.14	0.17	0.12	0.28

Table 4.5: Serum Triglyceride level at 16 week

Feed Groups	Concentration (mg/dL)
LFD-S	220.8±3.2
HFD-S	222.8±2.2
LFD-M	198.1±2.9
HFD-M	194.4±10.4
LFD-C	194.8±2.4
HFD-C	251.9±2.6
LFD-B	208±4.5
HFD-B	255±3.4
LFD-L	209.2±1.6
HFD-L	278.2±1.8

Table 4.6: Serum total cholesterol level at 16 week

Feed Groups	Concentration (mg/dL)
LFD-S	165.5±5.5
HFD-S	170.8±5.6
LFD-M	195.7±1.3
HFD-M	195.6±7.6
LFD-C	199.4±1.7
HFD-C	260.1±2.8
LFD-B	175.3±1.8
HFD-B	262.9±3.1
LFD-L	213.4±2.7
HFD-L	275.6±2.2

Table 4.7: Serum HDL level at 16 week

Feed Groups	Concentration (mg/dL)
LFD-S	59.5±1.3
HFD-S	46.8±3.6
LFD-M	55.1±2.6
HFD-M	39.4±1.0
LFD-C	54±2.3
HFD-C	39.7±2.7
LFD-B	54.7±0.4
HFD-B	39.8±2.5
LFD-L	56.4±2.3
HFD-L	43±2.0

Table 4.8: Serum LDL level at 16 week

Feed Groups	Concentration (mg/dL)
LFD-S	61.8±7.2
HFD-S	79.4±5.4
LFD-M	100.9±3.1
HFD-M	117.5±10.1
LFD-C	106.4±2.9
HFD-C	169±0.8
LFD-B	78.9±2.0
HFD-B	172±2.8
LFD-L	115±4.1
HFD-L	176.9±2.2

Table 4.9: Serum VLDL level at 16 week

Feed Groups	Concentration (mg/dL)
LFD-S	44.1±0.6
HFD-S	44.5±0.4
LFD-M	39.6±0.6
HFD-M	38.8±2.0
LFD-C	38±0.4
HFD-C	50.3±0.5
LFD-B	41.6±0.9
HFD-B	51±0.6
LFD-L	41.8±0.3
HFD-L	55.6±0.3

Table 4.10: Superoxide dismutase activity

Feed Groups	Activity (µmol/min/mg)
LFD-M	98.4±9.8
HFD-M	84.4±18.5
LFD-C	83.8±33.6
HFD-C	70.6±15.8
LFD-B	62.6±21.3
HFD-B	66.1±9.1
LFD-L	50.8±6.8
HFD-L	49.5±4.7

Table 4.11: Catalase activity

Feed Groups	Activity (µmol/min/mg)
LFD-M	833.7±104.3
HFD-M	628.4±69.6
LFD-C	1312±46
HFD-C	952.8±30.2
LFD-B	1902.3±475.7
HFD-B	1220.4±77.6
LFD-L	2497.1±115.8
HFD-L	1238.4±161.2

Table 4.12: Glutathione peroxidase activity

Feed Groups	Activity (µmol/min/mg)
LFD-M	4.1±1.6
HFD-M	2.8±0.3
LFD-C	6.4±1.6
HFD-C	4.5±0.1
LFD-B	7.4±0.5
HFD-B	7.1±0.8
LFD-L	8.3±0.4
HFD-L	5.2±0.4

CHAPTER –5

Discussion

DISCUSSION

Obesity is considered to be a major health concern in the developed as well as developing countries. Western type diets high in fat content as well as reýned cereals and sugars, combined with the changing lifestyle due to urbanization, are the causes of health threats in the form of obesity and associated metabolic disorders, including insulin resistance, type 2 diabetes mellitus (T2DM), and cardiovascular disease. Adipose tissue is the major site of triglyceride storage in the body and thus contributes significantly to nutrient metabolism in various tissues and influences plasma concentrations of free fatty acids and glucose. It has also been reported that the balance between fat consumption and oxidation rates is not so tightly regulated (Stubbs et al., 1995) and depends on the type of fatty acids (Yu et al., 2002). Recent studies have established that obesity is associated with systemic chronic inflammation and that this low-grade inflammation may play a causal role in obesity-associated insulin resistance, type 2 diabetes, and other complications. Obesity is characterized by a greater number of adipocytes (hyperplasia) and an increase in size (hypertrophy). Both adipocyte hyperplasia and hypertrophy can contribute to AT expansion; however, in adults, hypertrophy appears to predominate. Some of the consequences of hypertrophy include fatty acid flux, more production of ROS, increased leptin secretion, hypoxia and adipocyte death. The excess storage of triacylglycerols (TAG) from dietary intake results in excessive influx of free fatty acids into blood circulation. The excessive influx of FFA in blood causes disruption in the antioxidative metabolism in much tissue such as liver. Different fats are made up of different constituents and may have different effects on the antioxidative metabolism, lipid profile and diet induced complications. So it will be of great significance to compare the different type of fats.

5.1 To study the comparative effects of different types of fat rich diet on antioxidative enzymes and lipid profile

In the current animal study the feeding of fat rich diet having different types of fat i.e. Lard, Cow milk fat, Buffalo milk fat, Soyabean oil and mustard oil have different effects on the increase in body weight after the 16 week of feeding. The gain in the body weight

was more in case of mice fed with high fat diet containing lard, Buffalo milk fat, Cow milk fat than the corresponding low fat diets while the body weight gain was not higher in case of mice fed with high fat diet having either Soyabean oil or mustard oil than their corresponding low fat diet groups. It suggests that the Lard, Buffalo milk fat and Cow milk fat are more able to induce obesity as compared to Soyabean oil and Mustard oil. Zhao and coworker also did not observed much gain in body weight in rats fed with High Fat Diet containing Soyabean oil as compared to High Fat diet containing lard after 8 week of feeding (Zhao *et al.,* 2013). In case of Mustard oil also Cartea *et al*., 2010 has showed that there is less adiposity in mustard oil based high fat diets.

The cumulative feed intake was lower in the High Fat Diet groups than low Fat Diet groups. This may be due the excess fat present in the diet.

After the 16 week of experiment it was observed that the adipose tissue weight was higher in the group fed with HFD containing lard, Buffalo ghee and Cow ghee than the low Fat Diet. However the adipose tissue weight was not as higher in HFD containing Soyabean and Mustard than their corresponding diets. It reveals that the consumption of Soyabean oil and Mustard oil in diet does not cause more adiposity in mice. The fat mass accumulation in adipocytes seems to be in accordance with the body weight gains reflected in different high fat diet fed groups. Zhao *et al.,* 2013 also observed less adiposity when the soybean oil rich diet compared with lard rich diet. Cartea *et al.,* 2010 discovered less adiposity in mustard oil rich diet.

Feeding of different fat rich diets did not showed the potential effects on organ weights like liver, Kidney and spleen.

The Blood glucose level was more elevated in case of HFD containing lard, Buffalo ghee and Cow ghee and Mustard oil than the low Fat Diet while feeding of soyabean oil in High fat diet did not enhanced the blood glucose level up to significant extent. This is correlated with the fat deposition. Accumulation of fat in adipose tissue to a major extent causes insulin resistance due to which the level of blood glucose upregulates (Vincent *et al.,* 2005).

In our study , the feeding of different fat rich high fat diet showed decrease in enzyme activity of SOD and GPx while increased in Catalase activity compared to low fat diet routine of same fats. Antioxidant capacity of enzymes was decreased due to more ROS production in case of High fat diets. Vincent *et al.,* 2005 also reported that in erythrocytes SOD and GPx activity was decreased due to increase in ROS in case of consumption of excess of high fat diets.

In case of lipid profile, Yaqoob *et al.*, 1996 also showed increase in triglyceride, total cholesterol , levels when fed different high fat diet like coconut oil , olive oil, etc.while in our study the HFD-L, HFD-B, HFD-C, shows significant increase in serum lipids levels in comparison with their corresponding LFDs. This occurs due to high level of saturated fatty acids in comparison with mustard and soybean oil based diets.

5.2 Analysis of expression of genes related to Endoplasmic Reticulum stress in liver

Glucose-regulated protein (GRP-78), also known as Binding immunoglobulin protein (BiP) or heat shock 70 kDa protein 5 (HSPA5) is a protein encoded by the *HSPA5* gene. It is associated with activation of UPR response in ER stress condition. Its level had been observed higher after induction of ER stress which leads to accumulation of unfolded protein in lumen of endoplasmic reticulum (Kawasaki N. *et al*., 2012). In our study, the GRP78 expression was significantly higher in HFD-L and HFD-M compared to other LFD and HFD groups. Zhao et al., 2013 reported the increased expression of GRP78 in lard based high fat diet. The GRP78 tries to maintain the folded protein intact during ER stress generated in high fat based diets.

Buffalo milk and ghee contains high saturated fats (98%) compared to cow milk and ghee. DNA damage-inducible transcript 3, also known as C/EBP homologous protein (CHOP), is a transcription factor that is encoded by the *DDIT3* gene. During endoplasmic reticulum stress, CHOP can induce activation of Ero1, causing release of calcium from the endoplasmic reticulum into the cytoplasm resulting in apoptosis. ATF6 is a protein encoded by the *ATF6* gene and involved in the unfolded protein response. It is an endoplasmic reticulum (ER) stress-regulated transmembrane transcription factor that activates the transcription of ER molecules. Here in study, we observed the

significant increase in the expression of Ddit3 and ATF6 in buffalo based high fat diet and cow based high fat diet respectively compared to all other Low fat and high fat diet group even though lard contains high saturated fats. It suggests that the DDit3 and ATF6 are protecting normal cells by inducing apoptosis in affected cells in buffalo based high fat diets. ATF4 expression was increased in HFD-M and HFD-C which indicates the unfolding of proteins is increasing in these diets but increased expression of ATF4 would suggests that it is increasing the protein-protein interaction to compensate the level of unfolded protein during ER stress condition.

CHAPTER –6

Summary and Conclusion

SUMMARY AND CONCLUSIONS

- Å The body weight of C57BL/6 male mice after 16 week of feeding was higher in high fat diet groups (35% fat) in comparison with low fat diet groups (7% fat) of Cow ghee, Buffalo ghee and lard. However, body weight of the mice fed on high fat diet having mustard and soyabean oil was not statistically different from the group fed on low fat diet.
- Å Blood glucose level was significantly higher in all high fat diet groups in corresponding to their low fat diet groups.
- Å In case of mustard oil based high fat diet (HFD-M) the liver weight was significantly lower than corresponding low fat diet. The kidney weight was more elevated in case of lard based high fat diet (HFD-L) and cow ghee based high fat diet (HFD-C) than the HFD-M.
- Å The adipose tissue weight after 16 week of feeding is higher in all high fat diet groups than their corresponding low fat diet groups except in case of mustard based high fat diet which have lower adipose tissue weight than its corresponding low fat diet group.
- Å Serum Total cholesterol levels was significantly higher in high fat diet groups of cow ghee, buffalo ghee and lard than their corresponding low fat diet groups .More interestingly the total cholesterol in HFD-L, HFD-C,HFD-B was higher than the HFD-M and HFD-S.
- Å Serum triglycerides levels was significantly higher in high fat diet groups of cow ghee, buffalo ghee and lard than their corresponding low fat diet groups and mustard oil and soyabean oil based high fat diet groups.
- Å Serum LDL level was significantly higher in high fat diet groups of cow ghee, buffalo ghee and lard than their corresponding low fat diet groups and mustard oil and soyabean oil based high fat diet groups.
- Å Serum VLDL level was significantly higher in high fat diet groups of cow ghee, buffalo ghee and lard than their corresponding low fat diet groups and mustard oil and soyabean oil based high fat diet groups.
- Å Serum HDL levels after 16 week of feeding was significantly lower in all high fat diet groups with corresponding to their low fat diet groups.

- Atherogenic Index is significantly higher in all high fat diet groups than their corresponding low fat diet groups. Also HFD-C, HFD-B, HFD-L have higher atherogenic index than HFD-S, HFD-M.
- Superoxide dismutase activity in liver was assessed lower in all high fat diet groups corresponding to their low fat diet groups.
- Catalase activity was significantly lower in HFD-L corresponding to its low fat diet group, while in other high fat diet group catalase activity was lower than their corresponding low fat diet groups.
- Glutathione peroxidase activity was lowered in all high fat diet groups than corresponding low fat diet groups.
- Relative mRNA expression of 78 kD glucose regulated protein(Grp78) gene was significantly higher in HFD-M as compared to LFD-M, and HFD-L as compared to LFD-L. Overall expression of Grp78 is higher in lard based LFD and HFD groups.
- Relative mRNA expression of C\EBP homologous protein (CHOP) was significantly higher in HFD-B corresponding to LFD-B and all other groups.
- Relative mRNA expression of activating transcription factor 4(ATF4) and activating transcription factor 6(ATF6) was higher in high fat diet groups except HFD-B, to their corresponding low fat diet groups.
- The present study suggest that cow ghee, buffalo ghee and lard based high fat diets are able to disrupt the antioxidative metabolism by reducing the activity of antioxidative enzymes.
- The feeding above three types of diets also have negative impact on the lipid profile (TC,TG,HDL,VLDL,LDL).
- Comparatively mustard oil and soyabean oil based high fat diet have less harmful effects on lipid profile and antioxidative enzyme activity than cow ghee, buffalo ghee and lard based high fat diet.
- Further the feeding of mustard oil and soyabean oil based high fat diets have less negative effect on mRNA expression of endoplasmic reticulum stress marker genes in comparison with other high fat diets.
- The differential effects might be due to the consequence of varying proportions of SCFAs, SFAs, MUFA, PUFA and also certain lipid soluble minor constituents in different fats/oils for which further studies are warranted.

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Appendices

APPENDIX-I

Phosphate Buffer Solution (PBS 1x500mL): 4 g of NaCl (137 mM), 0.1 g of KCl (2.7 mM), 0.575 g of Na_2HPO_4 (8.1 mM) and 0.1 g of KH_2PO_4 (1.5 mM) were dissolved in distilled water and make upto 500 mL for 1x concentration and finally adjust the pH 7.4 with 2M NaOH and stored at 4 ^{0}C.

1% Agarose gel: 1g of standard agarose was dissolved in 100 ml of 1X TAE buffer by heating in micro oven. It was used for running RNA and cDNA on the gel.

Tris-Acetate buffer (pH = 8) (100 ml)

Sr. No.	Chemicals	Quantity
1.	Tris (50X)	24.2gm
2.	EDTA (0.5M)	1.86gm
3.	Glacial acetic acid	5.5ml

Total volume made was made to 100 ml.

APPENDIX-II

No.	Equipments	Company
1	Centrifuge	Remi Laboratory
2	CO_2 Incubatorï water jacketed	Thermo electron corporation Forma series-II
3	Light microscope	CK-RFL, Olympus
4	Weighing Balance	Sartorins
5	pH meter	Genei. Pvt. Ltd, India
6	Power supply	Genei Digital, PS 500, Bangalore
7	Serological Water Bath	Popular India
8	Sonicator	Branson Sonic Power Company, Danbury
9	Spectrophotometer	Specord 200, Analytikjena, GmbH, Germany and Spectronic-601, Milton Roy, USA
10	ELISA plate reader	Microscan (MS5605A) Electronic co-operation of india Ltd. India
11	Nanodrop	ND 1000
12	Real time	BioServe Biotechnologies India Pvt. Ltd.