BENGHAZI UNIVERSITY
FACULTY OF MEDICINE

EFFECT OF PHYSICAL AND METABOLIC STRESS ON SERUM ADIPONECTIN AND LIVER IN MALE MICE

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT FOR THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY TO THE FACULTY OF MEDICINE.

BENGHAZI UNIVERSITY.
BENGHAZI, LIBYA.

By

SARA ALI SHOAIB

Bachelor's Degree In Medicine And Surgery.

Faculty Of medicine , Benghazi University

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2011

جـامعـــة بنغازي

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تأثير الاجهاد البدني والأيضي على الأديبونيكتين في الدم وعلي الكبد في فئران المختبرات

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2011

DEDICATION

TO MY

DEAR FAMILY

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Abbreviations

AdipoR1 = Adiponectin receptor 1.

AdipoR2 = Adiponectin receptor2.

ADP = Adenosine -5´-diphosphate.

AMP = Adenosine Monophosphate.

ANOVA = One-way Analysis of Variance.

ANSA = 1-amono-2-naphthol-4- sulfonic acid.

AP-1 = Actvating Protein-1.

Apo-E = Apolipoprotein E.

APPL = Adaptor Protein Containing Pleckstrin Homology Domain, Phosphotyrosine Binding Domain And A Leucine Zipper Motif).

ASP = Plasma *Acylation-Stimulating Protein*,

ATF = Activating Transcription Factor.

AT = Adipose tissue

ATP = Adenosine Triphosphate.

ATP III = The Adult Treatment Panel III

ATPase = Adenosine Triphosphatase.

BAT = Brown (multilocular) Adipose Tissue

BTB = Blood Testis Barrier.

BSA = Bovine serum albumin.

CAM = Cell Adhesion Molecule.

C/EBP = CCAAT/enhancer binding protein

CETP = *cholesteryl ester transfer protein*

CHOP-10 = C/EBP homologous protein

DNA = Deoxyribonucleic Acid.

DTNB = 5,5-dithiobis(2-nitrobenzoic acid).

EDTA = Ethylene Diamine Tetraacetic Acid.

eNOS = Endothelial Nitric Oxide synthase.

FA = Fatty acid

FAS	=	Fatty acid synthase
fAd	=	full-length adiponectin
gAd	=	C-terminal globular domain
GI	=	Gastroinestinal.
G3PDH	=	Glycerol-3- Phosphate Dehydrogenase
G-6-P	=	Glucose-6-phosphate.
G6Pase	=	Glucose-6-Phosphatase
G6PDH	=	Glucose-6-phosphate dehydrogenase.
GCL	=	Glutamate-Cysteine Ligase.
γ-GCS	=	Gama Glutamylcysteine Synthetase.
GPX	=	Glutathione peroxidase.
GSH	=	Reduced Glutathione.
GSR	=	Glutathione Reductase.
GSSG	=	Oxidized Glutathione.
HDLs	=	High-Density Lipoproteins
HF	=	High-Fat
HFD	=	High Fat Diet
HGF	=	*Hepatocyte Growth Factor*
HGP	=	Hepatic Glucose Production
HIF-1	=	Hypoxia Inducible Factor-1.

HL = Hepatic Lipase

H2O2 = Hydrogen Peroxide .

HSL = Hormone-Sensitive Lipase Enzyme

ICE = Interleukin-1β-convertase.

IDLs = Intermediate-density lipoproteins

IGF-1 = Insulin-Like Growth Factor 1.

IL-1 = Interleukin-1.

IL-1β = Interleukin-1 beta.

IL-6 = Interleukin-6.

iNOS = Inducible Nitric Oxide Synthase.

IR = Insuline resistance

IRS-1 = Insulin Receptor Substrate-1

IRS-2 = Insulin Receptor Substrate-2

IU = International Unit.

LC-CoA = long-chain acyl-CoA)

LDH = Lactate dehydrogenase.

LDLs = low-density lipoproteins

LPL = Lipoprotein Lipase.

LPO = Lipid peroxidation.

LSD = Least-Squares Differences.

MAP = Mitogen-Activated Protein kinases.

MDA = Malondialdehyde.

MIF = Macrophage *Migration Inhibitory Factor* .

MPT = Mitochondrial Permeability Transition.

MS = Metabolic syndrome.

NAD^+ = Nicotineamide adenine dinucleotide (oxidized).

NADH = Nicotineamide adenine dinucleotide (reduced).

$NADP^+$ = Nicotineamide adenine dinucleotide phosphate (oxidized).

NAFLD = Non-Alcoholic Fatty Liver Disease .

NAPH = Nicotine amide adenine dinucleotide phosphate (reduced).

NEFAs = Non-Esterified FAs

NF-kB = Nuclear Factor kappa-light-chain-enhancer of activated B cells.

NHANES III = The Third National Health And Nutritional Survey

NO = Nitric Oxide.

PAI-1 = Plasminogen Activator Inhibitor 1

PE = Phophatidylethanolamin.

PEPCK = phosphoenolpyruvate carboxykinase

Pi = Inorganic phosphate.

PI3K = Phosphoinositide-3-Kinase

PLs = Phospholipids.

PPAR-α = Peroxisome Proliferator-Activated Receptor-A

PS = Phosphatidylserine.

PUFA = Polyunsaturated Fatty Acid.

PX1 = Pannexin 1

RBCs = Red Blood Cells.

RER = Rough Endoplasmic Reticulum.

RNA = Ribonucleic Acid.

RNS = Reactive Nitrogen Species.

ROOH = The lipid hydroperoxide

ROS = Reactive Oxygen Species.

SD = Stander Devotion.

SDS = Sodium Dodecyl Sulfate.

SER = Smooth Endoplasmic Reticulum.

SOD = Superoxide Dismutase.

SPSS = Package for Social Sciences.

SREBP-1 = sterol regulatory element binding protein-1.

TAG = Triacyglycerol.

TBA = Thiobarbituric Acid.

TBARS = Thiobarbituric Acid Reactive Substances.

TCA = Trichloroacetic Acid.

TGF-β = Transforming growth factor *β*

THP1 cells = *(Human acute monocytic leukemia cell line): This product is used to test leukemia cell lines in immunocytochemical analysis of protein-protein interaction, andimmunohistochemistry.*

TNF-α = *Tumor Necrosis Factor α*

TRIS = *Tris(Hydroxymethyl)Aminomethane.*

3T3-L1 = *a cell line derived from 3T3 cells that is used in Biological research on* adipose *tissue.*

UCP2 = *Uncoupling Protein-2*

VLDL = *Very low density lipoprotein*

VEGF = Vascular endothelial growth factor *protein*

WAT = *White (Unilocular) Adipose Tissue*

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CHAPTER 1

INTRODUCTION AND AIM OF STUDY

1.0. INTRODUCTION

Stress is a term in psychology and biology, first coined in the 1930s.

It refers to the consequence of the failure of an organism- human or animal to respond appropriately to emotional or physical threats, whether actual or imagined (Kennard., 2008).

Stress is one of the most important health and social problems. Nowadays, human are exposed to many physical and psychological stress. These are responsible for various pathological conditions such as cardiovascular, hormonal and gastrointestinal diseases (Schellenbaum et al., Swain et al., 2000). Stress has also been implicated as a cofactor in the severity and progression of a number of ailments (Eskiocak et al., 2006).

Stress symptoms commonly include a state of alarm and adrenaline production, short-term resistance as a coping mechanism, and exhaustion. Irritability, muscular tension, inability to concentrate and a variety of physiological reactions such as headache, elevated heart rate, inability to relax, feeling lonely, isolated or depressed, aches and pains, diarrhea or constipation, nausea, dizziness, chest pain, and increased alcohol and nicotine consumption are also mentioned in the literature (Barclay et al., 2005; Seyle, 1950; Glavas et al., 2007).

The autonomic nervous system provides the rapid response to stress commonly known as the fight-or-flight response, engaging the

sympathetic nervous system and withdrawing the parasympathetic nervous system (Tsigos et al., 2002).

There are three types of stress :

1-Physical stress (cold, heat, dark)

2- Metabolic stress (high fat diet ,high sucrose diet)

3-psychological stress .

There are many levels to study stress and its effect on body, one of recently studied one is investigation done on serum adiponectin and liver level.

Adiponectin was originally identified as a protein induced during adipogenesis (Scherer, P. E., 1995– Nakano, Y.,1996). Low serum adiponectin levels were demonstrated in murine models of obesity and insulin resistance (Hu E,1996). In humans, serum adiponectin levels are inversely correlated with body weights and adiposity (Arita Y, 1999 – Weyer C,2001), suggesting important roles of adiponectin in the development of insulin resistance in obese subjects. In addition,adiponectin is reportedly involved in protection against atherosclerosis (Okamoto Y.,2002 – Yamauchi T.,2003). Low serum levels of adiponectin are associated with atherogenesis in obese and insulin-resistant subjects (Hotta K.,2000- Kubota N.,2002). Therefore, to understand the pathophysiology of metabolic syndrome associated with insulin resistance and to develop novel therapeutic targets for the metabolic syndrome, it is essential to elucidate the mechanisms regulating adiponectin production and the resultant serum adiponectin levels.

Previous studies have demonstrated stress influence on the serum level of adiponectin and on liver , such as cold exposure may Suppresses serum adiponectin in some study (Imai junta.,2006) and in other increase adiponectin levels (Yoda. ,2001)., and high fat diet decrease adiponectin levels (Hotta et al. 2001).

But at the interaction of physical and metabolic stress on body weight and serum adiponectin and fasting blood sugar and liver ,the underlying mechanisms are largely unclear .

Therefore, the purpose of this study was to investigate the effects of the interaction between physical and metabolic stress on serum adiponectin and liver and interaction of both in mice.

1.1. Aim of the Study:

The present study was designed to develop an animal model through the exposure to different type of stress . Such a model may be helpful to gain an insight of the altered histology and biochemical perturbations occurring due to the exposure to different type of stress .

Aims of the thesis

1. In this study, we stimulated several forms of chronic (2-week) stress in mice, either alone or in combination with a diet that is high in fat **.**

2 . To study the effect of high fat diet alone and cold stress alone on liver ,serum adiponectin level and,fasting blood sugar. and combination of both.

3. The relationship between stress and obesity. In response to stress, some people lose weight, whereas others gain. stress exaggerates diet-induced obesity.

CHAPTER 2

LITERATURE AND REVIEW

2.0. LITERATURE AND REVIEW

2. 1 . Stress:

Stress is a non-specific response of a body known to alter the physiology-yical homeostasis of the organism resulting in a various endocrine and visceral responses. The immune system plays an important role in biological adaptation, contributing to the maintenance of homeostasis and to establishment of body integrity. (Boenisch ED., 2004) The term "stress" is used here in the classic sense as a state of threatened homeostasis.

Stressors can be broadly classified into two groups:

External Stressors: External stressors include adverse physical conditions (such as pain, uncomfortably hot or cold temperatures) or stressful psychological environments (such as poor working conditions or abusive relationships).

Internal Stressors: Internal stressors can also be physical (infections, inflammation) or psychological. An example of an internal psychological stressor is intense worry about a harmful event that may or may not occur.

Internal psychological stressors are rare or absent in most animals except humans. Internal stressors can be classified as under: Stressors may be of short term (acute) or long term(chronic).

Acute Stress: Acute stress is the reaction to an immediate threat, commonly known as the fight or flight response. The threat can be any situation that is experienced, even subconsciously or falsely, as a danger.

Chronic Stress: Frequently, however, modern life possesses on-going stressful situations that are not short-lived and the urge to act (to fight or to flee) is suppressed, then it will be chronic .

Stress can be classified into three groups:

Environmental or physical stress : (such as cold, hot, dark),

Metabolic stress : (such as high fat diet)

Psychological stress:

Stress decreases physical and mental tolerances and disturbs homeostasis, and subsequently causes diseases or aggravates existing illness. Of the many types of stresses, oxidative stress induces numerous diseases and contributes to aging and brain damage. Stress decreases physical and mental tolerances and disturbs homeostasis, and subsequently causes diseases or aggravates existing illness. Therefore, reduction of stress is important for the prevention of diseases and improvement of quality of life.

Stress is one of the basic factors in the etiology of number of disease and the pathogenesis of numerous human diseases. Some of the prominent stress-related health problems include immune dysfunction, heart disease and gastrointestinal (GI) diseases.

Stressors can alter immune function by several potential mechanisms. One of these mechanisms involves alterations of the endocrine system that in turn modulate immune function (Blalock., 1994; Savino and Dardenne., 1995).

Stressful condition leads to the formation of excessive free radicals which are the major internal threat to cellular homeostasis for aerobic organisms (Yu BP. ,1994).

Various studies have reported activation of the hypothalamic-pituitary-adrenal axis, the sympathetic-adrenal- medullary axis (Maier et al., 1994; Mills et al., 1997; Azpiroz et al., 1999), and the hypothalamic-pituitary-

thyroid axis (Silberman et al., 2002) by stressors resulting in hormonal stress responses.

There are reports about interactions between the thyroid axis and adrenocortical function (Sanchez-Franco et al., 1989; Ku¨hn et al., 1998; Lo et al., 1998). Moreover, the modulation of immune function by glucocorticoids (Wilckens and De Rijk., 1997), and thyroid hormones (Madden and Felten., 1995) is well known.

It has been hypothesized that the genotype or phenotype of an animal influences its hormonal reaction to stress stimulations, which in turn alters the animal's behavioral adaptability and well being (Siegel., 1995; Mench and Duncan., 1998). Understanding the genetic basis for different individual responses to stress is critical in preventing harmful management practices .

The stress system coordinates the adaptive response of the organism to real or perceived stressors (Chrousos GP., 2000). Activation of the stress system leads to behavioral and peripheral changes to improve the ability of the organism to adjust homeostasis and increase its chances for survival (Şahin E., 2004).

2.1.1 Cold stress:

More than 20 years ago, Selye (1973) recognized that the physiological system is activated by stress and that protecting and restoring the body can also damage the system. (Scorza FA, 2008; Andraus W, 2007). Exposure to low temperatures is considered to be an important stressing physical agent.(Gallo Cde B., 2009 ; Yuksel S., 2008).

The physiological components of stress response to cold are metabolic, circulatory and hormonal (Yüksel Ş., 2006).

Cold stress has been demonstrated to cause :

- Cold stress elicits shivering and muscle movement to maintain body temperature, and this action increases production of reactive oxygen species. (Siems WG, 1994)

-Lower body temperature during cold water immersion may inhibit enzyme activity. Thus, metabolism of glutathione, which is important in protecting various cells against oxidative stress and plays a part in cellular protein and immune function, may be impaired during cold water immersion. The ratio of oxidised glutathione to glutathione is increased after short-term whole body cold exposure in human beings and mice. ((Siems WG, 1994-Teramoto S., 1998) However, winter swimmers have a higher concentration of glutathione and a greater activities of glutathione peroxidase and catalase than do healthy controls. (Siems WG., 1999) These findings indicate that glutathione metabolism and function might be impaired during acute cold water immersion but can be preserved during chronic or repeat immersion.

- By summing the previous two point the cold stress cause oxidative damage to several tissues by altering the enzymatic and non-enzymatic antioxidant status, protein oxidation and lipid peroxidation (Şahin E., 2004).

- Activation of hypothalamic-pituitary-adrenal axis and subsequently release of corticosteron from the adrenal cortex into the blood stream (Owens MJ., 1991). The elevation of endogeneous corticosterone due to the stress response has been reported to accelerate the generation of free radicals (McIntosh LJ., 1996).

- Longterm cold exposure is known to induce increases in mitochondrial volume and density, capillary diameter, aerobic enzyme activity and tissue oxygen consumption (Selman C., 2002).

2.1.2 High Fat Diet (HFD)

Fats

Fats are composed of glycerol esterified to three fatty acids. Fats are composed of glycerol esterified to three fatty acids. Fats are derived from three primary sources: **(1)** the diet; **(2)** de novo biosynthesis, particularly in liver; and **(3)** storage depots in adipocytes.

Processes by which these sources are utilized in animals are summarized in **Figure 2.1. 1**. Breakdown of fats by lipases yields fatty acids and glycerol.

Triglycerides are the main form of fat in our bodies and in our diets. They provide us with energy, insulation, and protect our internal organs from damage. They also enable our bodies to metabolize proteins and carbohydrates more efficiently. Despite the many benefits triglycerides have, too much in our blood circulation can cause major health problems, such as heart disease. Knowing the right fats to eat can help reduce overall cholesterol levels and help us to maintain a healthy body. Triglycerides can be further divided into the following categories: (Jennifer Moll., 2008)

a) Saturated Fats**.**

b) Monounsaturated Fats**.**

c) Polyunsaturated Fats**.**

d) Essential Fatty Acids .

e) Hydrogenated Fats**.**

(b) Fat Absorption and Transport:

Dietary fat is hydrolyzed in the lumen of the small intestine (mostly by pancreatic lipase) to yield glycerol, free fatty acids, monoacylglycerols, and diacylglycerols. The hydrolysis products of this digestion are combined back into triacylglycerols (fats) in the endoplasmic reticula and Golgi complexes of the intestinal mucosa cells.

Fats are combined with apoproteins to form chylomicrons, which transport the fats through blood and lymph **(Figure 2.1.2).** Chylomicrons are thus the transport vehicle for dietary cholesterol. Note that fats in chylomicrons are digested in capillaries (to produce chylomicron remnants), like the VLDLs described below. Free fatty acids are rarely found in the bloodstream. Rather, they are complexed to serum albumin.

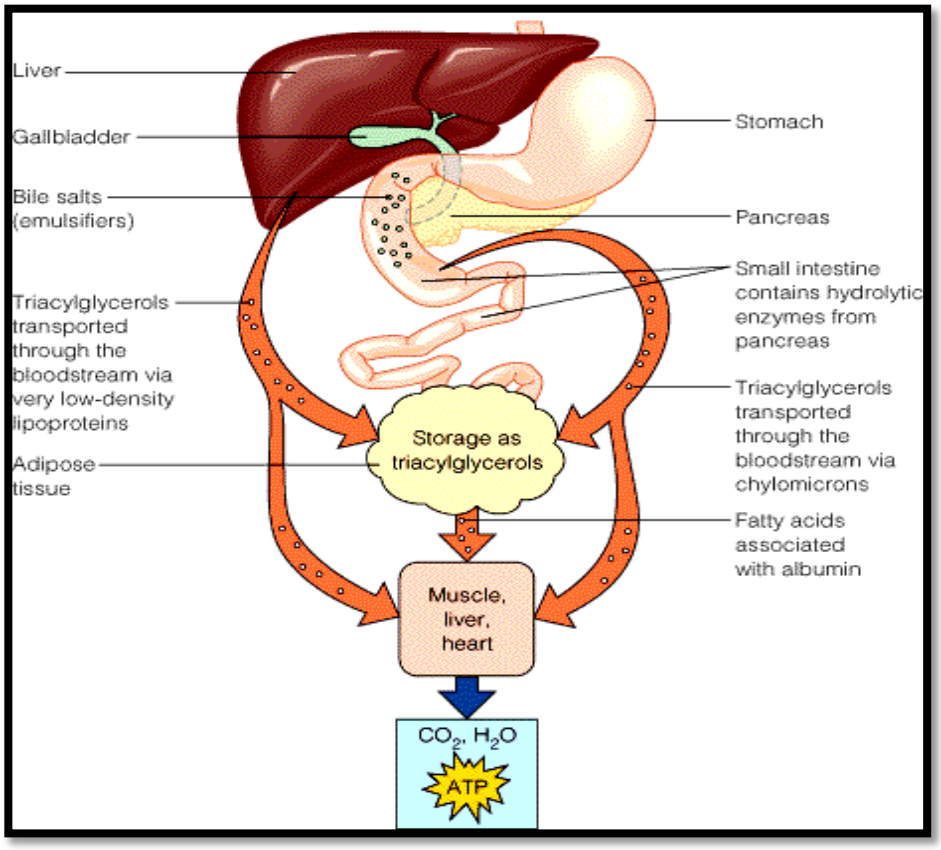


Figure 2.1. 1 : Overview of fat digestion, absorption, storage, and mobilization in the human. (Christopher K. Mathews., 1999).

The liver also plays an important role in fat metabolism. Fats synthesized in the liver are combined with another set of apoproteins to form very low density lipoproteins (VLDLs), which are hydrolyzed at peripheral tissues at the inner surface of capillaries.

Hydrolysis of fats in capillaries by lipoprotein lipase yields intermediate-density lipoproteins (IDLs) from VLDLs and chylomicron remnants from chylomicrons **(Figure 2.1.2).** IDLs are taken up by the liver and further processed to low-density lipoproteins (LDLs). LDLs are the primary form by which cholesterol is transported to tissues and high-density lipoproteins (HDLs) serve to transport cholesterol from tissues back to the liver**.**

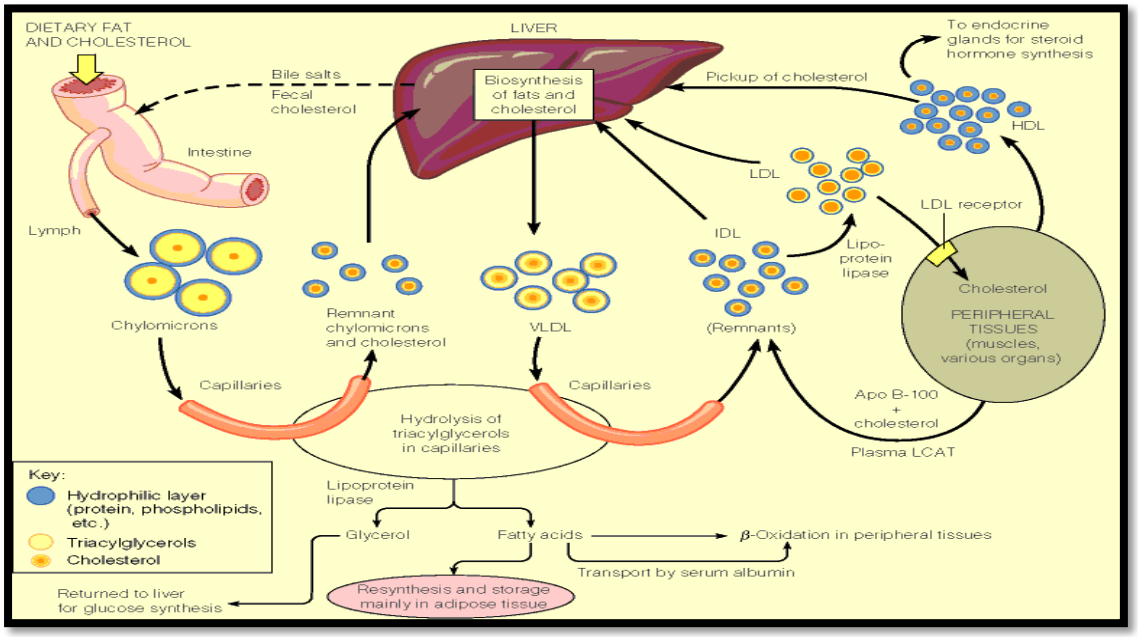


Figure 2.1.2 : Overview of lipoprotein transport pathways and fates (Christopher K. Mathews. ,1999).

High Fat Diet:

The researchers had been using fat-enriched, so-called high-fat (HF) diets, to generate obese rodent models. The first description of such a nutritional intervention dates back to the 1940s. Subsequent studies have revealed that HF diets promote hyperglycemia and whole body insulin resistance, and numerous researchers have examined their effects on muscle and liver physiology and on insulin signal transduction. Based on this experience, it is generally accepted that HF diets can be used to generate a valid rodent model for the metabolic syndrome with insulin resistance and compromised Beta-cell function (Oakes ND., 1997).

An important downside, however, is the definition of the term “high fat diet” itself. Although more than 650 publications [Medline query using the term “high fat diet AND (rat OR mouse) AND (diabetes OR insulin resistance)”, October 2005] have used this approach, neither the exact fat content nor the exact fat composition of the diets employed is standardized.

A multitude of different HF diets have been used with relative fat fractions between 20% and 60% energy as fat, and the basic fat component varies between animal-derived fats, e.g., lard or beef tallow, and plant oils, e.g., corn or safflower oil.

Importantly, many researchers have employed well-defined, semi-purified HF diets, in which the fat component replaces carbohydrate and/or protein, but others have simply added fat to a standard rodent chow. This obviously leads to an unbalanced diet composition with respect to all macro- and micronutrients.

Consequently, various diets with very different fatty acid compositions are summarized under the term HF diet in the literature. This has inevitably led to a considerable variability in the results reported.

Different effect of high fat diet:

a) Obesity:

Prolonged feeding with fat-enriched diets induces an increase in body weight in susceptible rats in the range of 10% to 20% over standard chow-fed controls. Obesity induction is most effective when the diet is started at a young age and continues for several weeks .Body weight gain during the feeding period is gradual .Although an increase in body weight can be appreciated after as little as 2 weeks, the diet-induced phenotype

becomes most apparent after several, i.e., more than 4 weeks of HF feeding. (Buettner.,2007)

Although no consistent fat type effects can be detected when looking at HF diets based on mammal and plant fats , some authors have described obesity resistance and less hypertrophy of visceral fat pads when employing fish oil-based diets . This might be connected with an increased lipid oxidation in these animals due to the fish oil-induced activation of peroxisome proliferator-activated receptor _gama (Jump DB.,2002- Duplus E.,2000).

It is known that a certain fraction of the animals subjected to the HF diet will not become obese. Recent studies have demonstrated that obesity-susceptible animals are hyperphagic, possibly due to a central resistance to the anorexigenic action of insulin (Clegg DJ.,2005) and a decreased hypothalamic expression of anorexigenic peptides such as -melanocyte stimulating hormone and cocaine and amphetamine- regulated transcript (Tian DR.,2004); rats remaining lean on an HF diet eat the same amount of calories as standard chow-fed controls (Farley C.,2003). These diet-induced obesity-resistant animals may turn into important models for the genetic basis of weight gain.

(b)Blood Glucose, Insulin Levels, and Insulin Sensitivity:

HF diet effects on blood glucose levels are described discrepantly. Normoglycemia, slight hyperglycemia, and the development of type 2 diabetes have been reported with different diet regimes . From the data published so far, one can conclude that prolonged, i.e., several weeks, feeding with both animal and plant fat-enriched diets will eventually lead to moderate hyperglycemia in most rat and mouse strains. With the diet

types mentioned above, the elevation of fasting glucose levels is usually accompanied by a moderate to distinct increase in fasting plasma insulin levels .

As with obesity, fish oil-fed animals generally do not develop such signs of systemic insulin resistance (Storlien LH., 1987). Matching with these biochemical parameters, hyperinsulinemic-euglycemic clamp experiments consistently have demonstrated wholebody insulin resistance in animal and plant fat-fed rodents, whereas fish oil feeding was coupled with sustained insulin sensitivity (Storlien LH., 1996 ; Storlien LH., 2002).

Although it has been stated that saturated fats lead to an enhanced development of insulin resistance in the HF setting (Rivellese AA., 2002), it should be remembered that the experimental basis for such an assumption is not very strong in rodent models. In the most cited study (Storlien LH., 1991) directly comparing dietary fat types, the diet labeled saturated contained predominantly unsaturated fatty acids (19% energy saturated, 12% energy monounsaturated, and 28% energy polyunsaturated), and other comparative studies have also failed to confirm a clear association between fat saturation and insulin action .(Ikemoto S., 1996).

The **Roland Buettner group** (Roland Buettner.,2007) were not able to detect significant correlations between the saturation level of free fatty acids and insulin action as estimated from whole-body glucose disposal after an insulin challenge.

Insulin resistance was shown after as little as 2 weeks of a lard-based HF diet, indicating the rapid dietary induction of this disorder (Youngren JF 2001). The development of overt diabetes in these animals is controversial. In **Roland Buettner group's** personal (unpublished)

experience, some Wistar rats held on a lard-based diet for more than 12 months show fasting glucose levels above 150 mg/dL and postprandial glucose levels of more than 300 mg/dL; therefore, these animals can be classified as diabetic. **Roland Buettner group's** (Roland Buettner.,2007) could not found reliable predictors of such diabetic development, which makes it difficult to use this approach for diabetes modeling. Other researchers have described similar results, e.g., with high-olive oil diets in C57BL/6J mice (Surwit RS., 1988)(Tsunoda N., 1998), whereas Chalkley (Chalkley et al.,2002) were not able to induce overt diabetes by HF feeding of Wistar rats with a safflower oil-based diet.

(c) Lipids:

Comparable with the effect on blood glucose levels, most authors report fasting hypertriglyceridemia when using HF diets based on animal fats and plant fats, whereas fish oil-based chow tends to lower plasma triglycerides. (Buettner ., 2007)

Data on total cholesterol levels under HF diets are inconsistent, and definite statements about the putative induction of hypercholesterinemia by a pure HF diet, i.e., without addition of cholesterol, do not seem possible from the literature at present. The only exception is fish oil-based diets; there, a hypocholesterolemic effect is clearly established (Luo J., 1996 ;Kim HJ., 1999).

(d) Adipokines:

Adipokines such as leptin, adiponectin, or resistin are recognized as systemic factors influencing insulin sensitivity. From the studies published so far , these parameters mostly mirror human obesity in HF-

fed rats. Leptin and resistin levels tend to be elevated, and adiponectin levels are slightly down-regulated in dietary obese rodents. However, because of the small number of studies giving detailed descriptions of the diet composition, fat type effects cannot be accurately specified . (Buettner ., 2007)

2.1.2.1 High Fat Diets and Adipose Tissue:

It is difficult to define an unanimous phenotype of the HF diet adipocyte, but some changes of adipocyte morphology and metabolism have been found consistently throughout the literature when using animal- and plant-derived HF diets.

Adipocyte number and size are increased (Belzung F.,1993, Corbett SW., 1986), and epinephrine-stimulated lipolysis is reduced (Tepperman HM., 1995). An enhanced uncoupling protein expression in brown and white adipose tissue and a decrease in the expression levels of liposynthetic genes (Matsuda J., 1997 and Takahashi Y., 1999) might provide a partial defense against the HF-induced lipid storage.

Interestingly, gene expression profiling has recently revealed an up-regulation of inflammatory genes in adipocytes of diet-induced obese mice (Moraes RC., 2003), which resembles the proinflammatory state described in human obesity.

Animal- and plant-derived HF diets also induce changes in insulin action in adipose tissues. The stimulation of glucose uptake is reduced in brown and white fat (Lavau M.,1979)(Storlien LH ., 1986).

Decreases in insulin receptor autophosphorylation (Watarai T., 1988) and an activation of glycogen synthase kinase-3 (Eldar-Finkelman H., 1999) have been described as molecular basis for these effects, but

relatively little is known about the exact changes in adipocyte insulin signaling in this model.

Animals overexpressing diacylglycerol acyltransferase, the rate-limiting enzyme of triglyceride synthesis, in their adipose tissue become obese under an HF diet but are resistant to diet-induced insulin resistance, pointing at the importance of an efficient fat storage in adipose tissue. (Chen HC., 2002)

Animals lacking hormone-sensitive lipase, the enzyme controlling lipolysis from adipose tissue, remain lean when fed with an HF diet; they also show lower glucose and fasting insulin levels. This was explained by an increase in thermogenesis and energy expenditure and an impaired differentiation of white adipose tissue (Harada K., 2003).

Taken together, animal- and plant-derived HF diets lead to adipocyte hypertrophy, hyperplasia, and insulin resistance.

The role of adipocytes as important regulators of whole-body energy and glucose metabolism has been confirmed by HF diet interventions in animals with tissue specific transgenic manipulations. Contrasting its clear beneficial effects in whole-animal lipid metabolism and the skeletal muscle, the impact of fish oil on adipose tissue function remains unclear

High-fat-diet (HFD)-induced obesity also suppresses adiponectin expression by increasing cellular levels of catecholamine and PKA-mediated activation of CREB.. (Meilian LIU., 2010) **(Figure 2.1.3).**

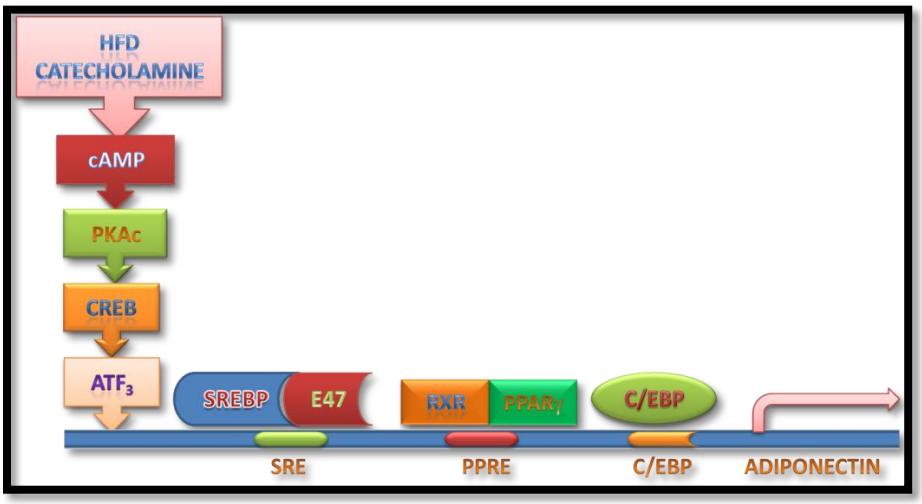


Figure 2.1.3: Effect of high fat diet on adiponectin level.(Meilian LIU and Feng LIU.,2010)

2.1.2.2 High Fat Diets and the Liver:

It is well established that the obesity-inducing HF diets based on animal and plant fats also lead to hepatic steatosis (Yaqoob P., 1995). From clamp studies, it is clear that this condition is associated in vivo with hepatic insulin resistance, i.e., an impairment of insulin's ability to lower hepatic glucose output (Haque M., 2002), and interventions designed to improve hepatic fat clearance, e.g., the hepatic overexpression of uncoupling protein-1, have demonstrated improvements of whole-body insulin resistance (Ishigaki Y., 2005).

However, insulin signaling studies in HF-fed rats have shown that the classic disorders observed in muscle and fat are not necessarily replicated in the liver.

IRS-1 and IRS-2 proteins and their phosphorylation are not altered, and phosphoinositide- 3-kinase activity associated with IRS-1 and IRS-2 is

increased (Yaqoob P., 1995;Anai M., 1999).

Also, recent studies have demonstrated sustained insulin action in isolated steatotic livers (Buettner R., 2004) and important regulatory effects of systemic and central nervous factors on hepatic glucose output (Obici S., 2002 ;Liu L., 1998), which questions whether insulin resistance is a truly intrinsic trait of diet induced hepatic steatosis, or may be secondarily enhanced, at least partially, by systemic factors.

Recent work has drawn attention to the activation of nuclear factor B by HF diets in the liver, which might contribute both to the development of non-alcoholic inflammatory hepatic disease and diet-induced disorders of hepatic glucose metabolism (Cai D., 2005).

Differing potencies of specific diets to activate hepatic nuclear factor _B, therefore, might explain the somewhat controversial results with respect to hepatic insulin action.

HF diets can induce hepatic steatosis and signs of hepatic insulin resistance in the whole animal; this closely resembles the human obese state. The possible dietary activation of hepatic inflammatory pathways may be part of the link between diet-induced fat deposition and non-alcoholic steatohepatitis.

2.2. Adipose tissue (AT):

Vertebrates in general and mammals in particular, have adipose tissue (AT), which allows them to store excess calories as lipids (triacylglycerols– TAG). These are hydrophobic and can be stored in large quantities without water as a solvent and contain twice as much energy per unit mass than other nutrients. Adipose tissue has always been known as an energy reserve and thermal insulator. For a long time, these two properties were applied to the study of energy metabolism regulation and

little attention was given to analysis of its other capabilities (Fonseca-Alaniz MH et al., 2007).

2.2.1. Adipocyte:

Fat cells or adipocytes are derived from undifferentiated fibroblast-like mesenchymal cells, although under certain conditions histologists believe that fibroblasts may give rise to adipose cells**(Figure 2.2.1).**

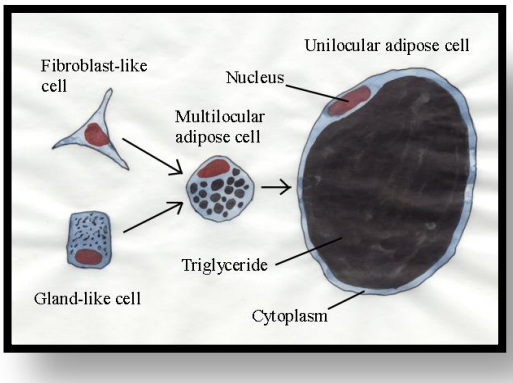


Figure 2.2.1: Origen of fat cells (http://www.aps.uoguelph.ca/ANSC*2340/LEC18/LEC18.html)

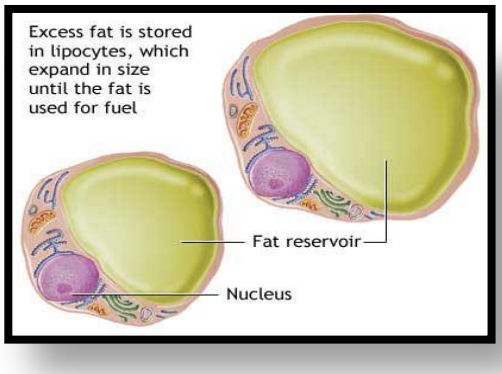


Figure 2.2.2: Fat cells http://medicineworld.org/images/blogs/7-2007/fat-cells-14560.jpg

Adipose cells are fully differentiated and don't undergo cell division. They function in the synthesis and storage of triglycerides, the only cells that are specialized and perfectly adapted to store lipids without this compromising their functional integrity **(Figure 2.2.2).** They have the enzymatic machinery necessary to synthesize fatty acids (a process known as lipogenesis) and to store TAG during periods of abundant energy supply and to mobilize them via lipolysis when there is a calorie deficit. (Ahima RS ., 2000).

The central nervous system takes part in regulation of these two processes by means of direct or indirect neural activity (for example, initiating behavior to seek and consume nutrition). Other regulatory systems (digestive and endocrine) participate by means of nutrients and hormones depending on requirements at any given moment. (Ahima RS ., 2000).

The autonomous nervous system acts directly on Adipose tissue through its subdivisions, the sympathetic and parasympathetic systems. The sympathetic system promotes catabolic actions (lipolysis), via β-adrenergic stimulation, which activates the hormone-sensitive lipase enzyme (HSL) (Pénicaud L, 2000). The parasympathetic system organizes anabolic actions by increasing insulin production and increasing glucose and fatty acid capture (Kreier F., 2002).

In addition to adipocytes, AT contains a matrix of conjunctive tissues (collagen and reticular fibers), nerve fibers, vascular stroma, lymph nodes, immune cells (leukocytes, macrophages), fibroblasts and preadipocytes (undifferentiated adipose cells) (Ahima RS., 2000).

2.2.2. Histology of Adipose tissue:

The adipose tissue is one type of connective tissue proper and classified into two types according to whether it is composed of unilocular or mult-ilocular adipocytes. Other differences between the two types of adipose tissue are color,vascularity, and metabolic activity (Leslie p . Gartner &James L . Hiatt., 2007).

(A) White (Unilocular) Adipose Tissue (WAT):

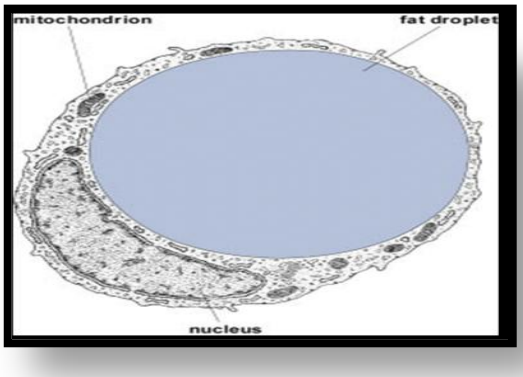


Figure 2.2.3. **White adipose cell with a large single fat droplet**(W.B.Saunders, 1971).

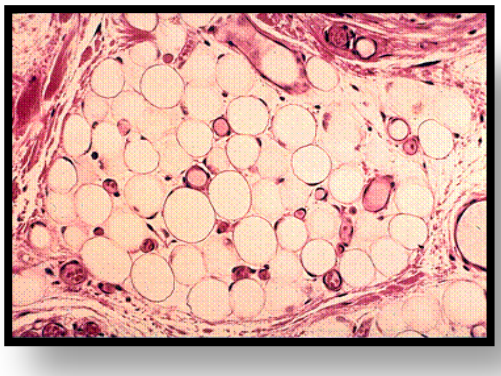


Figure 2.2.4: Adipose Tissue (White) (Color Atlas of Cytology, Histology,and Microscopi Anatomy 4th edition 2003)

Each unilocular fat cell contains a single lipid droplet, giving the adipose tissue composed of such cells a white color **(Figure 2.2.3).**

White adipose tissue is heavily supplied with blood vessels, which form capillary networks throughout the tissue. The vessels gain access via connective tissue septa that partition the fat into lobules **(Figure 2.2.4).**

The plasma membranes of the unilocular adipose cells contain receptors for several substances, including insulin, growth hormone, norepinephrine, and glucocorticoids, that facilitate the uptake and release of free fatty acids and glycerol.

Unilocular fat is present in the subcutaneous layers throughout the body. It also occurs in masses in characteristic sites influenced by sex and age. In men, fat is stored in the neck, in the shoulders, about the hips, and in the buttocks. As men age, the abdominal wall becomes an additional storage area. In women, fat is stored in the breasts, buttocks,

hips, and lateral aspects of the thighs. Additionally, fat is stored in both sexes in the abdominal cavity about the omental apron and the mesenteries (Leslie p Gartner &James L Hiatt., 2007).

(B) Brown (multilocular) Adipose Tissue (BAT):

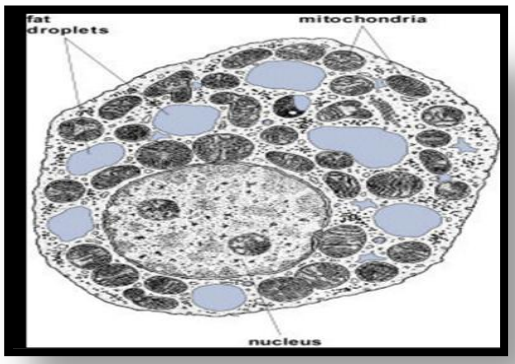


Figure 2.2.5: **Brown adipose cell with many small lipid droplets** (W. B. Saunders, 1971).

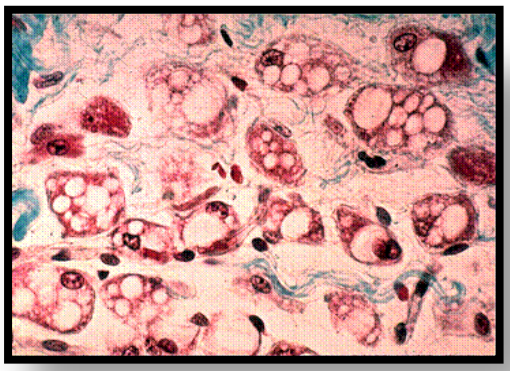


Figure2.2.6:Adipose Tissue (Brown). (W. B. Saunders, 1971).

Brown adipose tissue (Brown Fat) is composed of multilocular fat cells, which store fat in multiple droplets **(Figure 2.2.5).** This tissue may appear tan to reddish brown because of its extensive vascularity and the cytochromes present in its abundant mitochondria **(Figure 2.2.6).**

Multilocular adipose tissue has a lobular organization and vascular supply similar to those of a gland. Brown fat tissue is very vascular because the vessels are located near the adipocytes.

Unmyelinated nerve fibers enter the tissue, with the axons ending on the blood vessels as well as on fat cells, whereas in white fat tissue, the neurons end only on the blood vessels.

Although it has long been known that multiocular fat is found in many mammalian species, especially those that hibernate, and in the infants of most mammals, it was unclear whether multiocular fat exists in adult human.

However, in the newborn human, brown fat is located in the neck region and in the interscapular region.

Brown adipose tissue is associated with production of body heat because of the large number of mitochondria in the multilocular adipocytes composing this tissue. These cells can oxidize fatty acids at up to 20 times the rate of white fat, increasing body heat production three fold in cold environments. Sensory receptors in the skin send signals to the temperature regulating center of the brain, resulting in the relaying of sympathetic nerve impulses directly to the brown fat cells.

The neurotransmitter norepinephrine activates the enzyme that cleaves triglycerides into fatty acids and glycerol, initiating heat production by oxidation of fatty acids in the mitochondria. (Leslie p ., 2007).

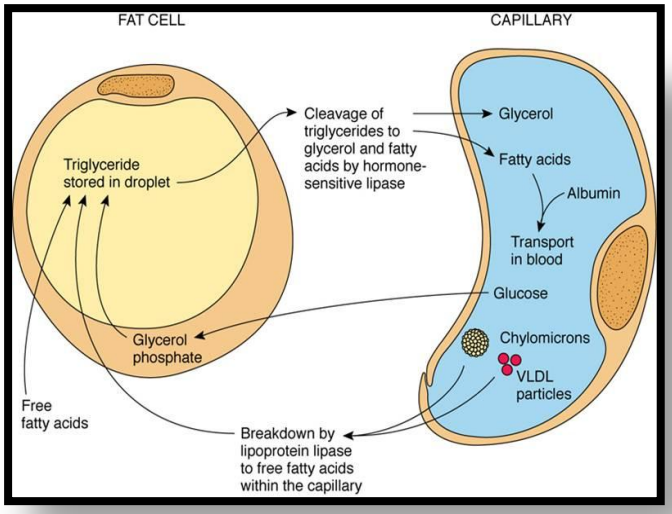
Thermogenin is a transmembrane protein located on the inner membrane of mitochondria , permits back flow of protons instead of utilizing them for synthesis of Adenosine triphosphate (ATP); as result of uncoupling oxidation from phosphorylation , the proton flow generates energy that is dispersed as heat(Leslie p . Gartner &James L . ,Hiatt 2007).

2.2. 3. Adipose tissue functions:

The metabolic role of adipose tissue:

Adipose tissue play role in glucose and lipid metabolism and how these are regulated. Adipose tissue may also play a significant role in the

whole-body production of the amino acids, alanine and glutamine (Frayn KN .,1999) **(Figur2.2.7).**



Figur2.2.7: The metabolic role of adipose tissue (Leslie p . Gartner &James L . ,Hiatt 2007).

(a) Lipid metabolism:

For many years adipose tissue was regarded as a metabolically inert tissue, its sole purpose being the storage of fat for insulation purposes and provision of a protective cushion explained by Its low rate of oxygen consumption (Coppack SW.,1990 ;Frayn KN.,1995). However, over the last two decades it has become clear that not only is adipose tissue involved in metabolism but that it plays an extremely important role as a regulator of the flow of energy-providing substances. Adipose tissue does indeed store triglyceride (TG) but, in the healthy individual, it is an efficient director of non-esterified fatty acids (NEFA) either into adipose tissue for storage or into the circulation as an energy supply for other tissues, such as heart and skeletal muscle.

Glucose metabolism:

In contrast to its major role in lipid metabolism, adipose tissue appears to play a very minor role in the whole-body disposal of a glucose load and contributes little to the total amount of lactate released by peripheral tissues in vivo (Frayn KN.,1989 ;Marin P.,1987).

The endocrine role of adipose tissue:

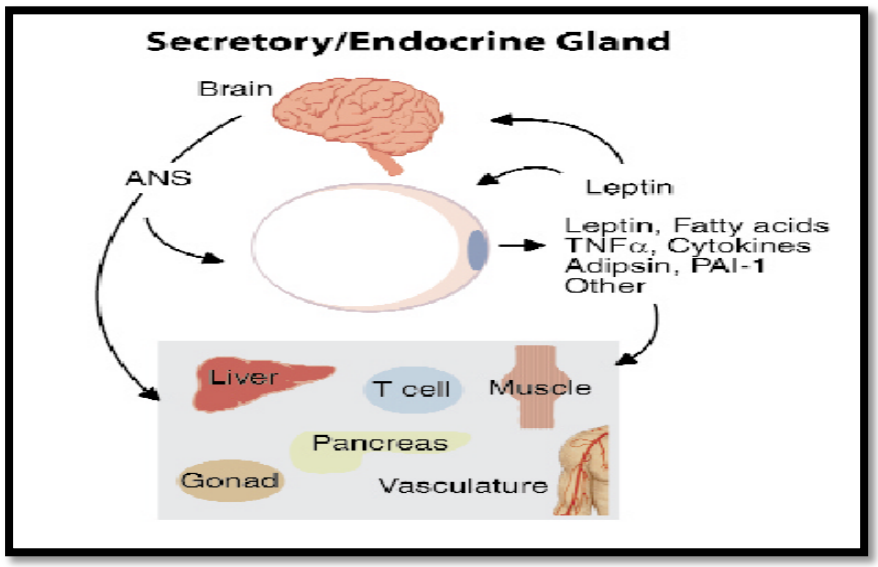


Figure 2.2.8: The endocrine role of adipose tissue (Hollung K., 2003) .

Over the last 15 years, with the discovery of capacity of white adipose tissue to secrete hormones, great importance has been attributed to its endocrinal role **(Figure 2.2.8).**

These hormones, known as adipokines, have revolutionized the conception of its biological function, consolidating the idea that it is not just a supplier and storer of energy, but a dynamic organ and central to

metabolic regulation given the structural diversity of adipokines and the variety of functions so far identified, it can be stated that they include everything from proteins and non-protein factors produced and secreted by white adipose tissue (Miriam Helena .,2007) .**(Table 2.2.1).**

Table 2.2.1 - Protein and non-protein factors produced and secreted by white adipose tissue (Miriam Helena .,2007):

Substance	Biological effect
Leptin	**Signals to the CNS about the body's energy stocks**
Adiponectin	**Increases sensitivity to insulin, is anti-inflammatory and attenuates the progression of atherosclerosis**
Resistin	**Increases insulin resistance**
TNF-α	**Lipolytic, increases energy consumption and reduces sensitivity to insulin**
Interleukin-6	**Proinflammatory, lipolytic, reduces sensitivity to insulin**
Adipsin	**Activates the alternative complement pathway**
ASP	**Stimulates triacylglycerol synthesis in WAT**
Angiotensinogen	**Precursor of angiotensin II, involved in regulating arterial blood Pressure**
PAI-1	**Inhibits plasminogen activation, blocking fibrinolysis**
Tissue factor	**Initiates the coagulation cascade**
VEGF	**Stimulates vascular proliferation (angiogenesis) in WAT**
Visfatin	**Insulinomimetic predominantly produced by**

	visceral fat
Monobutyrin*	**Vasodilator and inducer of vascular neoformation.**
TGF-β	**Regulates a series of processes in WAT, including proliferation of preadipocytes and differentiation, development and apoptosis of adipocytes**
IGF-1	**Stimulates proliferation and differentiation of adipocytes**
HGF	**Stimulates differentiation and development of adipocytes**
MIF	**Immunoregulator with paracrine action in WAT**
LLP†	**Hydrolysis stimulating enzyme in the TAG of lipoproteins(chylomicron and VLDL)**
CETP†	**Transfers cholesterol esters between lipoproteins**
Apo-E†	**Protein component of lipoproteins, especially VLDL**
Prostaglandins*	**Regulators of many cellular processes, active during inflammation, blood coagulation, ovulation and secretion of gastric acid**
Estrogens*	**Produced by the action of aromatase, this is the principal source of estrogen in men and postmenopausal women**
Glucocorticoids*	**Generated by the action of 11 hydroxysteroid dehydrogenase, typeII, which transforms cortisone into cortisol in WAT**
Apelin	**Its biological actions are not very clear yet, but are related to control of the body's energy stores**

* Non-protein substances. † Proteins without hormonal action

2.3. Adiponectin:

2.3.1. Discovery of Adiponectin:

Adiponectin was independently isolated from human plasma as gelatin-binding protein-28 (Nakano Y. et al., 1996). It was discovered during gene-expression profiling of human adipose tissue conducted during the human cDNA project, which analysed visceral and subcutaneous adipose tissues to elucidate the molecular mechanism of obesity-related diseases (Maeda,1996, Maeda1997). Unexpectedly, genes expressed in subcutaneous and visceral adipose tissue, 20% and 30% respectively, were bioactive secretory proteins, i.e. adipocytokines such as leptin and TNF-α. More surprisingly, 40% of the genes expressed in adipose tissue were novel and the most abundant among them, was termed adiponectin, (Maeda et al .,1996 ; Arita et al., 1999) .

2.3.2. Adiponectin structure and function:

(A) Adiponectin structure:

Adiponectin is one of the most abundant plasma proteins. Located on chromosome 3q27, a locus for diabetes susceptibility (Kissebah et al., 2000 and Takahashi et al.,2000).

Adiponectin is a secretory protein contains 244 amino acids,consist of a signal peptide, a collagen like domain at its N-terminus and a globular domain at its C-terminus **(Figure2.3.1).**

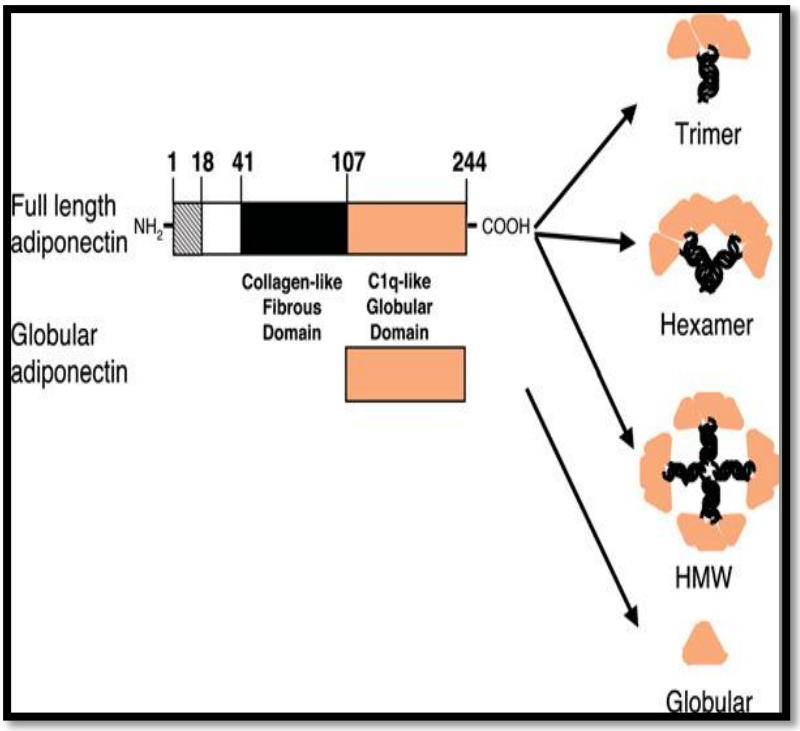


Figure 2.3.1: Domains and structure of adiponectin (Yoshihisa OKAMOTO., 2006).

The adiponectin gene product is a 30 kDa protein .Full-length adiponectin requires post-translational modifications for activity (e.g. hydroxylation and glycosylation) and is secreted from adipocytes in three major size classes trimers that are 90 kDa in size (the basic unit), low molecular weight hexamers (180 kDa) and high molecular weight isoforms (12-18mers) that can be more than 400 kDa in size **(Figure 2.3.2)**(Pajvani UB, et al., 2003; Schraw T., et al.2008).

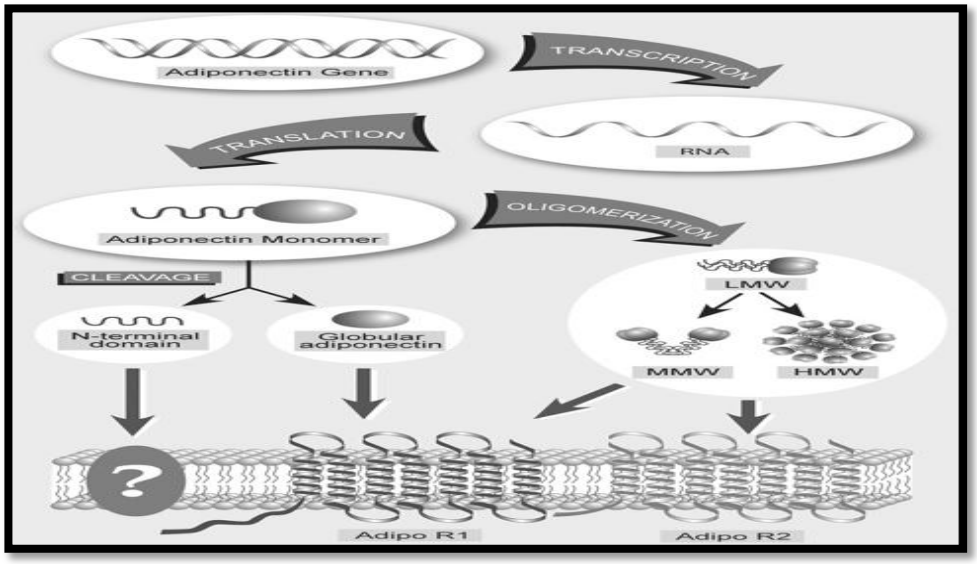


Figure2.3.2: Regulation of adiponectin synthesis and function
(Fang and G. Sweeney.,2006)

(B) Adiponectin function:

Adiponectin has antidiabetic properties due to insulin-mimetic and insulin-sensitizing actions, while anti-inflammatory and anti-atherosclerotic effects have also been consistently reported.

Differences in the functions of the various isoforms of adiponectin are:

(a) Both low molecular weight and high molecular weight adiponectin

(1) Induce apoptosis in nondifferentiated THP1 cells (a human acute monocytic leukemia cell line).

(2) Reduce expression of macrophage scavenger receptor A messenger RNA.

(3) Activate AMP kinase.

(4) Low molecular weight form reduces lipopolysaccharide-mediated interleukin-6 release and suppresses nuclear factor κB (NFκB) activation.

(5) High molecular weight adiponectin induces interleukin-6 secretion in human monocytes and THP1 cells and does not suppress lipopolysaccharide-induced interleukin-6 secretion (Neumeier M, et al., 2006). These disparate results could reflect cell-type specificity and other experimental differences.

(b) The C-terminal globular domain of adiponectin:

(1) Affects systemic glucose metabolism.

(2) Exert effect on the microvasculature and heart (Fruebis J, et al., 2006)(Hopkins TA, et al., 2006).

(c) The globular and full-length oligomers:

(a) In endothelial cells, they exert comparable effects in suppressing increased ROS production induced by increased glucose levels (Ouedraogo R, et al., 2006).

(b) fAd (full-length adiponectin) multimers may be cleaved to liberate a fragment containing the C-terminal globular domain (gAd), which exhibits potent metabolic effects, particularly in skeletal muscle (Ceddia, R.B., 2005)(Fruebis, J., 2001).

Circulating adiponectin has been found mainly in HMW, MMW and, to a lesser extent, LMW, whereas the presence of circulating gAd remains controversial(Fruebis, J.,2001 - Xu, A.,2005).

The localized cleavage of fAd to produce gAd by tissue specific proteases or at sites of inflammation will be of great physiological relevance. (Waki, H., 2005)

2.3.3. Secretion Of Adiponectin:

Adiponectin is secreted from adipose tissue directly into the blood stream. Adiponectin is very stable in blood, and the half-life for its clearance from blood is 32 min (Spranger et al., 2006).

The plasma range of adiponectin in human subjects is determined by An ELISA (**3 –30** μg/ml), accounting for 0.01% of total plasma protein (Arita Y., 2002), with significantly lower concentration found in obese subjects than non-obese subjects (Arita et al.,1999). Serum levels of adiponectin are approximately twofold higher in females than in males, which probably contribute to sex-related differences in some of its vascular protective effects. (Arita et al.,1999).

Plasma adiponectin concentration appear to vary somewhat between species, Wild type mice had plasma adiponectin concentrations ranging from 10 to 30 μg/ml(Combs et al., 2004), and horses ranged between 1.3 and 2.0 μg/ml (Gordon and McKeever, 2005; Gordon et al., 2007).

2.3.4. Metabolism &Excretion Of Adiponectin:

In the circulation, adiponectin oligomers are extremely stable and do not undergo exchange from one form to another, even in the face of an insulin or glucose challenge (Pajvani UBX, Combs TP et al., 2003).
Pajvani and Combs et al. (2003), reported that the circulating HMW adiponectin has a longer half life than LMW adiponectin (9 vs. 4.5 h) although HMW multimers may be cleared more rapidly than LMW adiponectin following metabolic challenges (Pajvani UBX, Combs TP et al., 2003).

A recombinant form of adiponectin (C39S), that is unable to form oligomers larger than trimers and is sensitive to proteolytic cleavage following secretion, appears to be more biologically active than HMW adiponectin and it is subject to more rapid clearance from the circulation . Together with the above observations that suggest an important role for HMW adiponectin in insulin sensitivity, these findings have led to the proposal that HMW adiponectin may be converted to a biologically active form in response to metabolic challenge, through reduction and proteolytic cleavage (Pajvani UB.,2004). Reduction followed by proteolysis is an emerging mechanism of ligand activation (Hogg PJ., 2003).

In support of such a model for adiponectin activation, a small amount of a C-terminal globular domain-containing fragment of adiponectin has been detected in human serum following immunoprecipitation (Fruebis J., 2001).

Moreover, leucocyte elastase, which is secreted by monocytes, has recently been identified as an enzyme capable of cleaving the collagenous domain of adiponectin to produce such fragments (Waki H.,2004).

Although little is known about the process of adiponectin clearance, adiponectin has been detected in urine from type 2 diabetic subjects and healthy males (Koshimura J.,2004).

Urinary adiponectin levels, which are around three orders of magnitude lower than serum levels, were significantly elevated in patients with macro-albuminuria and correlated with urinary albumin. These observations support a model where leakage of circulating adiponectin,

through the damaged kidneys of these patients, is largely responsible for the high urinary adiponectin, although additional physiological and and pathophysiological factors may also be involved (Koshimura J.,2004).

2.3.5. Adiponectin receptor:

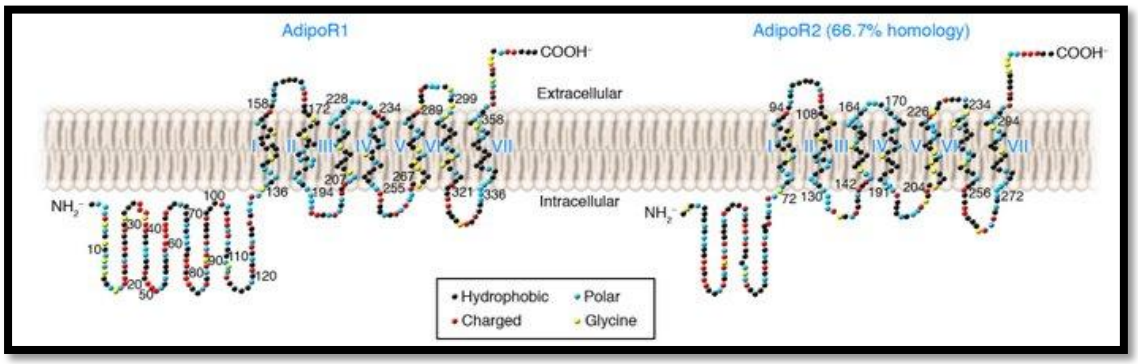


Figure2.3.3: Structure of adiponectin receptors (Takashi Kadowaki ., 2006).

A few years ago, Yamauchi et al.(2003) cloned two different isoforms of adiponectin receptor, AdipoR1 and AdipoR2. Both isoforms are expressed in many cell types, including adipocytes **(Figure 2.3.3)** (Karbowska J., 2005) (Fasshauer M., 2004) (Yamauchi T., 2003) .

Adiponectin binds to the C-terminal extracellular domain of AdipoR1 whereas the N-terminal cytoplasmic domain interacts with an adaptor protein APPL (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and a leucine zipper motif).

Since adiponectin receptors are expressed in fat cells, adiponectin may play an important role in the regulation of adipose tissue metabolism via autocrine and/or paracrine manner. (Yamauchi T 2003)

In human tissues AdipoR1 is expressed mainly in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. (Yamauchi T 2003)

Moreover, it has been demonstrated that two types of adiponectin receptor have different binding affinity for globular and full-length adiponectin. AdipoR1 is a high-affinity receptor for globular adiponectin but a very low-affinity receptor for full-length adiponectin, whereas AdipoR2 is an intermediate affinity receptor for globular and full-length adiponectin (Yamauchi T., 2003).

AdipoR1 and R2 belong to a new family of membrane receptors predicted to contain seven transmembrane domains but structurally and topologically distinct from G-protein coupled receptors (Yamauchi T. ,2003). In 2004, Lodish's group proposed that T-cadherin was also a receptor for high molecular-weight forms of adiponectin (Hug C., 2004).

However, the two receptors act through different pathways:

(a) AdipoR1 reduced HGP production by activating the AMPK pathway and by decreasing the expression of enzymes involved in gluconeogenesis (glucose-6-phosphatase, phosphoenolpyruvate carboxykinase1) and in liver steatosis (SREBP-1).

(b) AdipoR2 had no effect either on HGP or on the expression of the gluconeogenic enzymes and steatotic factors but increased the expression of glucokinase, an enzyme involved in increased hepatic glucose delivery **(Figure 2.3.4).**

In addition, increased expression of AdipoR2 but not of AdipoR1 resulted in increased expression of PPARα and its target genes such as acyl-CoA oxidase and UCP2 leading to increased fatty-acid oxidation and to decreased hepatic triglyceride content.

Insulin resistance in different mouse models results from increased production of TNFα or of chemokines such as MCP-1 leading to an increased oxidative stress.

Liver over-expression of AdipoR2, but not AdipoR1, decreased the expression of TNFα and MCP-1 and reduced oxidative stress.

Therefore, AdipoR1 and R2 have different roles in glucose metabolism as demonstrated in study by Yamauchi (2003).

Moreover, under high-fat conditions, they could display quite opposing roles, AdipoR1 being favorable for metabolism and AdipoR2 being mostly harmful.

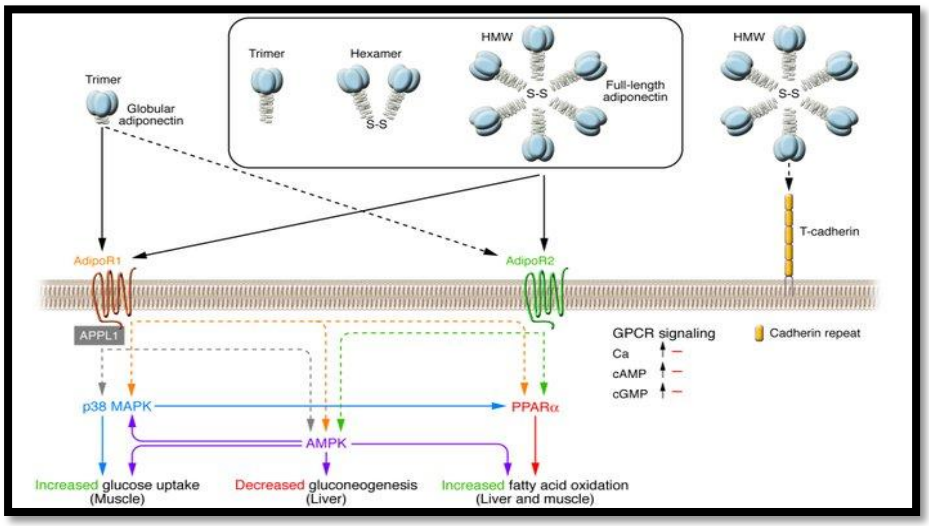


Figure 2.3.4: Signal transduction by adiponectin receptors. (Takashi Kadowaki .,2006)

2.3.6. Mechanism of Action of Adiponectin:

A. Insulin-sensitizing actions:(Figure 2.3.5)

1. Adiponectin reduces tissue TG content and up-regulates insulin signaling.

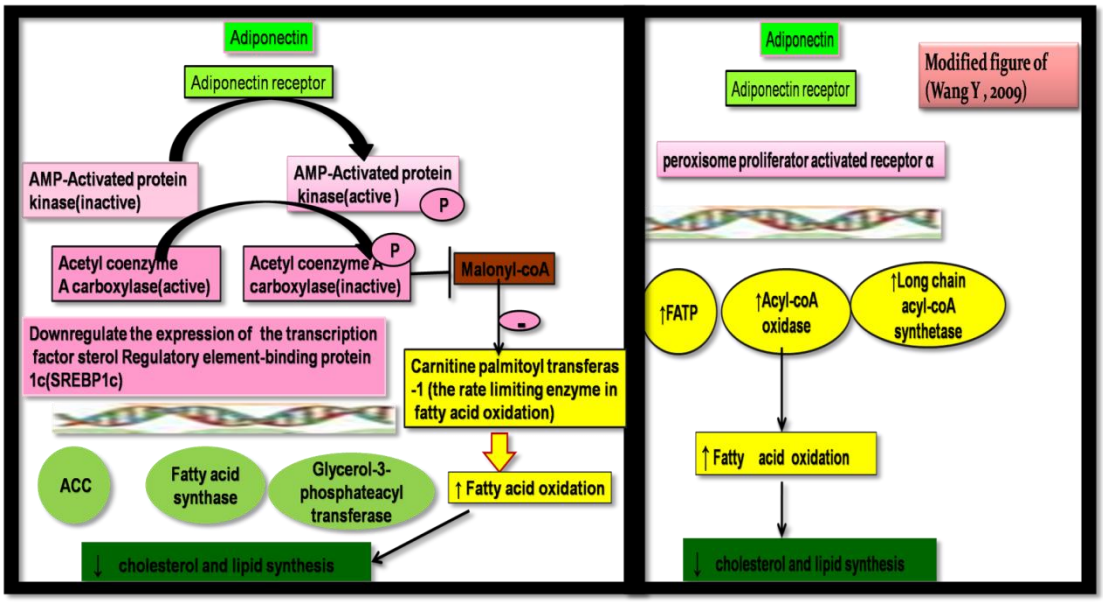


Figure 2.3.5 Insulin sensitization action of adiponectin (Modified figure of Wang Y, et al., 2009).

Interestingly, in skeletal muscle, adiponectin increases the expression of molecules involved in fatty-acid transport such as CD36, in combustion of fatty-acid such as acylcoenzyme A oxidase, and in energy dissipation such as uncoupling protein2. These changes lead to decreased tissue TG content in skeletal muscle (Yamauchi T.,2001).

Increased tissue TG content has been reported to interfere with insulin-stimulated phosphatidylinositol (PI) 3-kinase activation and subsequent glucose transporter 4 translocation and glucose uptake, leading to insulin resistance. Thus, decreased tissue TG content in muscle may contribute to improved insulin signal transduction (Yamauchi T,2001).

2. Adiponectin activates PPAR-α:

In 2001, Yamauchi hypothesized that adiponectin could activate PPAR-α, he suggested that adiponectin increased fatty-acid combustion and energy consumption, presumably via PPAR-α activation at least in part which led to decreased TG content in the liver and skeletal muscle and thus coordinately lead to increased in vivo insulin sensitivity **(Figure 2.3.6).**

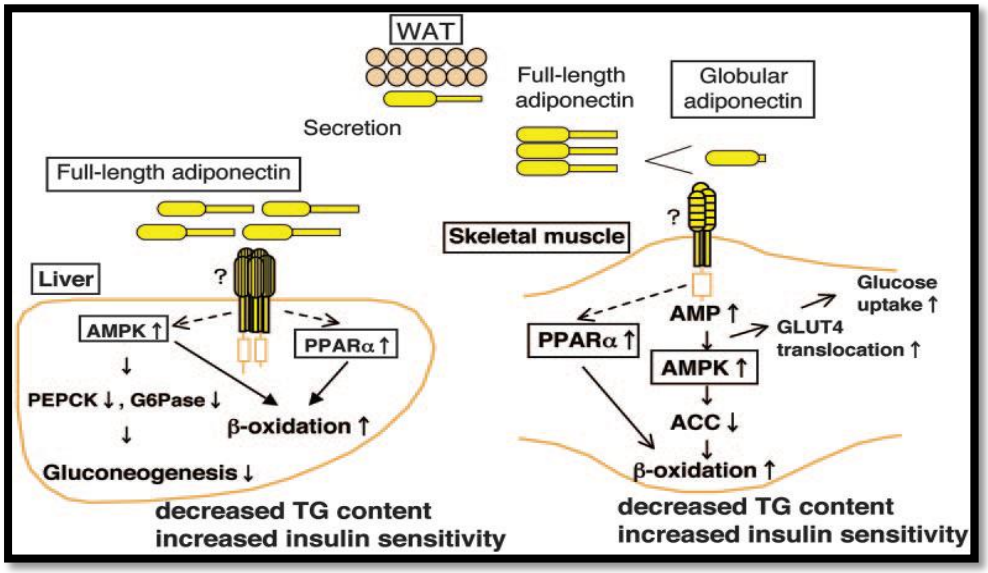


Figure2.3.6: Adiponectin can activate AMPK and PPAR-α in the liver and skeletal muscle (Takashi .,Kadowaki 2005).

3. Adiponectin activates AMP kinase:

Yamauchi T (2002), hypothesized that adiponectin may stimulate β-oxidation and glucose uptake via AMP-activated protein kinase (AMPK).

Globular adiponectin and full-length adiponectin stimulate phosphor- rylation and activation of AMPK in skeletal muscle **(Figure 2.3.7),** whereas only full-length adiponectin does so in the liver.

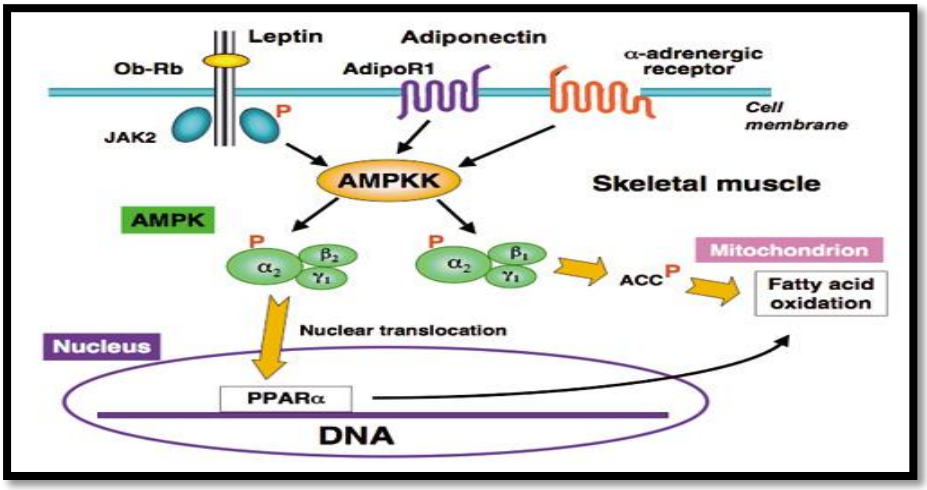


Figure 2.3.7: Regulatory role of AMPK in fatty acid oxidation in skeletal muscle
(http://www.nips.ac.jp/eng/contents/publication/guide/html/2008/res/developmental.html, 2008)

In parallel with its activation of AMPK, adiponectin stimulates phosphor- rylation of acetyl coenzyme-A-carboxylase (ACC), fatty-acid combustion, glucose uptake, and lactate production in myocytes, and causes reduction in molecules involved in gluconeogenesis in the liver, which can account for the acute glucose-lowering effects of adiponectin in vivo(Yamauchi T., 2002).

More recently, AMPK was reported to be involved in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes (Wu X.,2003).

B. Antiatherosclerotic actions:

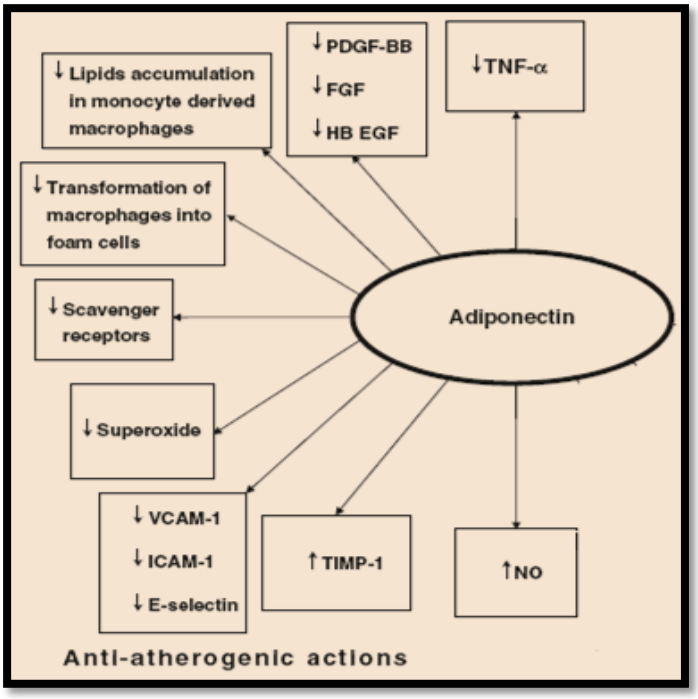


Figure 2.3.8: Anti-atherogenic action of of adiponectin (Andrzej Wie_cek et al., 2007)

Adiponectin has been reported to have direct antiatherosclerotic effects (Matsuzawa Y., 2003) (Kumada M., 2004). Adiponectin was demonstrated to strongly inhibit the expression of adhesion molecules, including intracellular adhesion molecule-1(ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), and E-selectin **(Figure 2.3.8).**

Adiponectin was also shown to inhibit TNF-α induced nuclear factor-kB activation through the inhibition of IkB phosphorylation (Ouchi N., 2000). Suppression of nuclear factor-kB by adiponectin might be a major molecular mechanism for the inhibition of monocyte adhesion to endothelial cells (Ouchi N.,1999).

Adiponectin also inhibits the expression of the scavenger receptor class A-1 of macrophages, resulting in markedly decreased uptake of oxidized low-density lipoprotein by macrophages and inhibition of foam cell formation (Ouchi N., 2001).

2.3.7. Adiponectin modulates carbohydrate and lipid metabolism in the liver:

The liver, where adiponectin receptor AdipoR2 is expressed, is one of the adiponectin target organs (Yamauchi T., 2003). Several studies have shown that adiponectin modulates hepatic carbohydrate and lipid metabolism **(Figure 2.3.9).**

Long-term treatment with adiponectin improved insulin sensitivity and reduced triglyceride content in the liver (Yamauchi T., 2001). Adiponectin suppresses hepatic glucose production by down-regulation of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) gene expression, thus decreasing plasma glucose levels (Yamauchi T., 2002;Berg AH., 2001;Combs TP., 2001).

An inhibitory effect of adiponectin on gluconeogenesis is probably mediated by AMPK phosphorylation. In the liver, AMPK activation is necessary for adiponectin-dependent inhibition of PEPCK and G6Pase gene expression and glucose production (Yamauchi T., 2002). Adiponectin has no effect on glycogen content and synthesis, glucose uptake or glycolysis in the liver (Berg AH., 2001;Combs TP., 2001).

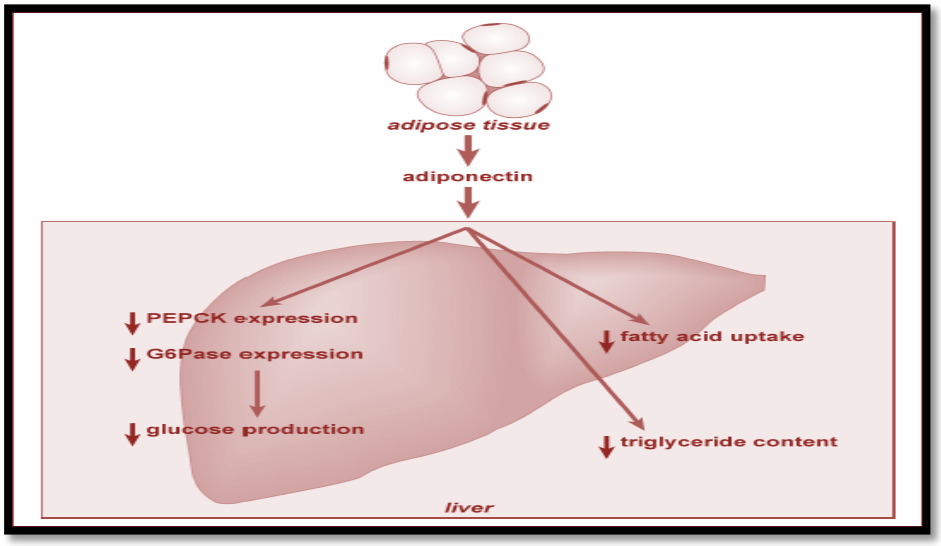


Figure 2.3.9: Effects of adiponectin on carbohydrate and lipid metabolism in the liver.(J. Karbowska, Z. Kochan, 2005).

2.4. The Metabolic syndrome (MS):

The metabolic syndrome, also called insulin resistance syndrome, has been described in many ways, in part owing to the lack of a "gold standard" diagnostic test.

The Adult Treatment Panel III (ATP III) (Bethesda., 2002) defines adult MS as 3 or more of the following abnormalities: hypertriglyceridemia, low HDL, high fasting glucose, excessive waist circumference, and hypertension, on the basis of associations with adverse cardiovascular outcomes derived from large research trials.(Bethesda. , 2002).

Adults with MS are at greater risk for cardiovascular disease (Isomaa B., 2001) and diabetes mellitus (Haffner SM., 1992) .

Ford et al,(Ford ES., 2002) using the Third National Health and Nutritional Survey (NHANES III), estimated the syndrome affected 25% of US adults.

The MS has not been well characterized in children and adolescents in terms of criteria, prevalence, or clinical implications, although studies have examined MS abnormalities (Ronnemaa T., 1991;Cook S., 2003).

2.4.1 Risk Factors:

The risk factors for MS included Age and menopause [Niaura R.,2000; Mesch VR., 2006], Race [Mugo M., 2004 ; Procopiou M., 2005], Disturbances in sex hormones (e.g., polycystic ovary syndrome (POS)[Hopkinson ZE., 1998], hyperandrogenism in pre- and postmenopausal women [Korhonen S., 2003 ; Golden SH., 2004]), energy excess (higher carbohydrate [Kang H., 2006], high fat, low food fiber [Williams DE., 2000;Giugliano D., 2006], high meat intake [Baxter AJ., 2006]), Family history (diabetes, hypertension, obesity [Chan JC., 1996 ; Hunt KJ., 2000]), Overweight [Chan JC., 1996], Life styles (tobacco use [Tahtinen TM., 1998], alcohol consumption [Kiechl S., 1996 ;Yamada Y., 2003], physical inactivity [Eriksson J., 1997 ; Carroll S., 2000]),Snoring and obstructive sleep apnea syndrome [Leineweber C, 2003 , Sasanabe R., 2006], Pychosocial and personality factors (lower social class [Pickering T.,1999 ;Brunner EJ., 1997],Difficulty in coping with stress [Pickering T., 1999 ; Vitaliano PP, 2002], Higher hostility level [Niaura R., 2000]).

Different risk factors may affect susceptibility to **MS** through both common and unique pathways. For example, higher hostility level or difficulty coping with stress may increase susceptibility to **MS** by over

activating sympathetic nervous system; High-fat diet may cause overweight/obesity and exacerbate beta-cell dysfunction; POS shares clinical similarities with the MS such as insulin resistance and visceral adiposity.

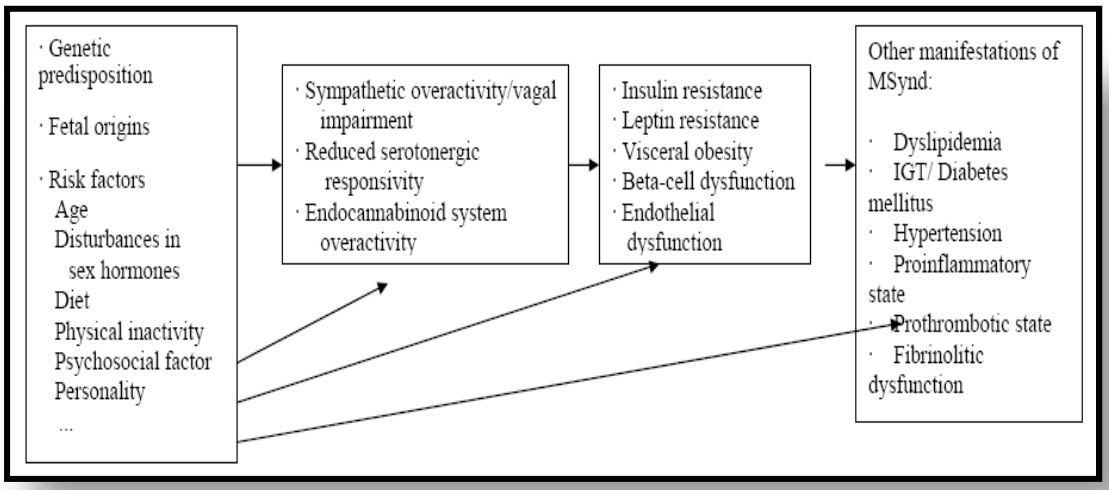


Fig. 2.4.1. A comprehensive framework for the etiology of the metabolic syndrome. (Amy Z. Fan., 2007)

2.4.2 Adipose tissue and metabolic syndrome

White adipose tissue, the body's major energy store, Adipose tissue is composed of TAGs (triacylglycerols), produced mainly by

a) FAs (fatty acids) derived from chylomicrons and circulating VLDLs [very-LDL (low density lipoproteins)], released through the action of LPL (lipoprotein lipase), an insulin-stimulated enzyme.

b) glucose provides the glycerol backbone for TAG.

In humans, FAs can also be synthesized from glucose, although the rate of synthesis is lower than in rodents.

Adipose tissue is also able to release **NEFAs** [non-esterified FAs ('free FAs')];during lipolysis, TAGs are hydrolysed in a reaction catalysed by

HSL (hormone-sensitive lipase), which is, in turn, regulated by numerous factors and hormones. Catecholamines, by binding to β-adrenergic receptors, stimulate lipolysis, whereas insulin inhibits this process (Gil-Hern´andez, A. 2002).

TAG accumulation is enhanced by the preferential channeling of nutrients into adipose tissue, rather than into muscle or other tissues for fairly immediate oxidation.

Overall, it would seem reasonable to assume that alterations which limit lipolysis and oxidation of FAs, and those which stimulate lipogenesis (the two processes are often linked), are the cause of, or at least are related to, obesity (Astrup, A.,1994; Sampath, H.2006).

Obesity is associated with the MS (metabolic syndrome) (Reaven, G. M., 1995) .IR (insulin resistance) would appear to be the major common finding in subjects with obesity, glucose intolerance or Type 2 diabetes, hypertension or dyslipidaemia, and it has been claimed to be the initial factor triggering a metabolic cascade which is also influenced by genetic and environmental factors [Reaven, G. M., 2005; Haslam, D.W., 2005; Goldstein, B. J., 2002].

The MS can best be explained by viewing abdominal adipose tissue as an endocrine organ that releases excess NEFAs and adipokines into the circulation.

(1). The portal theory:

Release of NEFAs by adipocytes is greater in central obesity than in lower-body obesity,with no concomitant increase in oxidation by peripheral tissues (Jensen, M. D.,1997; Sprangers, F., 2001).

First, increased blood NEFAs inhibit the uptake of glucose by muscle (Belfort, R., 2005). [Although the pancreas manufactures extra insulin, there is not enough to counter the hyperglycaemia, thus explaining the paradox of fasting hyperglycaemia despite increased plasma insulin levels, which is known as IR(Zammit, V. A., 2001)].

Hyperglycaemia and increased circulating NEFAs provide the correct substrates for increased manufacture of TAGs by the liver (Gil-Campos, M., 2004).

The 'portal theory' posits one of the major mechanisms behind the dyslipidaemia , which is the increased flux of NEFAs from adipose tissue to the liver via the portal vein when visceral TAG stores are increased, which is related to IR and the lack of inhibition of HSL (Bergman, R. N .,2000).

Based on visceral obesity with insulin resistant, one of component of the MS , the failure of insulin to suppress HSL stimulates the release of NEFAs from lipolytically active visceral fat. This increased flux of NEFAs, channelled to the liver via the portal circulation, stimulates hepatic TAG synthesis, apoB100 formation and, ultimately, the assembly and secretion of VLDL(Marsh, J. B., 2003).

NEFAs promote increased TAG synthesis in the liver, which can Lead to the secretion of VLDL**(Figure 2.4.2).**

In peripheral tissues, VLDL particles are exposed to LPL, which hydrolyses the TAG of VLDL particles, generating NEFAs. **Under normal conditions,** these NEFAs are taken up by muscle and adipose tissue for energy use or storage. The resulting remnant particles are hydrolysed further by HL (hepatic lipase) to form LDL.

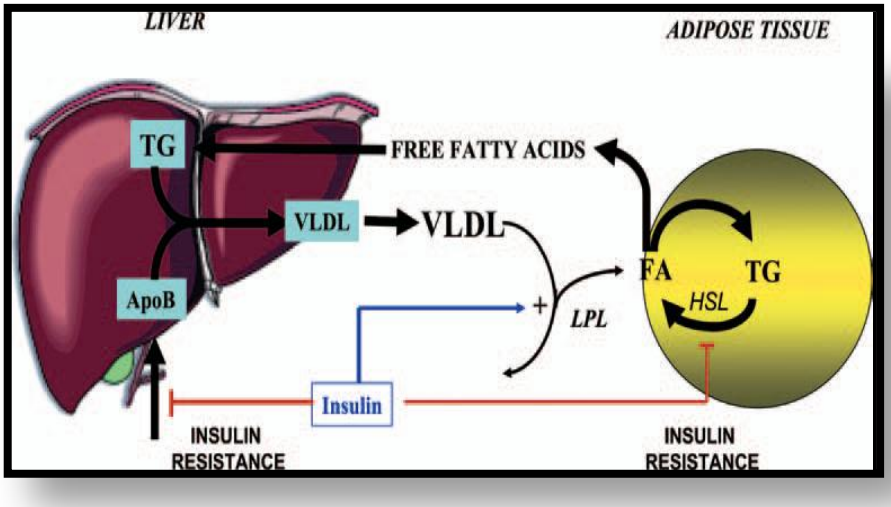


Figure 2.4.2: Increased synthesis of VLDL associated with IR (Concepci´on M. AGUILERA., 2008).

In contrast, **in individuals afflicted by the MS,** the failure of insulin to activate LPL favours the accumulation of TAG-rich lipoproteins in the circulation [Sprangers, F., 2001 , Chen, X., 1999], which in turn enhances the exchange of TAG from TAG rich lipoproteins to LDL and HDL, with a reciprocal transfer of cholesteryl esters to TAG-rich lipoproteins.

TAG-enriched cholesteryl ester-depleted LDL are better substrates for HL-mediated TAG lipolysis, which in turn leads to the formation of small-dense LDL **(Figure 2.4.3).** Mechanistically, small-dense LDL particles enter the arterial wall more easily, bind to arterial wall proteoglycans more avidly and are highly susceptible to oxidative modification, leading to macrophage uptake, all of which may contribute to increased atherogenesis (Grundy, S. M., 2005).

Elevated LC-CoA(long-chain acyl-CoA) tissue levels are involved in the increase in plasma TAG concentrations associated with IR. In the liver, LC-CoA stimulates the synthesis of TAG-rich lipoproteins, whereas in peripheral tissue they reduce plasma lipoprotein clearance via inhibition of LPL (Syvanne, M., 1997).

A low HDL-C level is even more common in patients with IR than hypertriglyceridaemia.

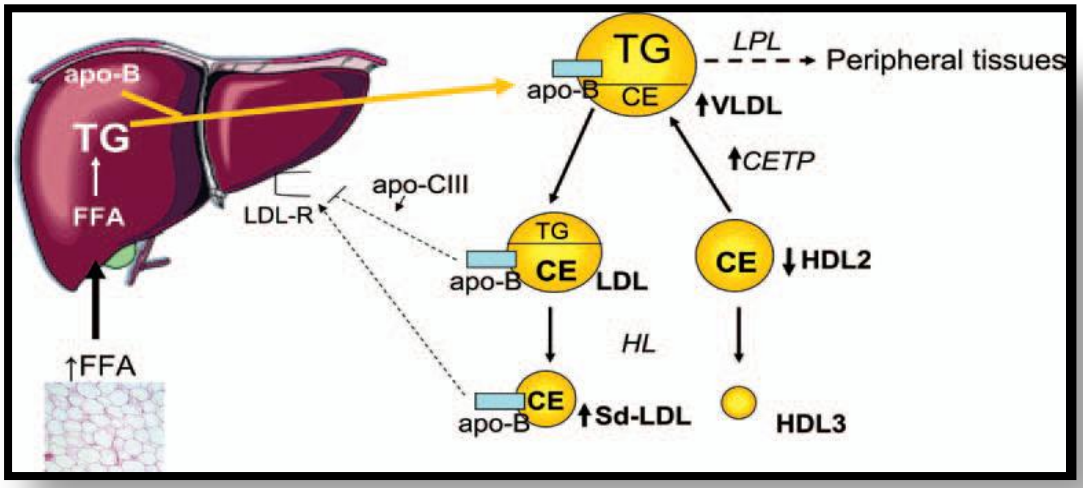


Figure 2.4.3: Mechanisms of NEFAs involved in dyslipidaemia associated with the MS (Amy Z. Fan.,2007)

The 'portal theory' proposes a link between visceral adipose tissue to IR and theMS and is based on the direct effects of NEFAs on the liver. Intra-abdominal tissue adipocytes are much more insulin-resistant than their subcutaneous counterparts, suggesting, as we commented before, that NEFA delivery to the liver via the portal vein is increased when visceral TAG stores are increased (Marsh, J. B. ,2003).

Elevated NEFA levels increase hepatic gluconeogenesis and lower peripheral tissue glucose uptake, prompting a further increase in the hyperinsulinaemia typically found in the MS (Wajchenberg, B. L., 2000 ; Bergman, R. N., 2007) **(Figure 2.4.4).** Moreover, it was found in the early 1960s that **NEFAs** restrain glucose use in muscle, as increased production of acetyl-CoA in muscle tissue mitochondria inhibits pyruvate dehydrogenase, a glucose-oxidation-limiting enzyme (Goldstein, B. J., 2002). Also, it has been shown that elevated intracellular LC-CoA

levels are associated with IR, and that these compounds are the equivalent of NEFAs at the intracellular level (Ruderman, N. B., 2003 ; Ruderman, N. B., 1997). Accumulation of free radicals derived from mitochondrial oxidation of LC-CoA gives rise to endothelial dysfunction and the gradual decline in insulin production by *β*-cells (Nolan, C. J., 2006 ; Hosokawa, H., 1997). Functional defects in pancreatic *β*-cells have been identified even prior to diabetes diagnosis, especially in individuals with central obesity and the MS.

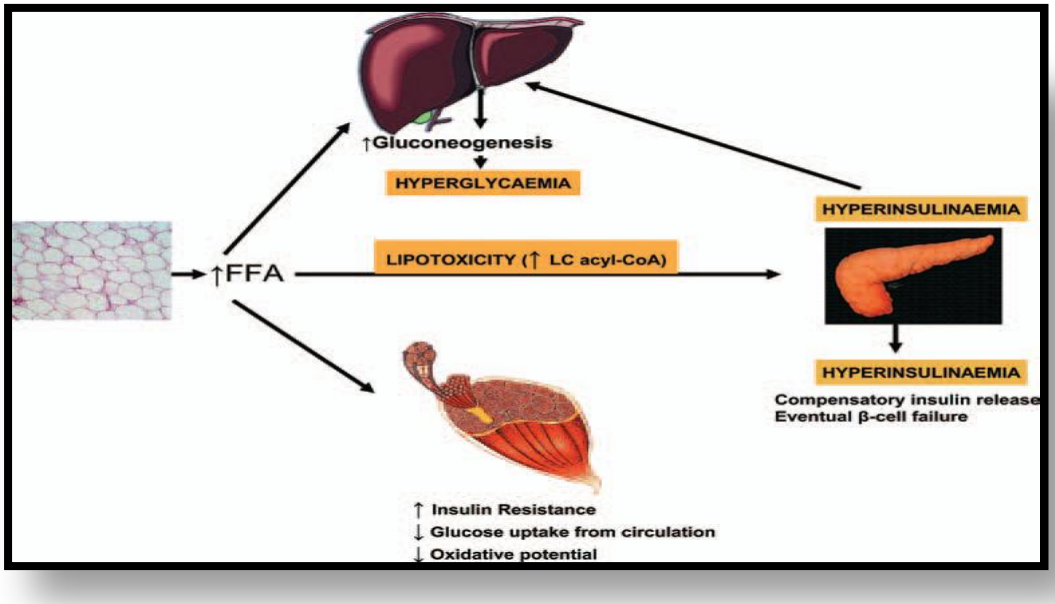


Figure 2.4.4: Influence of NEFAs on the development of some typical features of the MS (Amy Z. Fan., 2007)

(2). The Endocrine Hypothesis:

Ravussin and Smith (Ravussin, E. and Smith, S. R., 2002) have put forward an alternative to the classical paradigm or 'portal hypothesis' to explain the features of the MS. The 'endocrine' paradigm, developed in parallel with the ectopic fat storage syndrome hypothesis, posits that adipose tissue secretes a wide variety of endocrine hormones and

adipocytokines that regulate energy metabolism and, especially, lipid metabolism. From this viewpoint, adipose tissue plays a critical role as an endocrine gland, affecting the functions of distant organs including the CNS (central nervous system), skeletal muscle and liver. Hormone changes may thus precede any change in metabolites such as NEFAs or plasma glucose.

Adiponectin displays clear antisteatotic activity in non-adipose tissues, together with major insulin-sensitizing, anti-atherogenic and anti-inflammatory properties (Gil-Campos, M.,2004 ;Stefan, N. 2002). Plasma concentrations are inversely correlated with the amount of body fat in obesity, Type 2 diabetes and, in general, in all states characterized by IR, including coronary heart disease (Arita, Y.,1999). Plasma adiponectin levels correlate negatively with BMI (body mass index), insulin and TAG levels, and positively with HDL-C, in obese adults (Diez, J. J.,2003). Adiponectin also enhances whole-body insulin sensitivity by increasing FA oxidation, prompting a decline in circulating FA levels as well as in muscle and liver TAGs (Stefan, N.,2002) **(Figure 2.4.6).**

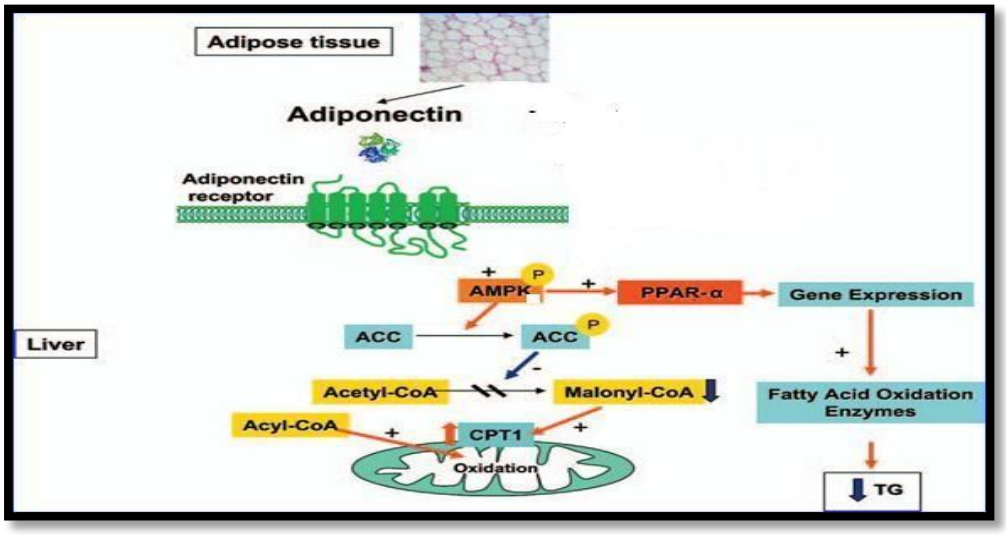


Figure 2.4.5 **Regulation of adiponectin-mediated cellular FA oxidation** (Amy Z. Fan., 2007)

Adiponectin stimulates insulin receptor tyrosine kinase activity by activating oxidative phosphorylation mediated by UCPs (uncoupling proteins). Adiponectinknockout mice fed on a carbohydrate-rich diet develop IR and display impaired PI3K activity (Yamauchi, T.,2002). adiponectin also activates AMPK, stimulating glucose utilization and FA oxidation [(Gil-Campos, M., 2004 ; Maeda, N., 2002 ; Fruebis, J., 2001), but see (Erratum ., 2004)] **(Figure 2.4.5).** Administration of full-length or globular adiponectin in mice increases AMPK-dependent phosphorylation in skeletal muscle; in the liver, this activation is achieved only with the full-length form (Lam, T. K., 2005). As indicated above, AMPK activation prompts an increase in acyl-CoA oxidation. The expression of adipoR1 is associated with increased phosphorylation of AMPK,ACC and p38MAPK(mitogen-activated protein kinase), whereas, in liver cells, there is an increase in phosphorylation of AMPK and ACC. Adiponectin also increases the activity of PPAR-α, a transcription factor expressed in the liver, which plays an essential role in regulating FA oxidation[(Gil-Campos, M., 2004 ;Yamauchi, T., 2003), but see (Erratum .,2004)].

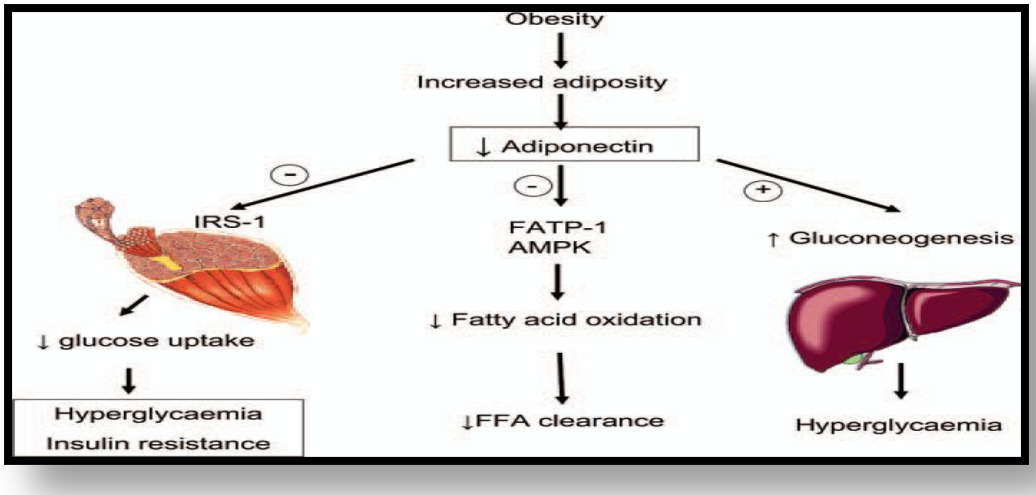


Figure 2.4.6 Influence of obesity on adiponectin secretion, decreased clearance of NEFAs and IR (Amy Z. Fan., 2007)

2.5. Asparatate aminotransferase (AST) Alanine aminotransferase (ALT) and alkaline phosphatase (ALP):

ALT and AST enzymes catalyze the transfer of amino groups between pyruvate and oxaloacetate, respectively. They are released when hepatocytes are injured (either acutely or chronically), and can be readily assayed in serum. While ALT is found predominantly in the cytosol of hepatocytes, AST is abundant both in the cytosol and mitochondria.

AST is found in cardiac muscle, skeletal muscle, kidneys, brain, and blood, and for this reason high levels of ALT is considered to be more specific to the liver. Diurnal fluctuations can be seen in both AST and ALT, and significant increases may be seen following vigorous exercise. Muscle injury from primary myositis or rhabdomyolysis can lead to significantly elevated ALT levels as well as AST levels.
It has been shown that acute muscle injury secondary to extreme exercise can demonstrate an AST to ALT ratio of 5:1, as opposed to a chronic process such as polymyositis where the ratio is closer to equal, reflecting the shorter half-life of AST (Nathwani RA., 2005). In addition these are often elevated in acute biliary obstruction.

Alkaline Phosphatase:

Alkaline phosphatases are metallophosphatases that are found in many tissues. They are predominantly present in the canalicular microvilli, as well as in bone and the small intestine. Alkaline phosphatase elevation occurs in conditions of extrahepatic biliary obstruction, infiltrative diseases of the liver, or due to certain medications. An elevated alkaline

phosphatase level can be a normal finding in the third trimester of pregnancy as well as in an adolescent growth spurt.(Laurel H.2005)

2.6. Oxidative stress:

Aerobic Organisms such as vertebrates and man in particular produce their energy from the oxidation of organic substrates by molecular oxygen.

The complete four-electron reduction of molecular oxygen leading to the formation of water occurs in the mitochondrial respiratory chain. Occasionally, molecular oxygen is partly reduced instead of proteins of respiratory chain; hence, superoxides and various reactive oxidant intermediates are produced leading to secondary oxidation (Liu, 1997).

Besides these physiological oxidations and their unavoidable secondary reactions, many substances contained in food and environment are oxidants either by themselves or by oxidant precursors (Ichihashi et al., 2003).

Finally, although organism adapt to any unstable situation by preventing undesirable reactions and repairing damaged molecules and tissues, the very few undesirable reactions that escape the prevention and repair systems accumulate little by little, and will invariably be deleterious after a long period of time (Tahara et al., 2001), thus confirming the free radical theory of ageing developed by Harman(2001).

If the organism fails to neutralize them, these oxidants accumulate and react with a variety of biomolecules, creating an undesirable situation known as oxidative stress. Therefore, oxidative stress corresponds to an

imbalance between the rate of oxidant production and that of their degradation (Sies, 1991).

Two classes of molecules mediate most of the reactions leading to oxidative stress: free radicals and reactive oxygen species (ROS) **(Figure 2.6.1).** Free radicals are molecules possessing at least one unpaired electron; for this reason, they are unstable and promote electron transfer, i.e., oxidations and reduction reactions . ROS including lipid radicals are formed by hydrogen abstraction, a process elicited by UV irradiation or the hydroxyl radical (•OH) (Tyrrell, 1995). These unstable intermediates derive from molecular oxygen ($\bullet O_2 \bullet$ or $^3O_2$).

In the case of molecular oxygen, the triplet (biradical) state can be produced by several biochemical oxidations involving peroxidases and lipoxygenases, by reactions between various ROS or in the presence of light, oxygen and a photosensitizer such as porphyrins, as it is the case in congenital erythropoietic porphyria (Mathews., 2000).

$$^3O_2 \xrightarrow{\text{Light + photosensitizer}} {}^1O_2$$

$$O_2\bullet^- + M^{(n+1)+} \rightarrow {}^1O_2 + M^{n+}$$

$$H_2O_2 + ONOO^- \rightarrow {}^1O_2 + NO_2^- + H_2O$$

$$H_2O_2 + ClO^- \rightarrow {}^1O_2 + Cl^- + HO$$

Other ROS derive from the secondary reduction of $^3O_2$ in the inner membrane of mitochondria (mainly from ubiquinol oxidation), where

oxygen receives one electron in the place of a metalloprotein of the respiratory chain (Liu., 1997).

The superoxide radical anion thus formed ($\bullet O_2^-$) can then dismutate into oxygen ($^3O_2$) and hydrogen peroxide (H_2O_2), either spontaneously at acidic pH or in a reaction catalyzed by superoxide dismutase (SOD) (Halliwell and Gutteridge., 1999).

(SOD)

$$2O_2\bullet^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

Superoxide and hydrogen peroxide can react with transition metals such as iron or copper to form the strong oxidant hydroxyl radical (•OH)(Buettner and Jurkiewicz, 1996):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\bullet$$

(Fenton reaction)

$$Fe^{3+}/Cu^{2+}$$

$$O_2\bullet^- + H_2O_2 \longrightarrow O_2 + OH^- + OH\bullet$$

(Metal ion-catalysed Haber–Weiß reaction)

Superoxide and hydrogen peroxide can be formed enzymatically by NADPH oxidase in activated neutrophils or macrophages during inflammatory processes (Babior et al., 2002).

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2\bullet^-$$

Generally speaking, all endogenous oxidase – enzymes that transfer two electrons from a substrate (AH_2) to molecular oxygen – produce superoxide and hydrogen peroxide (Halliwell and Guttering., 1999)

Oxidase

$$AH_2 + 2O_2 \longrightarrow A + 2H^+ + 2O_2^{\bullet -}$$

Myeloperoxidase, an enzyme present in neutrophils, but not in macrophages, can produce hypochlorous acid (HOCl) by oxidizing chloride ions with hydrogen peroxide (Halliwell and Guttering, 1999; Winterbourn et al., 2000):

Myeloperoxidase

$$H_2O_2 + Cl^- \longrightarrow HOCl + OH^-$$

Hypochlorous acid (or its conjugated base hypochlorite, depending on the pH), is a powerful oxidant towards various biomolecules, especially amino groups; furthermore, in acidic pH it readily decomposes to liberate the highly toxic chlorine gas, Cl_2 (Halliwell and Guttering, 1999; Klebanoff, 1999).

$$HOCl + H^+ + Cl^- \rightarrow H_2O + Cl_2$$

As mentioned above, hydrogen peroxide can react with hypochlorite to form singlet oxygen. Under physiological conditions, 1-2% of the consumed oxygen is converted to ROS. Nitric oxide (•NO), an intercellular messenger produced from oxygen by various nitric oxide synthases (Dawson and Dawson, 1996; Halliwell and Guttering, 1999):

Nitric oxide synthase

$$O_2 + \text{arginine} + NADPH \longrightarrow \bullet NO + \text{citrulline} + H_2O + NADP^+$$

This free radical, by reacting with superoxide –another radical– produces the strong oxidant peroxynitrite ($ONOO^-$) (Pryor et al., 1995).

$$O_2\bullet^- + \bullet NO \rightarrow ONOO^-$$

The main biological targets of free radicals and ROS are proteins, lipids and DNA. Protein oxidation leads to loss of function or premature degradation in proteasomes. Lipid peroxidation, occurring mostly in the plasma membrane or the stratum corneum – the most superficial layer of the epidermis – alters the physical properties of cell membranes or the epidermis, and thereby their biological function. Oxidation of DNA can lead to gene mutation, and thus abnormal protein synthesis, alteration in gene expression, apoptosis and cell death (Kohen and Nyska, 2002).

2.6.1. Endogenous defenses against oxidative stress:

Strict anaerobic organisms cannot live in the presence of oxygen, because they have insufficient defenses against the multiple secondary reactions induced by oxygen. All other organisms which can or must live in the presence of oxygen possess an efficient battery of antioxidant defenses able to trap reactive intermediates before they have time to oxidize biomolecules or reduce those which have been oxidized **(Figure 2.6.1)**(Blokhina et al., 2003).

Due to the great variety of reactive intermediates that must be neutralized, as well as the variety of oxidized biomolecules that must be reduced, there are many different antioxidants; moreover, there is a need for

antioxidants for both hydrophilic (cytosol, extracellular fluids) and lipophilic (membranes, lipids) phases (Wood et al., 2003).

The most efficient antioxidants are enzymes that catalyze the reduction of ROS:

1- Superoxide dismutase (SOD):

Catalyses the dismutation of superoxide ($\bullet O_2^-$) into hydrogen peroxide (H_2O_2) and oxygen ($3O_2$).

2- Catalase:

Catalyses hydrogen peroxide (H_2O_2) dismutation into water (H_2O) and oxygen ($^3O_2$).

3- Glutathione peroxidases (GPX) and reductase (GSR):

Reduce both hydrogen peroxide (H_2O_2) and organic (R-OOH) hydroperoxides. The oxidized glutathione cofactor (GS–SG) is then reduced (2 GSH) by glutathione reductase (GSR). Various GPX isoforms exist, which are specific for hydrophilic or lipophilic phases.

4- Metallothioneins:

Are small proteins with several cysteine residues which bind transition metal ions: these can both detoxify metals and avoid them catalyzing the Haber–Weiß and Fenton reactions that lead to the production of the hydroxyl radical (•OH).

5- Tripeptide glutathione and the selenoprotein thioredoxin:

Are efficient in reducing disulphur bridges (R–S–S–R) into thiols (R–SH + R–SH), as well as reducing thiyl radicals (R–S•); the

whole system involving thioredoxin, thioredoxin peroxidase and thioredoxin reductase is known as peroxiredoxin. Besides being an antioxidant by itself, glutathione is also the cofactor for GPX and GSR.

In addition other defense systems exist including :Ubiquinol (coenzyme Q, QH2) ,Lipoic acid, L-Ascorbic acid (vitamin C) ,Carotenoids , Tocopherols (vitamin E) . Selenium.

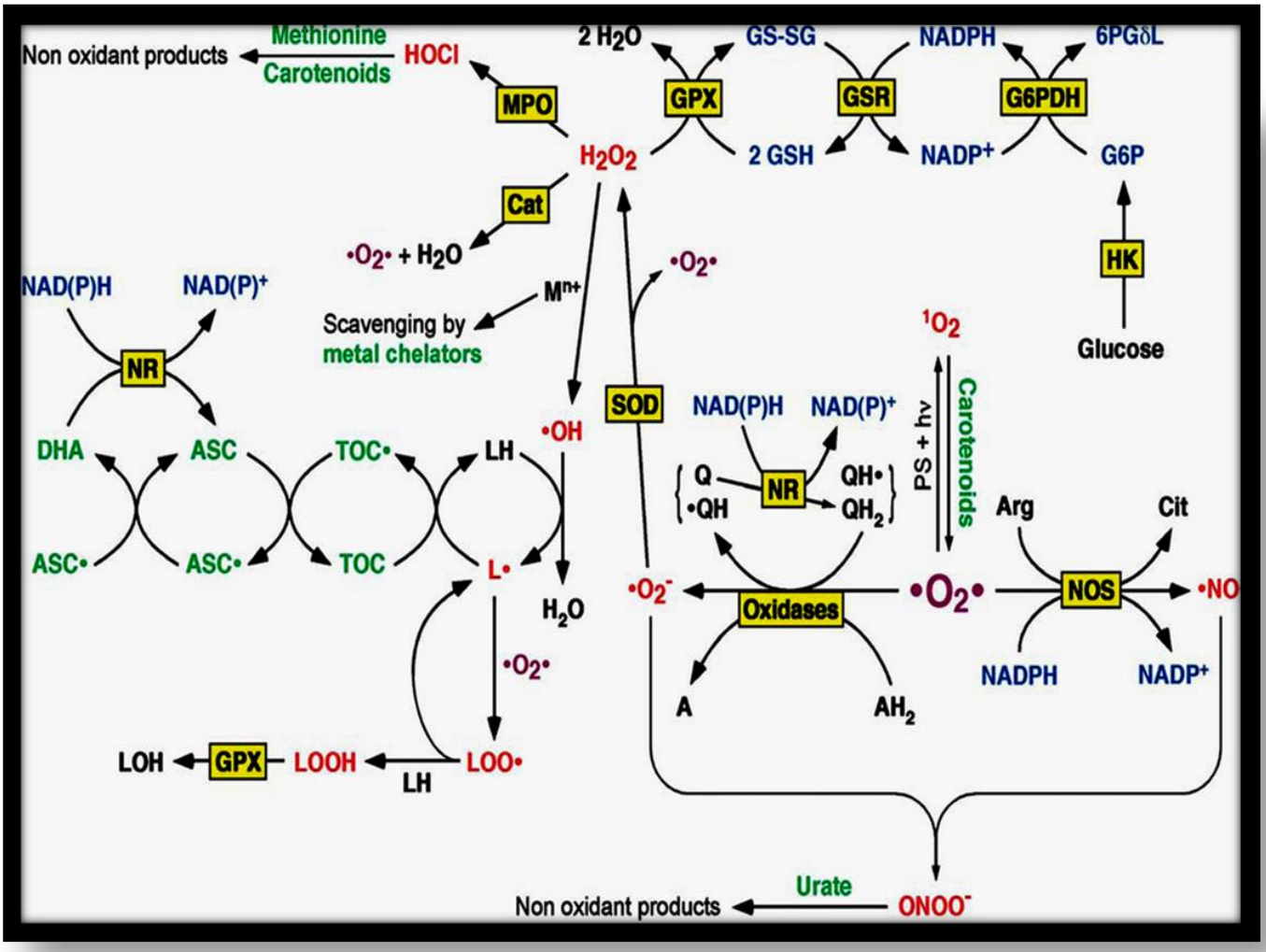


Figure 2.6.1: Overview of free radical and ROS formation and elimination (Sorg. , 2004).

2.6.2. Oxidative Stress and adiponectin:

There are abundant data from studies in mice to indicate that oxidative stress also regulates adiponectin secretion. Oxidative stress is defined as a persistent imbalance between the production of highly reactive molecular species (chiefly oxygen and nitrogen) and the capacity of antioxidant defense systems to inactivate or remove them. (Rosen P.,2001)

Oxidative stress is elevated in human obesity and insulin resistance. (Keaney JF.,2003). Results obtained from experiments in mice suggest that lipid accumulation in adipocytes, and a concomitant rise in ROS production, may be a key trigger for the development of insulin resistance via reduced adiponectin secretion (Furukawa S.,2004).

Exposure of cultured primary rat adipocytes to hyperglycemic conditions (15mM glucose, 100nM insulin) increases intracellular nutrient availability and ROS production, leading to a reduction in insulin sensitivity (Lu B., 2001). Similarly, exposure of 3T3-L1 adipocytes to hydrogen peroxide, a powerful oxidizing agent, reduces adiponectin mRNA expression within 10 minutes (Kamigaki M., 2006). The mechanism(s) linking ROS production to adiponectin secretion are currently under investigation, and studies in 3T3-L1 adipocytes have suggested roles for uncoupling protein-2 (UCP2), a protein which increases mitochondrial respiration, as well as the transcription factor CHOP-10, which interferes with the C/EBP-binding region in the promoter of the adiponectin gene (Chevillotte E.,2007).

2.6.3. Oxidative stress and high fat diet:

High-fat feeding-induced obesity led to lipid accumulation in visceral adipose tissue, as fat content data indicate, which could be associated with enhanced glycerol-3- phosphate dehydrogenase(G3PDH) and leptin gene expression, thus confirming previous data (Lopez IP., 2004).

Fatty acid synthase (FAS) adipose tissue gene expression is not significantly influenced by the diet. However, it does not mean that changes in FAS activity could not affect fatty acid synthesis from glucose.

Triglyceride accumulation in adipocytes seems to be a major source of oxidative stress in WAT and further adipocytokine dysregulation driving to metabolic syndrome (Trayhurn P, 2004).

In recent years, obesity has been associated with an inflammatory status (Hotamisligil GS., 1993 , Moreno-Aliaga MJ., 2005). On the contrary, insulin has shown to be anti-inflammatory and to suppress ROS generation and NADPH oxidase (Dandona P., 2001).

Therefore, in this way,the high fat diet model coursing with obesity and insulin resistance could be characterized by oxidative and inflammatory stress .

Inflammatory status related to obesity may be also originated by oxidative stress, which induces cell injury and could be able to dysregulate adipocytokine production and insulin sensitivity (Furukawa S., 2004; Soares AF., 2005).

Furthermore, oxidative stress in obesity may be generated by hypoxia in adipocytes (Trayhurn P., 2004) or by exacerbated nutrient oxidation, as it has been reported after glucose uptake (Talior I., 2003).

In any case, oxidative stress has been correlated with BMI in obese subjects (Uzun H., 2004). Furthermore, it has been reported that obesity per se may induce systemic oxidative stress.

However, contradictory results regarding intracellular redox state in adipocytes as compared with other tissues have been recently found (Furukawa S, 2004 and Galinier A, 2006).

Moreover, conflicting results have been observed regarding liver peroxidation. In a recent report (Carmiel-Haggai M, 2005), an increase in liver MDA in a genetic model of obesity fed a high-fat diet was reported when compared with fa/fa rats fed the control diet.

However, in other animal models of obesity (Furukawa S., 2004), lipid peroxidation is not altered in liver. Thus, the role of obesity induced oxidative stress in the liver, a chief organ regulating body metabolism, remains to be clarified.

This trial evidenced a correlation between liver MDA, a good estimate of lipid peroxidation, and different variables accompanying the common features of the cafeteria obesity model in rats (increased body weight, hyperleptinemia, and hyperinsulinemia). These results suggest that obesity induced by high-fat diets enhances oxidative stress not only in WAT (Furukawa S., 2004) or in plasma (Dobrian AD., 2001), but also in liver, which probably contributes to hepatic steatosis and other disorders.

A possible role of increased hepatic ROS and lipid peroxidation in causing inflammation and non-alcoholic fatty liver disease (NAFLD) is an important clinically relevant issue.

NAFLD is a major cause of liver-related morbidity and has been related to obesity in human patients in epidemiological studies (Hu KQ., 2004), but it also has been associated with other characteristics of the metabolic syndrome, such as impaired glucose tolerance and hyperinsulinemia (Marceau P., 1999). A two-hit theory best describes the progression from

simple steatosis to NAFLD, fibrosis, or cirrhosis (Day CP., 1998). These two hits consist of the accumulation of excessive hepatic fat primarily because of insulin resistance and oxidative stress because of ROS occurrence.

As described earlier, one of the main causes of NAFLD is the accumulation of excessive hepatic fat because of insulin resistance and the exacerbated lipid mobilization and oxidation that it is usually found in the liver of obese subjects (Unger RH. ,2003).

The cafeteria diet is a good model of insulin resistance and lipid accumulation in the liver, with increased HOMA index and liver weight and enhanced TG hepatic content.

Furthermore, excess accumulation of free fatty acids in liver and other non-adipose tissues commonly led also to cell dysfunction and lipotoxicity or lipid-induced cell death (Schaffer JE. ,2003).

The elevated levels of MDA in rats fed on a cafeteria diet, even the fa/fa rats (Carmiel-Haggai M., 2005), suggest increased lipid peroxidation in fat deposits that could be released and have detrimental effects on hepatocytes and other hepatic cells.

In hepatocytes, ROS and lipid peroxidation products further impair the respiratory chain, either directly or indirectly through oxidative damage to the mitochondrial genome. These features, in turn, lead to the generation of more ROS, and a vicious cycle ensues.

Mitochondrial dysfunction can also lead to apoptosis or necrosis depending on the energy status of the cell. Finally, ROS and lipid peroxidation products also activate stellate cells, thus resulting in fibrosis (Fromenty B., 2004).

On the other hand, adipokines have been implicated in the pathogenesis of type 2 diabetes and NAFLD, through their metabolic and pro-/antiinflammatory activity (Musso G., 2005).

2.6.4. oxidative stress and Cold Stress:

It is well known that cold exposure may be reflected in an elevated metabolic rate and possibly also increased production of reactive oxygen species, e.g. hydrogen peroxide (H2O2), hydroxyl radicals (HO·) and superoxide anion radicals ($O^{2-\cdot}$), which cause lipid peroxidation (Selman et al., 2000; Heise et al. ,2003).

Membrane injury following lipid peroxidation causes disruption of the tissue integrity (Bagchi et al. 1999). Therefore, to neutralize reactive oxygen species the body uses mainly enzymatic copper, zinc superoxide dismutase (Cu,Zn-SOD), catalase and selenium-dependent glutathione peroxidase (Se-GSH-Px) and non-enzymatic antioxidants, e.g. reduced glutathione (GSH).

Cold stress, which is characterized by the increase in metabolic rate, leads to alterations in some antioxidant enzyme activities. These alterations are explained as a mechanism resisting the negative effects of reactive oxygen species (Selman et al. 2000). It is reported that acclimation at temperatures below 10 ◦C for a period of more than 3 weeks would result in observable oxidative effects (Kennedy et al. 1977).

Several studies have investigated whether exposure to low temperatures results in compensatory changes taking place in the antioxidant defence system (Barja de Quiroga et al. 1991; Ohno et al. 1991; Bondarenko et al. 1999), although there has been no detailed and comparable study investigating the effects of chronic cold stress on protein modification and lipid peroxidation in different tissues.

Additionally **Gumuslu's group** could not find any study clarifying the tissue most affected by chronic cold stress according to the oxidative parameters. (Gumuslu et al. 2002).

2.7. Glucose-6-phosphate dehydrogenase (G6PD):

Glucose-6-phosphate dehydrogenase (G6PD) is a cytoplasmic enzyme present in all cells, where it plays the key role in regulating carbon flow through the pentose phosphate pathway. Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme of the pentose phosphate pathway, which results in the production of ribose-5-phosphate and NADPH**.(Figure 2.7.1)(Figure 2.7.2)**

In erythrocytes, defense against oxidative damage is heavily dependent on G6PD activity, which is the only source of NADPH.(Kletzien RF,. 1994).

Almost all cases of G6PD deficiency are caused by one amino-acid change due to a point mutation of the genomic DNA, and about 140 molecular abnormalities of the G6PD genotype have been identified (Cappellini MD., 2008).

The entire antioxidant system, as well as other reductant- requiring processes, relies on an adequate supply of NADPH because it is the principal intracellular reductant for all cells.

G6PD is the principle source of NADPH. Therefore, a decrease in G6PD activity leads to decreased NADPH and makes cells very sensitive to oxidant damage (Felix K., 2003).

These results have provided strong evidences showing that NADPH, which is mainly produced by G6PD, is of central importance to cellular redox regulation. On the other hand, any changes in G6PD activity will alter NADPH levels and thus impact the antioxidant system.

G6PD has been suggested to be an “antioxidant” enzyme because it provides NADPH to maintain glutathione (GSH) in its reduced form.
Increased G6PD activity may restore cellular GSH levels after its depletion by oxidative stress (Leopold JA., 2000).
Previous reports have demonstrated that NADPH produced by G6PD is required for both the production of ROS, including superoxide anions and NO, and the elimination of these ROS via glutathione peroxidase and catalase in different cell types (Spolarics Z: 1998)(Spolarics Z., 1999).

Recently, **Park J, 2005** revealed that G6PD is highly expressed in the adipocytes of several obese animal models, and its overexpression in the adipocytesprovoked the dysregulation of lipid metabolism and adipocytokine expression, resulting in insulin resistance (Park J., 2005).

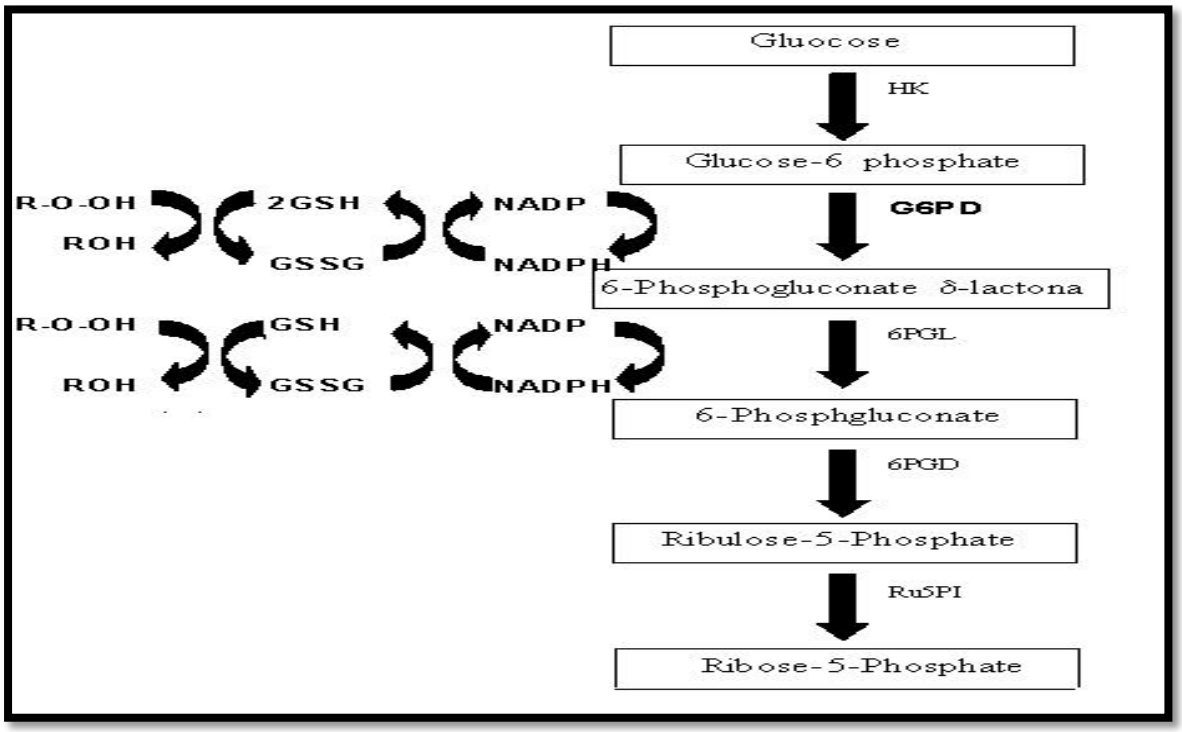


Figure 2.7.1: Pentose phosphate pathway (Judit Ola.,2002)

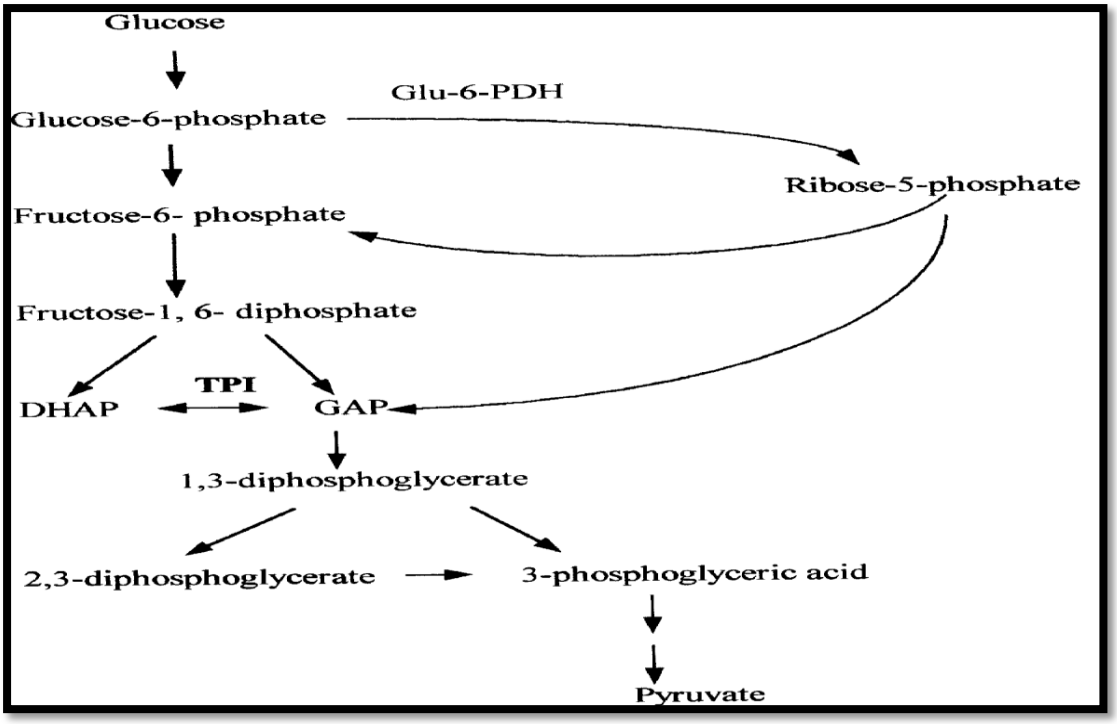


Figure 2.7.2: **Pentose phosphate pathway** (Javier Fernando Bonilla., 2007) **.**

2.8. Reduced glutathione :

Glutathione (gama_-glutamyl-cysteinyl-glycine; GSH) is the most abundant low- molecular- weight thiol .

a . GSH Synthesis:

The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, δ-glutamylcysteine synthetase (GCS) and GSH synthetase. This pathway occurs in virtually all cell types, with the liver being the major producer and exporter of GSH.

In the GCS reaction, the α-carboxyl group of glutamate reacts with the amino group of cysteine to form a peptidic _-linkage, which protects GSH from hydrolysis by intracellular peptidases. (Griffith, O. W. ,1999).

b. Roles of GSH:

Glutathione participates in many cellular reactions. **First,** GSH effectively scavenges free radicals and other reactive oxygen species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H2O2) directly, and indirectly through enzymatic reactions (Fang., 2002). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase). In addition, glutathione peroxidase (a selenium-containing enzyme) catalyzes the GSH-dependent reduction of H2O2 and other peroxides (Lei., 2002).

Second, GSH reacts with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostaglandins, and leukotrienes), and xenobiotics (e.g., bromobenzene and acetaminophen) to form mercapturates (Fang., 2002). These reactions are initiated by glutathione-S transferase (a family of Phase II detoxification enzymes).

Third, GSH conjugates with NO to form an S-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO (Fang., 2002). Recent evidence suggests that the targeting of endogenous NO is mediated by intracellular GSH (Andre.,2003). In addition, both NO and GSH are necessary for the hepatic action of insulin-sensitizing agents (Guarino., 2003).

Fourth, GSH serves as a substrate for formaldehyde dehydrogenase, which converts formaldehyde and GSH to S-formyl-glutathione (Townsend., 2003). The removal of formaldehyde (a carcinogen) is of physiological importance, because it is produced from the metabolism of

methionine, choline, methanol (alcohol dehydrogenase), sarcosine (sarcosine oxidase), and xenobiotics (via the cytochrome P450–dependent monooxygenase system of the endoplasmic reticulum).

Fifth, GSH is required for the conversion of prostaglandin H2 (a metabolite of arachidonic acid) into prostaglandins D2 and E2 by endoperoxide isomerase (Lu., 2002).

Sixth, GSH is involved in the glyoxalase system.

Finally, glutathionylation of proteins (e.g., thioredoxin, ubiquitin-conjugating enzyme, and cytochrome c oxidase) plays an important role in cell physiology (Townsend., 2003).

Adequate GSH concentration is necessary for the proliferation of cells, including lymphocytes and intestinal epithelial cells (Aw., 2003). Glutathione also plays an important role in spermatogenesis and sperm maturation (Sies., 1999). In addition, GSH is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses when the host is immunologically challenged (Townsend, 2003).

c. **Glutathione's Role As An Antioxidant:**

Glutathione is currently one of the most studied antioxidants. This is likely due to it being endogenously synthesized all throughout the body and it is basically found in all cells, sometimes in rather high concentrations. Investigations have highlighted many roles in which it is used including antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of

deoxyribonucleotide synthesis, regulation of immune responses, and regulation of leukotriene and prostaglandin metabolism (Sen CK:1999).

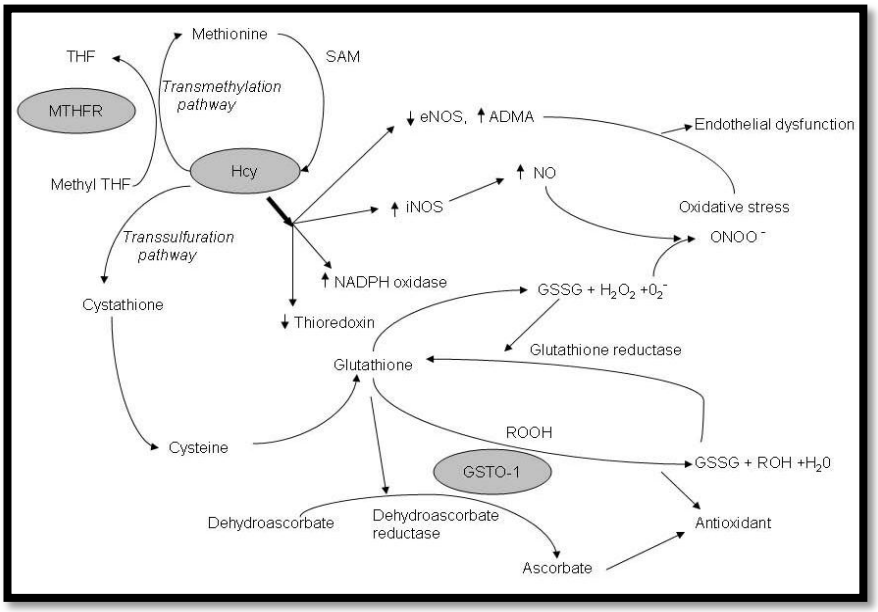


Figure.2.8.1: The metabolic pathways regulating oxidative stress(Leema Reddy

The increased ROS in hypertrophied adipose tissue is accompanied by a decrease in mRNA expression levels and activities of antioxidant enzymes such as glutathione peroxidase (GPX), Cu/Zn superoxide dismutase (Cu/Zn SOD), and catalase (Furukawa S,2004), which are essential for homeostasis of the redox state (Michiels C,1988) and are induced to detoxify ROS when cells are exposed to oxidative stress in other organs (Ray G.,2002).

Thus, dysregulation of antioxidant enzymes should lead to dysfunction of adipocytes. However, the precise regulatory mechanisms and roles of each antioxidant enzyme in adipocytes remain to be elucidated.

GPX is one of the antioxidant enzymes down regulated in hypertrophied adipose tissue. Seven members of the GPX family have been identified so far. Including GPX1 (Furukawa S.,2004), and GPX3 (Maeda K., 1997) that are expressed in adipose tissue.

GPX1 is the most characterized cytosolic and mitochondrial GPX (Chambers I.,1986), whereas GPX3 is the extracellular GPX recognized as serum GPX and is secreted mainly by the kidney (Maddipati KR.,1987). However, there is little or no information on the role of other GPXs in the homeostasis of redox state in adipocytes.

GPXs scavenge and inactivate hydrogen and lipid peroxides to water or lipid hydroxyls in a glutathione (GSH)-dependent reduction reaction in mammalian cells (Cohen G 1963, Drevet JR. 2006). Thus, GSH is an essential factor for the enzymatic function of GPXs on the reduction reaction and also an antioxidant itself to scavenge ROS. The intracellular content of GSH and the ratio of reduced GSH to oxidized GSH (GSH/GSSG ratio) are regulated through several steps to maintain homeostasis. GPXs facilitate the oxidation of GSH to GSSG upon the catalytic reaction to reduce H2O2. GSSG is recycled to GSH by GSH reductase (GSH-R). (Franco R., 2007 ,;Njalsson R., 2005 ; Rahman I., 2000).

2.9. Lipid peroxidation (LP) :

Lipid constitute about 50% of the mass of most animal cell membranes, nearly all of the remainder being protein. All of the lipid molecules in cell membranes are amphipathic (or amphiphilic)—that is, they have a hydrophilic (water-loving) or polar end and a hydrophobic (water-fearing) or nonpolar end.

The most abundant membrane lipids are the phospholipids. These have a polar head group and two hydrophobic hydrocarbon tails. The tails are usually fatty acids, and they can differ in length (they normally contain between 14 and 24 carbon atoms). One tail usually has one or more cis-double bonds (i.e., it is unsaturated), while the other tail does not (i.e., it is saturated).

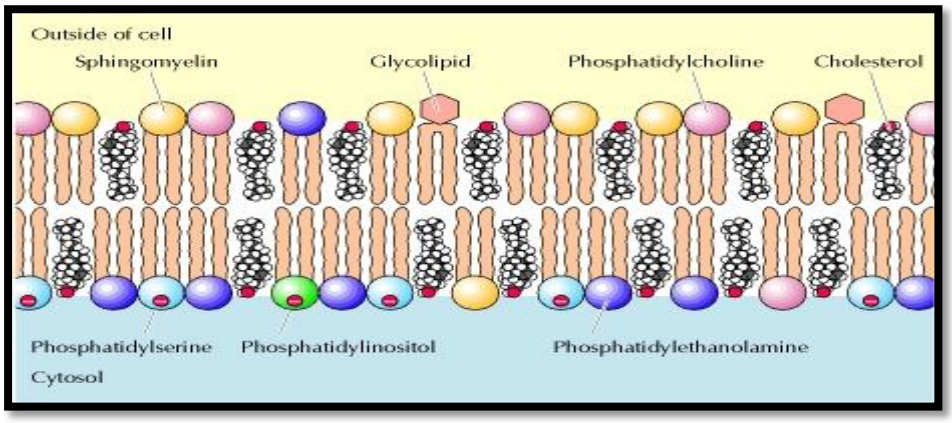


Figure 2.9.1 Lipid components of the plasma membrane. (The Cell, 2nd edition, 2000)

However cell membranes are rich sources of poly unsaturated fatty acids (PUFA) which are particularly susceptible to oxidation(Slater., 1984 Halliwell and Gutteride., 1984). The consequent oxidative destruction of PUFA is known as lipid peroxdation (Cheesman .,1993).

Lipid peroxidation (LP) is an autocatalytic process caused by oxidative stress (Gordana Juric-Sekhar., 2009).

LP is one of the most investigated consequences of ROS action on the membrane structure and function. The idea of LP as a solely destructive process has changed during the past decade. (Gordana Juric-Sekhar., 2009).

The peroxidation of lipids involves three distinct steps: initiation, propagation and termination. (Shewfelt and Purvis, 1995). **(Figure 2.9.2)**

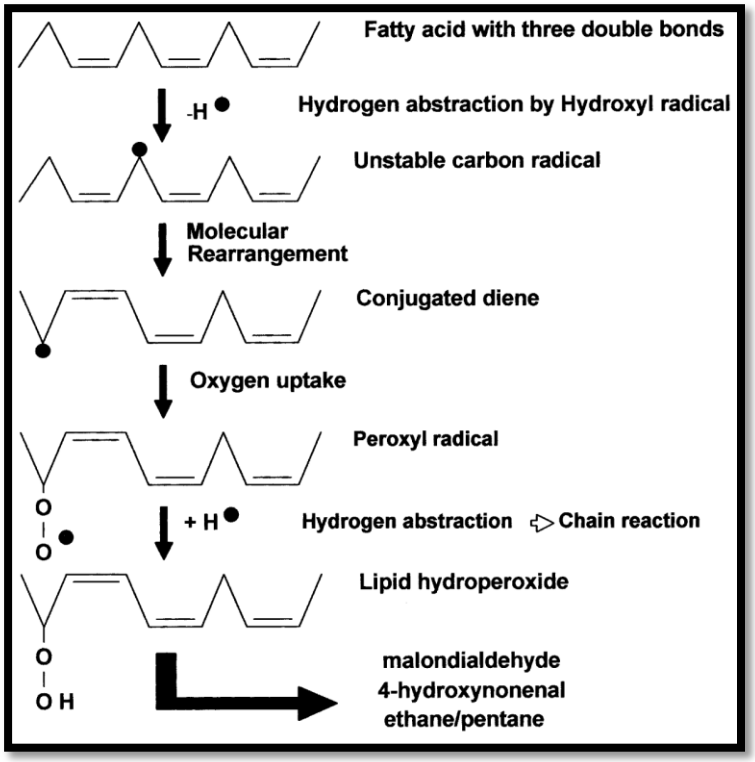


Figure 2.9.2: Basic reaction sequence of lipid peroxidation (I. S. Young2001).

The initiation reaction between an unsaturated fatty acid (e.g. linoleate) and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid in the case of linoleate this occurs at carbon-11 **(Fig. 2.9.3).**

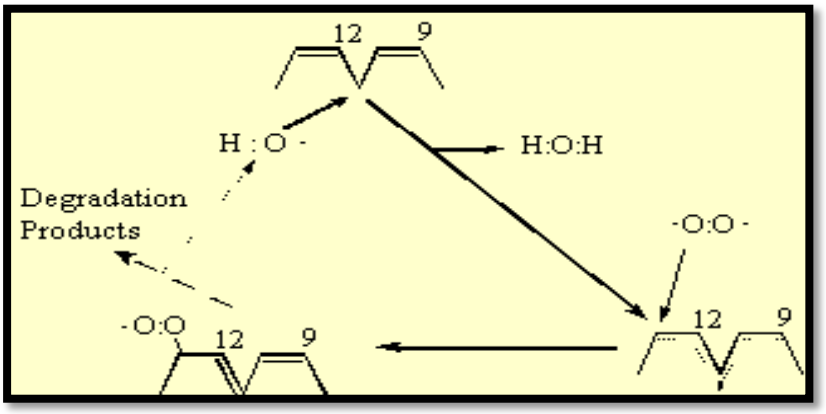


Figure 2.9.3: The peroxidation of linoleic acid. (I. S. Young2001).

The remaining carbon centred radical, forms a resonance structure sharing this unpaired electron among carbons 9 to 13.

In **the propagation reactions**, this resonance structure reacts with triplet oxygen, which is a biradical having two unpaired electrons and therefore reacts readily with other radicals. This reaction forms a peroxy radical .In the case of linoleate, addition occurs at either carbon-9 or -13 **(Fig 2.9.3).** The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centred free radical that can participate in a second H abstraction . Therefore, once one hydroxyl radical initiates the peroxidation reaction by abstracting a single H atom, it creates a carbon radical product (R) that is capable of reacting with ground state oxygen in a chain reaction.

The role of the hydroxyl radical is analogous to a "spark" that starts a fire. The basis for the hydroxyl radical's extreme reactivity in lipid systems is that at very low concentrations it initiates a chain reaction involving triplet oxygen, the most abundant form of oxygen in the cell.

The lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalysts because ROOH will participate in a Fenton reaction leading to the formation of reactive alkoxy radicals:

$$ROOH + Fe^{2+} \rightarrow OH^{-} + RO\bullet + Fe^{3+}$$

Therefore, in the presence of Fe, the chain reactions are not only propagated but amplified.

Among the degradation products of ROOH are aldehydes, such as malondialdehyde, and hydrocarbons, such as ethane and ethylene, that are commonly measured end products of lipid peroxidation.

The peroxidation reactions in membrane lipids are **terminated** when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals, such as those shown in the following reactions:

$$R\bullet + R\bullet \rightarrow R - R$$

$$R\bullet + ROO\bullet \rightarrow ROOR$$

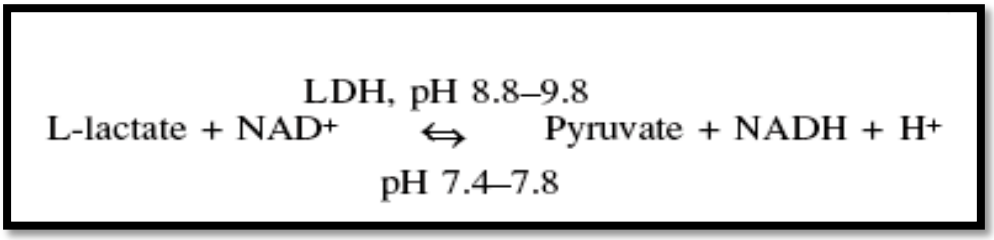
$$ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$$

Typically high molecular weight, cross-linked fatty acids and phospholipids accumulate in peroxidised membrane lipid samples.

2. 10. Lactate dehydrogenase:

Lactate dehydrogenase (LDH) reversibly catalyzes the oxidation of lactate to pyruvate by transferring hydrogen from lactate to the cofactor NAD^{+}.

The reaction represents the last step in the process of anaerobic glycolysis, and provides a mean for the regeneration of NAD^+ required for the reaction catalyzed by glyceraldehydes-3-phosphate dehydrogenase.



Lactate dehydrogenase is a cytoplasmic enzyme distributed widely in many tissues with high concentrations in heart and skeletal muscles, liver, kidney, erythrocytes, and testis.

In vertebrate, there are five major isozymic forms, LDH-A_4, LDH-A_3B, $LDHA_2B_2$, LDH-AB_3 and LDH-B_4, formed by the random combination of two types of polypeptide chains A and B into tetramers. The relative proportion of these five isozymic forms varies from tissue to tissue, with the LDH-A_4 isozyme predominating in cardiac muscle.

On the basis of the differences in their kinetic properties, the biological role of the LDH-A_4 isozyme had been proposed to be the conversion of pyruvate to lactate as the terminal reaction of anaerobic glycolysis, while the LDH-B_4 isozyme is thought to be involved in the production of pyruvate from lactate in actively respiring tissues such as heart (Dawson, et al., 1964).

Enzyme levels in various tissues (in U/g weight) are very high compared to those in serum: liver 9,000; heart 25,000; kidney 15,000; skeletal

muscle 9,000; lung 9,500 (Moss and Henderson, 1986). Thus, tissue levels are about 500 fold higher than those normally found in serum, and leakage of the enzyme from even a small mass of damaged tissue can increase the observed serum level of LDH to a significant extent.

A significant increase of LDH enzyme level was found in brain, liver and testis tissues, which indicates a high-energy demand due to depletion of oxygen.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Materials:

(a) Animals:

Sixty male mice were obtained from the Central Animal House of Al-Arab Medical University, Benghazi, Libya. All animals weighed between 25-30g. The animals were housed in cages in groups of twelve in each colony. The colonies were maintained at room temperature, and on a 12-hour light/dark cycle. All the animals had a free access to food and water.

Animals were divided randomly into five groups, in the following order:

Group 1:

Control group (c) in this group mice were on chow without any stress

Group 2:

High fat diet group(HFD) in this group mice on high fat diet wish prepared in our lab

Group 3:

High fat diet and cold group(HFD&C) in this group mice on high fat diet and were on cold stress(every day at 9:30AM mice putted in refrigerator at 4c for one hour)

Group 4:

Normal diet and cold group(N&C) in this group mice were on normal diet (chow) and were on cold stress(every day at 9:30AM mice putted in refrigerator at 4c for one hour).

Group 5:

Normal and dark group(N&D) in this group mice were on normal diet (chow) and were on dark stress(every day at 9:30AM mice putted in dark dry room for one hour).

(b) Equipments:

Livers were homogenized in Teflon glass homogenizer (Janke and Kunkel –type RW18).

Centrifugation was carried out in Bench centrifuge Barid &Tatlok London limited.

Thin tissue section (4 microns) of the liver were cut on Microtome-Leica RM2135.

Representative tissue pieces were passed from low to high alcohol concentration using a Jung histokinette 2000-leica.

The absorbance of different samples at different wave length was carried out by using spectrophotometer (spectro UV-Vis Double PC spectrophotometer, U.S.A).

Thermostatic oven (Thermostate Binder, Germany) make was used for incubating at $37^{o}C$ for various enzyme activities assays

3.2.Methods:

3.2.1. Measurement of weight of mice:

After one week of adaptation in our lab weights of mice in all group
First day of experiment.
After two weeks experiment.
After five weeks experiment.

3.2.2. Preparation of mice diet:

Preparation of high fat diet:

In all experiments, components for purified diets were mixed for 45 min, formed into a dough with the addition of one kilogram of fat to four kilogram of mice laboratory diet.
(High fat diet consisting of a mixture of one part fat diet and four parts chow was prepared.)

Diets were stored in tightly stopperd plastic containers and refrigerated at 4 ℃ for no longer than 2 weeks. Under these conditions, high-fat diets did not become rancid, and to minimize oxidation. In addition, fresh food was provided to animals daily.
The final mixture contained 20% fat, approximately 80% Chow
The source of the fat contained in high fat diet was from an animal source (sheep).

3.2.3 Removal of the mice liver

Following an overnight fast the animals were anaesthetized and the blood was drawed after that scarified. The livers were removed rapidly and adherent blood clots removed from the surface.

Representative pieces of mice livers were homogenized in a Potter Elvenhiem homogenizer (Janke and Kunkel – Type RW 18).

3.2.4. Preparation of liver homogenates:

The homogenate was prepared using a 1g of tissue in a volume made to 10 ml of ice cold 0.15 M KCl (10% w/v; pH adjusted to 7.4) using a Teflon Potter Elvenhjem homogenizer.

3.2.5 . Estimation of concentration of fasting blood glucose:

Estimation of adiponectin concentration in blood was carried out using Colorimetric method EnzyChromTM Glucose Assay Kit (EBGL-100) Quantitative Colorimetric/Fluorimetric Glucose Determination BioAssay Systems)

(a) Principle:

Glucose ($C_6H_{12}O_6$) is a key diagnostic parameter for many metabolic disorders. Increased glucose levels have been associated with diabetes mellitus, hyperactivity of thyroid, pituitary and adrenal glands. Decreased levels are found in insulin secreting tumors, myxedema, hypopituitarism and hypoadrenalism. Simple, direct and high-throughput assays for measuring glucose concentrations find wide applications in research and drug discovery. BioAssay Systems' glucose assay kit uses a single

Working Reagent that combines the glucose oxidase reaction and color reaction in one step. The color intensity of the reaction product at 570nm or fluorescence intensity at lem/ex = 585/530nm is directly proportional to glucose concentration in the sample.

(b) Sample:

Sensitive and accurate. Use as little as 20 µL samples. Linear detection range in 96-well plate: 5 to 300 µM (90 µg/dL to 5.4 mg/dL) glucose for colorimetric assays

Simple and high-throughput. The procedure involves addition of a single working reagent and incubation for 30 min at room temperature

(c) Reagents:

Tabel 3.1:Reagent used in Estimation of concentration of fasting blood glucose.

No	300µ M STD + H2O	Vol (µL)	Glucose (µM)
1	200 µL + 0µ L	200	300
2	120 µL + 80 µL	200	180
3	60 µL + 140 µL	200	90
4	0 µL + 200 µL	200	0

(d) Procedure:

Sample treatment: samples should be centrifuged for 5 min at 14,000 rpm prior to assay. Milk samples should be cleared by mixing 100 µL 6N HCl and 600 µL milk. Centrifuge 5 min at 14,000 rpm and transfer supernatant into a clean tube. Add 170 µL 6N NaOH per mL supernatant. Mix well and centrifuge again at 14,000 rpm. The supernatant can be assayed. The dilution factor in this procedure is n = 1.36.

Samples can be analyzed immediately after collection, or stored in aliquots at –20 °C. Avoid repeated freeze-thaw cycles. If particulates are present, centrifuge sample and use clear supernatant for assay.

1. Equilibrate all components to room temperature. During experiment, keep thawed Enzyme in a refrigerator or on ice.

2. Standards and samples: for 300 µM standard, mix 15 µL 300 mg/dL standard with 818 µL dH2O. Dilute standard in dH2O as follows Transfer 20 µL standards and samples into separate wells.

3. Working Reagent. For each reaction well, mix 85 µL Assay Buffer, 1 µL Enzyme Mix (vortex briefly before pipetting), and 1 µL Dye Reagent ina clean tube. Transfer 80 µL Working Reagent into each reaction well. Tap plate to mix.

4. Incubate 30 min at room temperature. Read optical density at 570nm (550-585nm).

(e) Calculation:

Subtract blank OD (water, #4) from the standard OD values and plot the DOD against standard concentrations. Determine the slope and calculate the glucose concentration of Sample,

[Glucose] =

$$\frac{OD_{SAMPLE} - OD_{BLANK}}{Slope} \quad (\mu M)$$

OD**SAMPLE**, OD**BLANK** are optical density values of the sample and water.

Conversions: 1 mg/dL glucose equals 55.5 μM, 0.001% or 10 ppm.

3.2.6. Estimation of adiponectin concentration in blood:

Estimation of adiponectin concentration in blood was carried out using enzyme immunoassay kit (catalog number Cat. No.: RD293023100R, BioVendor – Laboratorní medicína, a.s.).

(a) Principle

In the BioVendor Mouse Adiponectin ELISA, Standards, Quality Controls and samples are incubated in microplate wells pre-coated with monoclonal anti-mouse Adiponectin antibody. After 60 minutes incubation and washing, polyclonal anti-mouse Adiponectin antibody, conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 60 minutes with captured mouse Adiponectin. Following another washing step, the remaining HRP conjugate is allowed to react with the Substrate Solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to the concentration of mouse Adiponectin. A standard curve is constructed by plotting absorbance values against concentrations of

Standards, and concentrations of unknown samples are determined using this standard curve.

(b).Reagent

Tabel 3.2: Reagent used in estimation of adiponectin concentration in blood:

Quantity	State	Kit Components
96 wells	ready to use	Antibody Coated Microtiter Strips
13 ml	ready to use	Conjugate Solution
2 vials	lyophilized	Master Standard
2 vials	lyophilized	Quality Control High
2 vials	lyophilized	Quality Control Low
22 ml	concentrated	Dilution Buffer Concentrate (10x)
100 ml	concentrated	Wash Solution Concentrate (10x)
13 ml	ready to use	Substrate Solution
13 ml	ready to use	Stop Solution
1 pc	Product Data Sheet + Certificate of Analysis	

(C). Procedure

1. Preparation of reagents

All reagents need to be brought to room temperature prior to use.
Always prepare only the appropriate quantity of reagents for your test.
Do not use components after the expiration date marked on their label.

Assay reagents supplied ready to use:

Antibody Coated Microtiter Strips

Stability and storage:

Return the unused strips to the provided aluminium zip-sealed bag with desicant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8°C and protected from the moisture.

Conjugate Solution

Substrate Solution

Stop Solution

Stability and storage:

Opened reagents are stable 3 months when stored at 2-8°C.

Assay reagents supplied concentrated or lyophilized:

Dilution Buffer Concentrate (10x)

Dilute Dilution Buffer Concentrate (10x) ten-fold in distilled water to prepare a 1x working solution. Prepare only required amount of Dilution Buffer.

Example: 22 ml of Dilution Buffer Concentrate (10x) + 198 ml of distilled water for use of all 96-wells.

Stability and storage:

The diluted Dilution Buffer is stable 1 week when stored at 2-8°C.

Opened Dilution Buffer Concentrate (10x) is stable 3 months when stored at 2-8°C.

Wash Solution Concentrate (10x)

Dilute Wash Solution Concentrate (10x) ten-fold in distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Concentrate (10x)+ 900 ml of distilled water for use of all 96-wells.

Stability and storage:

The diluted Wash Solution is stable 1 month when stored at 2-8°C. Opened Wash Solution Concentrate (10x) is stable 3 months when stored at 2-8°C

Mouse Adiponectin Master Standard

Reconstitute the lyophilized Master Standard with 1 ml of Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasionally gently shaking (not to foam). The resulting concentration of the mouse Adiponectin in the stock solution is **8 ng/ml**.

Prepare set of standards using Dilution Buffer as follows:

Concentration	Dilution Buffer	Volume of Standard
8 ng/ml	**1.0 ml**	**Stock**
4 ng/ml	0.5 ml	0.5 ml of stock
2 ng/ml	0.5 ml	0.5 ml of 4 ng/ml
1 ng/ml	0.5 ml	0.5 ml of 2 ng/ml
0.5 ng/ml	0.5 ml	0.5 ml of 1 ng/ml
0.25 ng/ml	0.5 ml	0.5 ml of 0.5 ng/ml

2. Preparation of samples

The kit measures mouse Adiponectin in serum.

Samples should be assayed immediately after collection or should be stored at -20°C. Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

Dilute samples just prior to the assay 10 000x with Dilution Buffer, in two steps as follows:

Dilution A (100x):

Add 10 µl of sample into 990 µl of Dilution Buffer.

Mix well (not to foam). Vortex is recommended.

Dilution B (100x):

Add 10 µl of Dilution A into 990 µl of Dilution Buffer to prepare final dilution (10 000x). **Mix well** (not to foam). Vortex is recommended.

Stability and storage:

Samples should be stored at -20°. Avoid repeated freeze/ thaw cycles.

Do not store the diluted samples.

See Chapter 13 for stability of serum samples if stored at 2-8°C and effect of freezing/thawing on the concentration of mouse Adiponectin.

Note: It is recommended to use a precision pipette and a careful technique to perform the dilution in order to get precise results

3. Assay Procedure

1. Pipet **100 µl** of each individual concentration of Standards, Quality Controls, Dilution Buffer (=Blank) and diluted samples, preferably in duplicates, into the appropriate wells.

2. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

3. Wash the wells **3-times** with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

4. Add **100 µl** of Conjugate Solution into each well.

5. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

6. Wash the wells **3-times** with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

7. Add **100 µl** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.

8. Incubate the plate for **10 minutes** at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.

9. Stop the colour development by adding **100 µl** of Stop Solution.

10. Determine the absorbance by reading the plate at 450 nm. The absorbance should be read within 5 minutes following step 9.

Note: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine mouse Adiponectin concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Note 2: Manual washing: Aspirate wells and pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

(e) Calculation:

Most microplate readers perform automatic calculations of analyte concentration. The standard curve is constructed by plotting the mean absorbance at 450 nm (Y) of Standards against log of the known concentration (X) of Standards, using the four-parameter algorithm. Results are reported as concentration of mouse Adiponectin ng/ml in samples.

Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards.
The measured concentration of samples calculated from the standard curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay (e.g. 2.65 ng/ml (from standard curve) x 10 000 (dilution factor) = 26.5 μg/ml).

Note:
Typical analytical data of BioVendor Mouse Adiponectin ELISA are:

Sensitivity

Limit of Detection (LOD) (defined as concentration of analyte giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: Ablank + 3xSDblank) is calculated from the real Adiponectin values in wells and is 0.1 ng/ml.
*Dilution Buffer is pipetted into blank wells.

Limit of assay

Results exceeding Adiponectin level of 8 ng/ml should be repeated with more diluted samples (e.g. 20 000x). Dilution factor needs to be taken into consideration in calculating the Adiponectin concentration.

Specificity

The antibodies used in this ELISA are specific for mouse Adiponectin protein with no detectable crossreactivities to mouse cytokines: RELM-α,

RELM-β, Leptin, Leptin-receptor, Resistin; as well as for rat Leptin and human Adiponectin at 100 ng/ml.

Stability of samples stored at 2-8°C

Samples should be stored at –20°C. However, no decline in concentration of mouse Adiponectin was observed in serum and plasma samples after 14 days when stored at 2-8°C. To avoid microbial contamination, samples were treated with ε-aminocaproic acid and sodium azide, resulting in the final concentration of 0.03% and 0.1%, respectively.

3.2.7. Estimation of Alanine aminotranferase (ALT) activity in liver homogenate

Estimation of GPT in liver was carried out using a colorimetric kit **(**SPINREACT, S.A. Ctra.Santa Coloma, 7 E-17176 SANT ESTEVE DE BAS (GI) SPAIN)

(a) Principle:

Alanine aminotranferase (ALT) or Glutamate pyruvate transaminase (GPT) catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming glutamate and piruvate.

The piruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH:

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Glutamate} + \text{Piruvate}$$

$$\text{Piruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{Lactate} + NAD^{+}$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample[1].

(b) Sample:

Appropriate concentration of fresh tissue homogenate.

(c) Reagents:

Table 3.4: Reagents used for estimation of ALT

R1 Buffer	TRIS PH 7.8 100mmol/L Lactate-dehydrogenase(LDH) 1200U/L L-Alanine 500mmol/L
R 2 Substrate	NADH 0.18mmol/L α- Ketoglutarate 12mmol/L

Preparation:

Working reagent (WR)

Mix:

9 vol. (R1) Buffer + 1 vol. (R2) Substrate

Stability: 21 days at 2-8ºC or 72 hours at room temperature (15-25ºC).

(d) Procedure:

1. Assay conditions: Wavelength: . . . . . .340 nm

Cuvette: . . . . 1 cm light path

Constant temperature . . . . .25ºC / 30ºC / 37ºC

2. Adjust the instrument to zero with distilled water or air.

3. Pipette into a cuvette:

WR (mL)	1,0
Sample (µL)	100

4. Mix, incubate for 1 minute.

5. Read initial absorbance (A) of the sample, start the stopwatch and read absorbance at 1 minute intervals thereafter for 3 minutes.

6. Calculate the difference between absorbance and the average absorbance differences per minute (ΔA/min).

(e) Calculation:

$$\text{ALT (IU/mg protein)} = \frac{\Delta A \times \mathbf{Vt} \times \mathbf{1000}}{\mathbf{E} \times \mathbf{Vs} \times \mathbf{Tmin} \times \mathbf{mg\ protein}}$$

Where:

ΔA = Difference in absorbance between 1_{min} and $3_{min.}$;

V_t= Total volume of homogenate (ml); Vs= Volume of estimation (ml);

T_{min}= Time in minute;

Wt= weight of fresh tissue used in gram.;

E= molar absorption coefficient at 340 nm of $6.3x10^3$ per 1 cm pathlength

Referance; (Tietz Textbook of Clinical Chemistry, W.B. Saunders, Philadelphia, PA,Third edition 1999, pp. 652-653 ,656-657).

3.2.8 Estimation of Aspartate aminotransferase (AST) activity in liver homogenate

Estimation of GPT in liver was carried out using a colorimetric kit **(**SPINREACT,S.A. Ctra.Santa Coloma, 7 E-17176 SANT ESTEVE DE BAS (GI) SPAIN **)**

(a) Principle:

Aspartate aminotransferase (AST) formerly called glutamate oxaloacetate (GOT) catalyses the reversible transfer of an amino group from aspartate to α-ketoglutarate forming glutamate and oxalacetate. The oxalacetate produced is reduced to malate by malate dehydrogenase (MDH) and NADH:

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Glutamate} + \text{Oxalacetate}$$

$$\text{Oxalacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{Malate} + NAD^+$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of AST present in the sample.

(b) Sample:

Appropriate concentration of fresh tissue homogenate.

(c) Reagents:

Tabel 3.5:Reagent used in estimation of AST activity in liver homogenate

R1 Buffer	TRIS PH 7.8 80mmol/L Lactatedehydrogenase(LDH) 800U/L Malatedehydrogenase(MDH) 600U/L L-Aspartate 0.18mmol/L
R 2 Substrate	NADH 0.18mmol/L α-Ketoglutarate 12mmol/L

Preparation

Working reagent (WR)

Mix: 9 vol. (R1) Buffer + 1 vol. (R2) Substrate

Stability: 21 days at 2-8ºC or 72 hours at room temperature (15-25ºC).

(d) Procedure:

1. Assay conditions:

Wavelength: . . . . . 340 nm

Cuvette: . . . . . .1 cm. light path

Constant temperature . . . . . .25ºC /30ºC / 37ºC

2. Adjust the instrument to zero with distilled water or air.

3. Pipette into a cuvette:

WR(ml)	**1.0**
Sample(ml)	**.10**

4. Mix, incubate for 1 minute.

5. Read initial absorbance (A) of the sample, start the stopwatch and read absorbances at 1 minute intervals thereafter for 3 minutes.

6. Calculate the difference between absorbances and the average absorbance differences per minute (ΔA/min).

(e) Calculation:

$$\text{AST (IU/mg protein)} = \frac{\Delta A \times \mathbf{Vt} \times \mathbf{1000}}{\mathbf{E} \times \mathbf{Vs} \times \mathbf{Tmin} \times \mathbf{mg\ protein}}$$

Where:

ΔA = Difference in absorbance between 1_{min} and $3_{min.}$;

V_t= Total volume of homogenate (ml); Vs= Volume of estimation (ml);

T_{min}= Time in minute;

Wt= weight of fresh tissue used in gram.;

E=molar absorption coefficient at 340 nm of $6.3x10^3$ per 1 cm pathlength

Referance;(Tietz Textbook of Clinical Chemistry, W.B. Saunders, Philadelphia, PA,Third edition 1999, pp. 652-653 ,656-657).

3.2.9 Estimation of Alkaline Phosphatase (ALP) activity in liver homogenate:

Alkaline Phosphatase Assay Kit- BioVision (Catalog #K412-500; 500 Reactions; Store kit at –20°C)

(a) Principle:

Alkaline phosphatase (ALP) catalyses the hydrolysis of p-nitrophenyl phosphate at pH 10.4, liberating p-nitrophenol and phosphate, according to the following reaction:

p-Nitrophenylphosphate + H_2O p-Nitrophenol + Phosphate ⟶–ALP

The rate of p-nitrophenol formation, measured photometrically, is proportional to the catalytic concentration of alkaline phosphatase present in the sample.

(b) Sample:

Appropriate concentration of fresh tissue homogenate.

(c) Reagents

Table3.6. : Reagents used for estimation of alkaline phosphates

R1 Buffer	**D**iethanolamine (DEA) PH 10.4 Magnesium chloride	1mmol/L 0.5mmol/L
R 2 Substrate	**p-**Nitrophenylphosphate (Pnpp)	10mmol/L

(d) Procedure:

Alkaline Phosphatase Assay Protocol:

Preparation

Working reagent (WR)

Mix: 9 vol. (R1) Buffer + 1 vol. (R2) Substrate

Stability: 1 month at 2-8ºC or 10 days at room temperature

1. Assay conditions: Wavelength: . . . . . .410 nm

Cuvette: . . . . 1 cm light path

Constant temperature . . . . . 25ºC / 30ºC / 37ºC

2. Adjust the instrument to zero with distilled water or air.

3. Pipette into a cuvette:

WR(Ml)	**1.2**
Sample(µL)	**20**

4. Mix, incubate for 1 minute.

5. Read initial absorbance (A) of the sample, start the stopwatch and read absorbances at 1 min intervals thereafter for 3 min.

6. Calculate the difference between absorbances and the average absorbance differences per minute (ΔA/min).

(e) Calculation:

$$\text{ALP (IU/mg protein)} = \frac{\Delta A \times \mathbf{Vt} \times \mathbf{1000}}{\mathbf{E} \times \mathbf{Vs} \times \mathbf{Tmin} \times \mathbf{mg\ protein}}$$

Where:

ΔA = Difference in absorbance between 1_{min} and $3_{min.}$;

V_t= Total volume of homogenate (ml); Vs= Volume of estimation (ml);

T_{min}= Time in minute;

Wt= weight of fresh tissue used in gram.;

E= The extinction coefficient for p-nitrophenol at 410 nm is 18.5 $mM^{-1}cm^{-1}$

Referance;(Sakharov, I. Y., Makarova, I. E., and Ermolin, G. A. 1988. Purification and characterization of intestinal alkaline phosphatase from harp seal. Comparative Biochemistry and Physiology, B 90B:709-714.)

3.2.10 Estimation of Total protein in liver homogenate :

Total protein was estimated by the procedure of (Lowery et al., 1951).

(a) Reagents:

Solution A: 1volume of 5% (w/v) copper sulfate, 1 volume of 10% (w/v) sodium potassium tartrate and 8 volumes of water.

Solution B: 2% (w/v) sodium carbonate in 0.1N NaOH.

Solution C: A mixture containing 1 volume of solution A and 50 volumes of solution B was immediately prepared before use.

(b) Procedure:

Liver tissue was weighed and homogenized in glass homogenizer with Teflon pestle in 0.015 M KCl.

Two ml of solution C were added to 20µl of the homogenate in 18 × 150 mm test tube (corning), 0.2 ml of diluted Folin-Ciocalteau reagent was added to the mixture and the contents were mixed immediately. After 40 minutes, the absorbance of the developed blue color solution was measured at 700 nm against sample blank. A typical standard curve shown in figure [2.1] was prepared using 20 µl containing 10 to 90 µg BSA in each standard reaction.

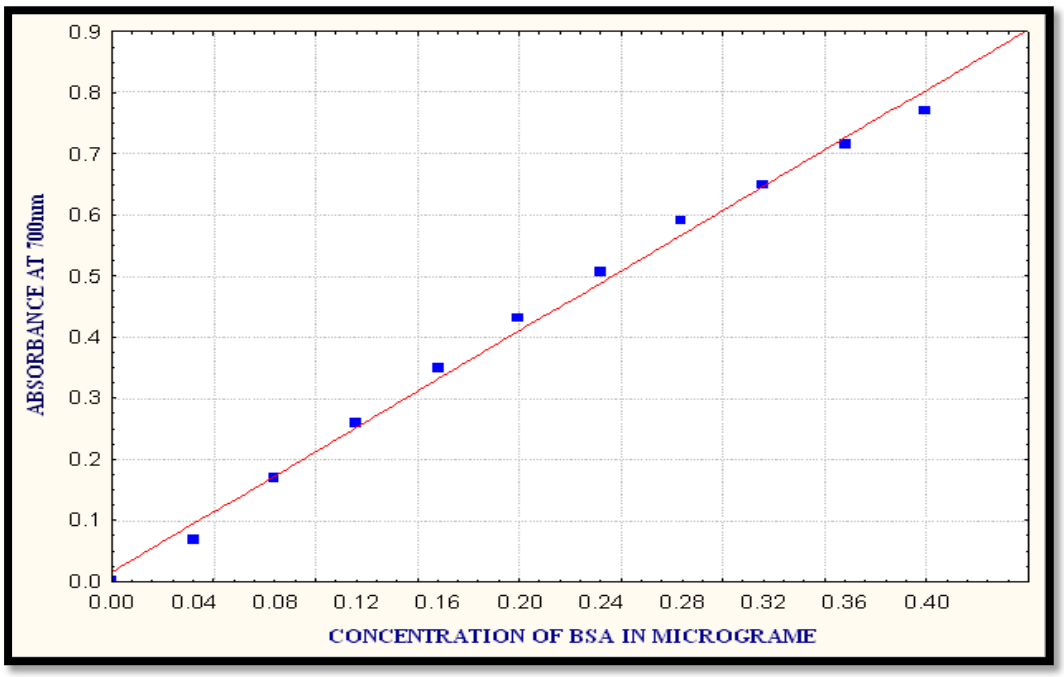
(c) Calculation: The total protein concentration in the homogenate was evaluated as follow:

$$\text{Protein concentration (mg/gm fresh tissue)} = \frac{\mathbf{C\mu g \times Vt}}{\mathbf{Wt \times Vs \times 1000}}$$

Where:

C = Concentration in microgram was calculated from standard curve;

$\mathbf{V_t}$ = Total volume of homogenate (ml); $\mathbf{V_s}$ = Volume used for estimation (ml); $\mathbf{W_t}$ = Weight of fresh tissue used in gram; **1000** = Factor to convert units from µg to mg.



<u>Figure 3.1:</u> Standard curve for total protein estimation.

3.2.11. Estimation of Glucose-6-Phosphate dehydrogenase (G6PDH):

The quantitative determination of G6PDH was performed using a kit supplied by RANDOX Laboratoirse Ltd., Cataloge number PD410.

(a) Principle:

The enzyme activity was determined by the measurement of the rate of change in absorbance at 340 nm due to the reduction of $NADP^+$ (Lohr and Waller 1974; Makarem, 1974).

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{Gluconate-6-P} + \text{NADPH} + \text{H}^+$$

(b) Reagents:

Table 3.7: Reagents were used for estimation of G6PDH.

CONTENT	**CONCENTRATION IN THE TEST**
***Buffer** -Triethanolamine Buffer -EDTA	31.7 mmol/L, pH 7.6
***NADP**	3.2 mmol/L
***Glucose-6-phosphate**	0.34 mmol/L
***Digitonin**	0.58 mmol/L

(c) Procedure:

Table 3.8 : Method was used for estimation of G6PDH.

0.2ml of the homogenate was added to 0.5 ml of digitonin. The reaction was left to stand for 15 minutes, centrifuged and an appropriate volume was taken from the supernatant for estimation as follow:	
Solution 1	1ml
Solution 2	0.03ml
Supernatant	0.015ml
The contents were mixed well, and the initial absorbance was read and timer was started simultaneously. Absorbance was noted after 1, 2, 3, 4 and 5 minutes. The difference in absorbance (ΔA) was determined.	
Solution 3	0.015 ml
The contents were mixed well, and the initial absorbance was read and timer was started simultaneously. Absorbance was noted after 1, 2, 3, 4 and 5 minutes. The difference in absorbance (ΔA) was determined.	

(d) Calculation:

The activity of G6PDH was calculated based on IU/mg protein.

$$\text{G6PDH (IU/ mg protein)} = \frac{\Delta A \times Vt \times 1000}{6.22 \times Vs \times Tmin \times \text{mg protein}}$$

Where:

ΔA= Difference in absorbance (A_{340nm} at 5_{min} – A_{340nm} at 1_{min});

V_t= Total volume of homogenate (ml); Vs= Volume of estimation (ml); T_{min}= Time in minutes; Wt= weight of tissue used in gram; $6.22x10^{-3}$ =Millimolar extinction coefficient of ß-NADPH at 340 nm (Noltmann et al., 1961).

3.2.12. Estimation of glutathione (GSH):

Estimation of reduced glutathione in liver was carried out using a colorimetric kit (catalog number GR2510, Biodiagnostic, Egypt).

(a) Principle:

The method was based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid)(DTNB) with glutathione (GSH) to produce a yellow compound. The extent of the reduced chromogen formation was directly proportional to GSH concentration. Its absorption was measured at 405 nm (Beutler et al., 1963).

(b) Sample:

Appropriate concentration of fresh tissue homogenate.

(c) Reagents:

Table 3.9: Reagents used for estimation of GSH.

i	**Trichloroacetic acid (TCA) [R1]**	**500 mmol/L**
2	Buffer [R2]	100 mmol/L
3	**DTNB [R3]**	**1mmo/L**

(d) Procedure:

Table 3.10: Procedure was used for estimation of GSH.

	Homogenate (ml)	Blank (ml)
Sample	0.5	/
Distell water	/	0.5
R1	0.5	0.5
Contents were mixed well and allowed to stand for 5 min at room tempreature The tubes were centrifuged at 3000 rpm for 15 min. then, the following were mixed.		
R2	1.0	1.0
R3	0.1	0.1
The contents were mixed well, and the absorbance was measured at 405 nm after 5-10 min against sample blank.		

(e) Calculation:

Concentration of reduced glutathione (GSH) was calculated on basis of mg/mg protein:

$$\text{GSH (mg / mg protein)} = \frac{\mathbf{Asample \times 66.6 \times Vt}}{\mathbf{mg\ protein \times Vs}}$$

Where: A_{sample}= Absorbance of sample; V_t= Total volume of homogenate (ml); V_s= Volume of estimation (ml); W_t= Weight of tissue used in gram; 66.6 = Extinction coefficient of reduced glutathione.

3.2.13. Assay of lipid peroxidation:

The assay for lipid peroxides in the liver was performed by thiobabituric acid reaction method as described by Ohakawa et al. (1979).

(a) Test principle:

The method was based on the reaction of one molecule of malondialdehyde (MDA) with two molecules of thiobarbituric acid (TBA) forming pink chromogen to be measured at532 nm.

(b) Reagents:

1- Sodium dodecyl sulfate (SDS) (8.1 % w/v) was prepared by dissolving 8.1g SDS in a 100ml distilled water.

2- Acetic acid (20% v/v) was prepared by mixing 20 ml of acetic acid with 100 ml distilled water and the pH was adjusted to 3.5 with two pellets of NaOH

3- Thiobarbituric acid (TBA, 0.8%) was prepared by dissolving 0.8 g in 100ml distilled water stable for one week at room temperature.

4- n-butanol and pyridine (15:1 v/v) was prepared by mixing 15 ml of n-butanol with 1ml of pyridine.

(d) Procedure:

The method consisted of the addition of the following into a centrifuge tube: 0.2 ml of the tissue homogenate, 0.2 ml of 8.1%of SDS solution, 1.5 ml of 20% acetic acid solution and 1.5 ml of 0.8 % solution of TBA. The volume was made up to 4 ml with distilled water and the solution was mixed and heated in a water bath at 95°C for 60 min.

The tubes were cooled with tap water, 1 ml of distilled water and 5 ml of the mixture of n- butanol and pyridine (15:1) were added and the contents were shaken vigorously. The tubes were centrifuged for 15min at 4000 rpm. After centrifugation, the organic layer was taken off and its absorbance was measured against blank at 532 nm.

(c) Calculation:

The level of MDA was calculated on bases of µM/g fresh tissue according to following equations:

$$\text{MDA (µM/g fresh tissue)} = \frac{C \times Vt}{Wt \times Vs}$$

C= Concentration of MDA in (µM), which was calculated from a typical MDA calibration curve generated using Malondialdehyde (NWLSS MDA01, Northwest Life Science Specialties) assay employing a standard procedure. (Botsoglou, 1994).

V_t= Total volume of homogenate in (ml).

Vs= Volume of estimation (ml).

W_t= Fresh weight of tissue used in gram.

3.2.14. Estimation of LDH:

Determination of lactate dehydregenase in tissue was carried out using a kinetic assay based kit (Centronic GmbH, Germany).

(a) Principle:

NADH and pyruvate were converted to lactate and NAD^+

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+$$

The decrease in NADH (NADH oxidation) was directly proportional to the LDH activity. The rate of the formed NAD^+ was determined by the decrease in absorbance at 340 nm (Klin, 1970 and 1972).

(b) Concentration of working solution:

Table 3.11: Concentration of different reagents used for estimation of LDH.

Buffer (R_1)	**Concentration**
*TRIS-Buffer, pH 7.5	50 mmol/L
Pyruvate	0.6 mmol/L
*Sodium Azide	0.09%
Starter (R_2)	**Concentration**
*NADH	0.18mmol/L

(c) Assay procedure:

Table 3.12: Method used for estimation of LDH.

The following were pipetted into a cuvette:	
Buffer (R_1)	1.0ml
Sample	0.02ml
Starter reagent (R_2)	0.200 ml
The contents were mixed well immediately after addition of the sample. The absorbance was measured after 30 seconds at 340 nm, and the measurements were repeated every minute for 5 minutes. The difference in absorbance was determined (ΔA/ min).	

(d) Calculation:

The level of LDH in tissue homogenate was evaluated as IU/mg protein according to:

$$\text{LDH (IU/mg protein)} = \frac{\Delta A \times \mathbf{Vt} \times \mathbf{1000}}{\mathbf{6.22} \times \mathbf{Vs} \times \mathbf{Tmin} \times \mathbf{mg\ protein}}$$

Where:

ΔA = Difference in absorbance between 1_{min} and $5_{min.}$;

V_t= Total volume of homogenate (ml); Vs= Volume of estimation (ml);

T_{min}= Time in minute; Wt= weight of fresh tissue used in gram.;

$6.22x10^{-3}$= Millimolar extinction coefficient of ß-NADPH at 340 nm. (Noltmann et al., 1961).

3.2.16. Estimation of phosphate:

Phosphate was estimated by the well-known method of Fiske and Subbarow as described by Marinetti (1962).

(a) Test principle:

The method was based on the reaction of phosphate ions with ammonium molybdate to form complex structures such as ammonium phosphomolybdate, which was converted to molybdenum blue using a heteropolymer, 1-amono-2-naphthol-4-sulfonic acid (ANSA) as a reducing agent, and absorbance was measured at 700 nm.

(b) Reagents:

1. Working standard solution: 10µg of inorganic phosphate /ml was prepared by diluting 5 ml of refrigerated stock solution (0.43g potassium dihydrogen phosphate /500ml distilled water) to 100ml with distilled water in volumetric flask.

2. Ammonium molybdate solution (2.5%w/v) was prepared by dissolving 2.5g of ammonium molybdate in 100 ml distilled water.

3. Perchloric acid (62%).

4. Reducing reagent:

(a) Sodium sulfite (20% w/v): 20g of sodium sulfite was dissolved in 100 ml of distilled water.

(b) Sodium metabisulfite (15%w/v): 15g sodium metabisulfite was dissolved in 100 ml of distilled water.

(c) Reducing reagent (1-amino-2-naphthol-4-sulphonic acid –ANSA): was prepared by taking 45ml of (15%w/v) sodium metabisulfite, 0.125gm of ANSA and 5 ml of (20%w/v)sodium sulfite, which was added dropwise to make up the volume to 50ml. The reagent was stable for a week at room temperature.

(c) Procedure:

100µl of liver tissue homogenate or its organic solvent extract aliquots were pipetted into 18×150mm test tubes (corning). 1.0 ml of perchloric acid (62%) was added and the sample was heated in a digester for 2 hr or until the sample became clear. After complete digestion, the samples were cooled at the room temperature.

Thereafter, 1.5 ml of ammonium molybdate (2.5%w/v), 0.2 ml of reducing agent, and 7.0 ml of distilled water were added to them. The contents were mixed after each addition, and the tubes were heated in a boiling water bath for 7 min. The intensity of developed blue color was read at 700nm. A typical calibration curve [Figure 2.2] was drawn by standard procedure taking 1 to 10 µg of phosphorus and the blank was prepared without sample aliquot.

(d) Calculation:

The inorganic phosphate level in homogenate was evaluated on basis of mg/g fresh tissue

$$\text{Phosphate (mg/gm fresh tissue)} = \frac{\mathbf{C\mu g \times Vt}}{\mathbf{Ve \times Wt \times 1000}}$$

Where:

C= Concentration of phosphate in µg which was evaluated from standard curve.

Vt= Total volume of the lower layer (ml).

$\mathbf{V_s}$= Volume taken for estimation (ml).

$\mathbf{W_t}$= Fresh weight of the tissue in gram.

1000= Factor to convert units from µg to mg.

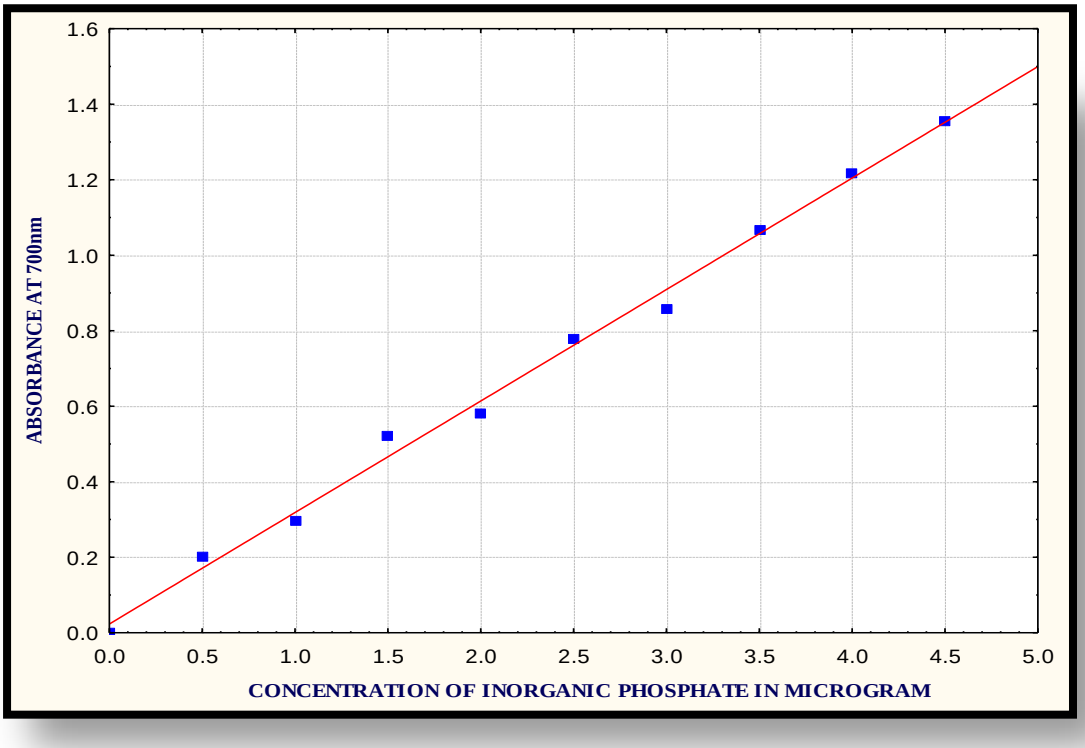


Figure 3.2.: Stander curve for inorganic phosphate.

3.2.17. Tissue fixing and sectioning:

Representative pieces of liver were fixed in Bouin´s reagent (75ml saturated aqueous picric acid 1.22% (w/v), 25 ml formalin and 5 ml glacial acetic acid) for 18 hr. The next day, they were washed twice in 50% (v/v) ethanol and twice in 70 % (v/v) ethanol to remove picric acid and then stored in 70 % (v/v) ethanol until processed as outlined below.

3.2.18 Paraffin processing:

The tissue was dehydrated in graded ethanol series and clearing was carried out using toluene. It was infiltrated using paraffin wax with a melting point of 57ºC. The automated rotary tissue processor was used that contained 12 solution stations. The fixed tissue was placed in baskets, which were then rotated at suitable time intervals through the dehydration, clearing agents and wax solutions with agitation. A summary of the cycles used to process the tissue is shown in table (3.4).

Table 3.13: Cycles used to process tissues for sectioning:

Duration	Solution	Station
1 hr	50% ethanol	**1**
1 hr	70% ethanol	**2**
1hr	90% ethanol	**3**
1hr	Absolute ethanol	**4**
1hr	Absolute ethanol	**5**
1hr	Toluene	**6**
1hr	Toluene	**7**
1hr	Wax	**8**
1hr	Wax	**9**
½ hr	Wax vacuum embedder 25 mmHg	**10**

Following these cycles, the tissue pieces were blocked in paraffin wax (melting point 57ºC).

3.2.19. Cutting and staining of the sections:

Paraffin 4μm thick sections were cut on a rotary microtome. Once a ribbon was formed it was floated on a water bath heated to 45°C and a single square lifted onto a slide. During the process, blocks to be cut were left on ice. Sections mounted on slides were incubated overnight in an oven at 45°C to dry. Sections were then dewaxed in toluene for 2 min, drained and the process repeated.

The drained sections were then taken to water gradually by using a decreasing alcohol series; agitating and drying each time. The hydrating fluids were 100% ethanol twice, 70% ethanol (v/v) and sterile water. Haematoxyline was used to stain the nuclei for 2 min, before the sample was drained and rinsed in water. Sections were then quickly immersed in 1% (v/v) acid alcohol [70% ethanol, 1 % HCl (v/v)] and rinsed in water, washed in alkaline rinse (0.15g lithium carbonate in 300ml sterile water) with agitation ten times and again rinsed in water. To stain the cytoplasm, sections were dehydrated by washing them ten times in 70% ethanol (v/v) with agitation and draining, and were dipped in eosin for 1min. Sections were dehydrated by three washes (with agitation) in absolute ethanol. Sections were then washed in toluene three times with agitation and drying. The resin DePex was then used to mount cover slips onto the slides, which were left to dry at room temperature.

3.3. Statistical analysis:

Data were analyzed by a commercially available Statistical Package for Social Sciences (SPSS) program for Windows software. P-values <0.05 were regarded as statistically significant. One-way Analysis of Variance (ANOVA) test was performed and post hoc multiple comparisons were done with least-squares differences (LSD).

Table. 4.1.1: Body weight in mice feeding on high fat diet (HFD)**(Mean ± S.D.).**

Group / Parameter	CONTROL N =5	HFD1 N =5	HFD2 N =5	HFD3 N =5
Body weight (gm)	32.50±0.79	31.20±0.71	31.30±1.89	31.20±0.71

N: Number of animals

* denotes significance at p≤0.05 when compared to control

HFD1= weight of of high fat diet group mice at first day of feeding

HFD2= weight of high fat diet group after two weeks of feeding high fat diet

HFD3= weight of high fat diet group after five weeks of feeding high fat diet

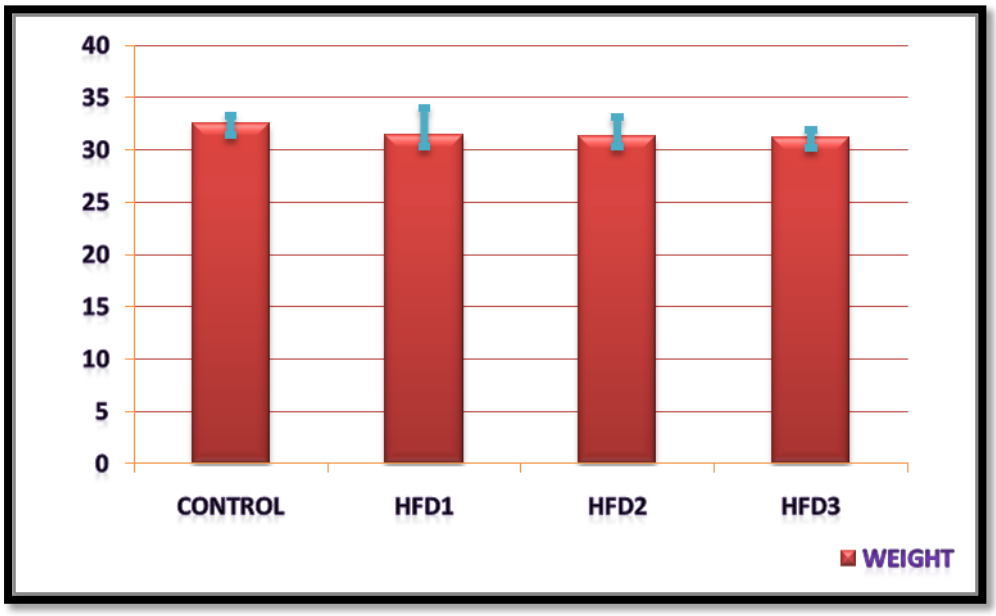


Fig .4.1.1: Histogram of Body weight in mice feeding on high fat diet .

Table. 4.1.2: Fasting serum glucose concentration in mice feeding on high fat diet (Mean ± S.D.)

Group / Parameter	CONTROL N =5	HFD1 N =5	HFD2 N =5	HFD3 N =5
FBS **(mg/dl)**	225±29	196±38	249±47	322±63*

N: Number of animals
* denotes significance at p≤0.05 when compared to control

HFD1= fasting serum glucose concentration of high fat diet group mice at first day of feeding

HFD2= fasting serum glucose concentration of high fat diet group after two weeks of feeding high fat diet

HFD3= fasting serum glucose concentration of high fat diet group after five weeks of feeding high fat diet

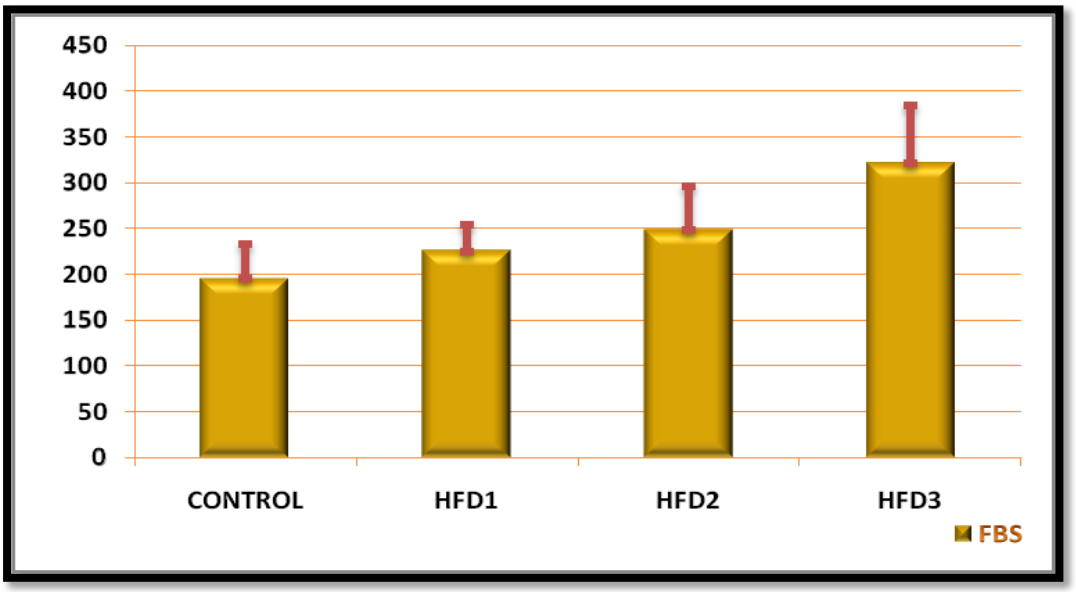


Fig .4.1 .2: Histogram of Serun glucose concentration in mice feeding on high fat diet .

Table. 4.1.3 :Adiponectin concentration in mice feeding on high fat diet (Mean ± S.D.)

Group / Parameter	Control 2WKS	Control 5WKS	HFD 2WKS	HFD 5WKS
Adiponectin μg/ml	2.67±**0.72**	2.60±**0.90**	3.60±**0.33***	3.50±**0.54**

N: Number of animals

***** denotes significance at p≤0.05 when compared to control.

Control 2wks=mean adiopnectin concentration after two weeks in control group.

Control 5wks= mean adiopnectin concentration after five weeks in control group.

HFD 2wks= mean adiopnectin concentration after two weeks in high fat diet group.

HFD 5wks = mean adiopnectin concentration after five weeks in high fat diet group.

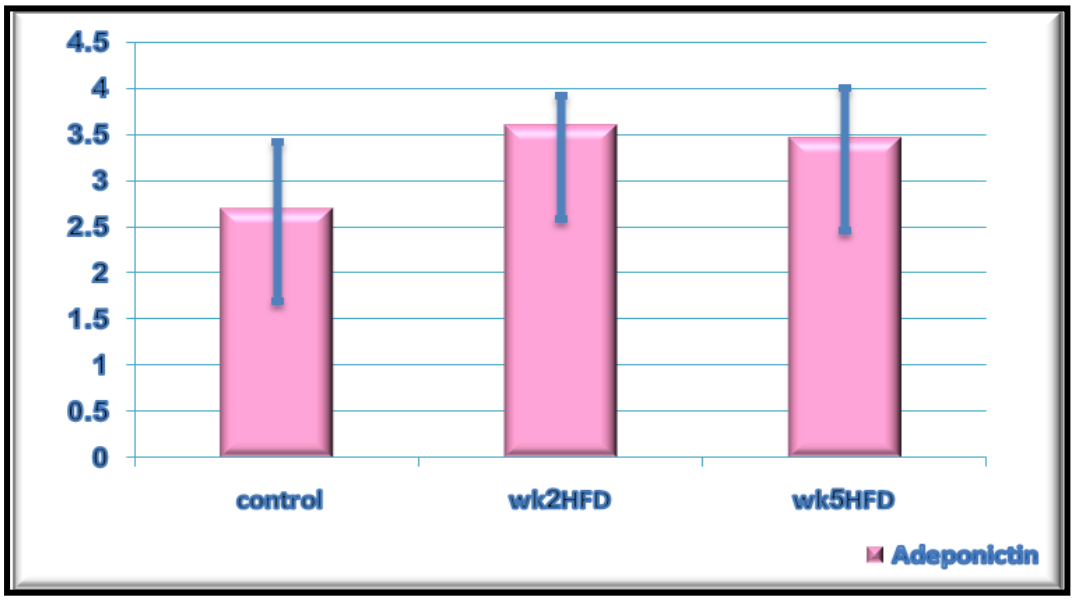


Fig .4.1.3 : Histogram of adiponectin concentration in mice feeding on high fat

Table.4.1. 4: Hepatic Glutamic-Pyruvic Transaminase (GPT) activity in mice feeding on high fat diet (Mean ± S.D).

Group / parameter	Control N =	HFD N =
GPT IU/mg protein	2.60±1.90	8.90±1.75*

N: Number of animals
* denotes significance at p≤0.05 when compared to control

GPT Glutamic-Pyruvic Transaminase

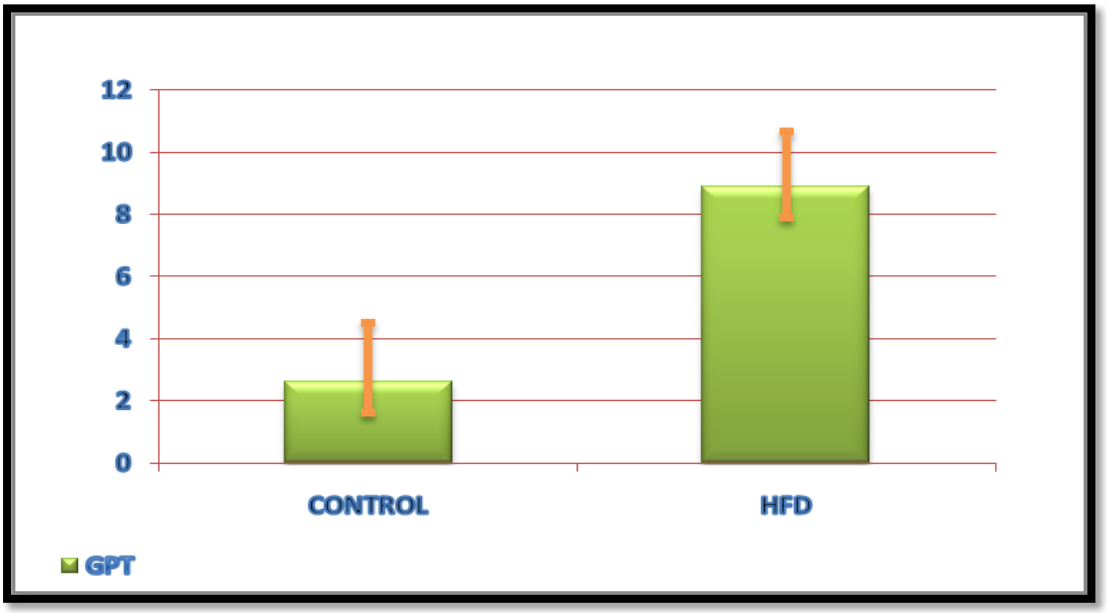


Fig.4.1. 4: Histogram of Hepatic GPT activity in mice feeding on high fat diet.

Table .4.1.5: Hepatic Glutamate-oxaloacetate transaminase (GOT) enzyme activity in mice feeding on high fat diet (Mean ± S.D).

Group / Parameter	Control N =5	HFD N =5
GOT(IU/mg protein)	4.7±1.3	7.8±2.5

N: Number of animals

* denotes significance at p≤0.05 when compared to control

GOT : Glutamate-oxaloacetate transaminase

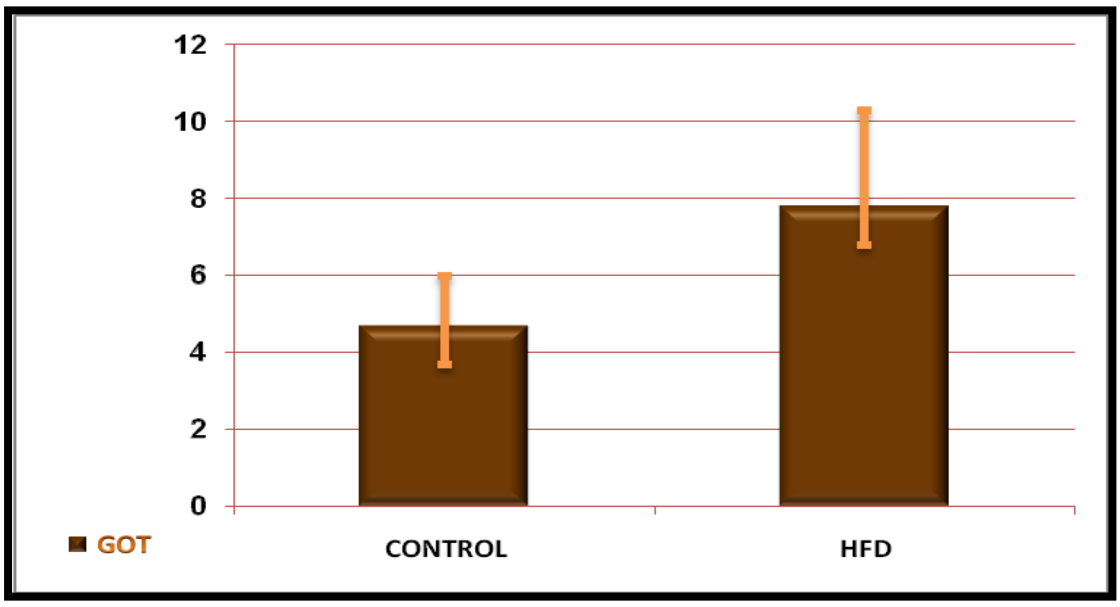


Fig .4.1 .5: Histogram of Hepatic GOT activity in mice feeding on high fat diet .

Table. 4.1.6: Hepatic alkaline phosphatase activity in mice feeding on high fat diet (Mean ± S.D.)

Group / Parameter	Control N=5	HFD N=5
ALKP (IU/mg protein)	3.90±**0.98**	1.65±**0.10**

N: Number of animals
* denotes significance at p≤0.05 when compared to control

ALKP: alkaline phosphatase

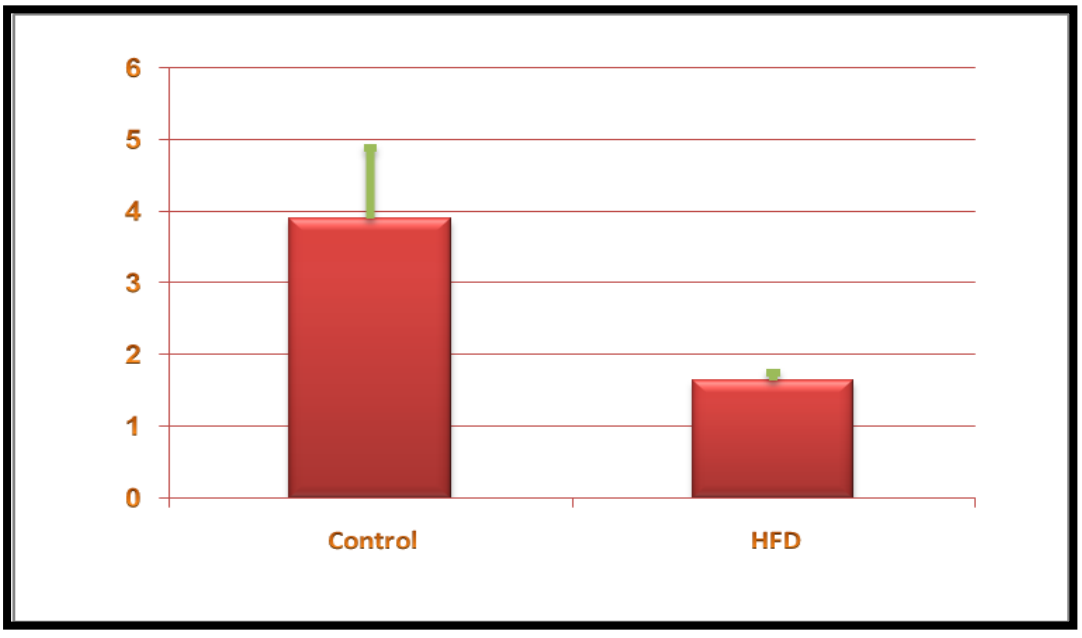


Fig .4.1.6: Histogram of Hepatic Liver alkaline phosphatase activity in mice feeding on high fat diet.

Table.4.1.7: Hepatic glucose 6-phosphate dehydrogenase activity in mice feeding on high fat diet (Mean ± S.D.).

Group / Parameter	Control N=5	HFD N=5
G6PDH (IU/mg protein)	519±31.7	285**±8.9***

N: Number of animals
* denotes significance at p≤0.05 when compared to control

G6PDH= glucose 6-phosphate dehydrogenase.

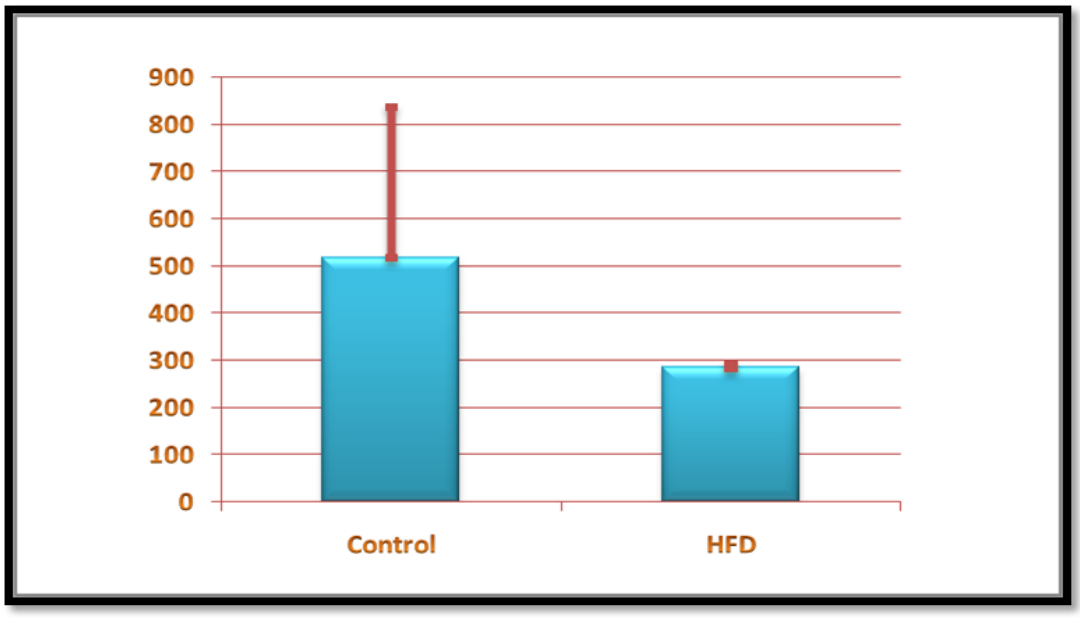


Fig .4.1.7.Histogram of Hepatic glucose 6-phosphate dehydrogenase in mice feeding on high fat.

Table.4.1. 8: reduced glutathione activity in mice feeding on high fat diet (Mean ± S.D.).

Group / Parameter	Control N=5	HFD N=5
GSH (mg/ mg protein)	24.9±**4.4**	21.0±**3.3**

N: Number of animals.
* denotes significance at p≤0.05 when compared to control.

GSH : reduced glutathione .

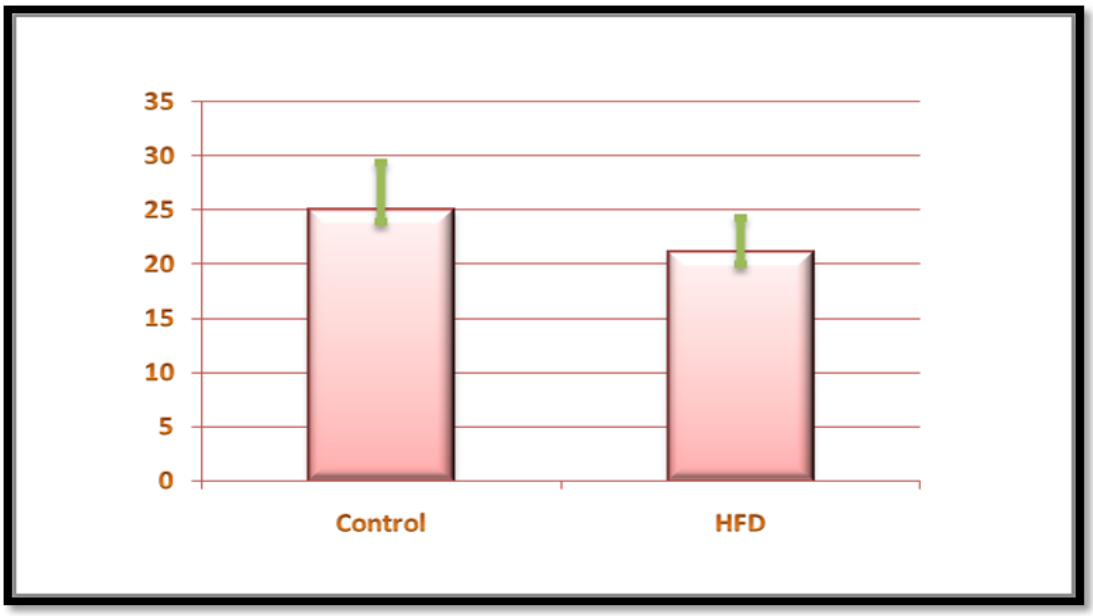


Fig .4.1.8:Histogram of Hepatic reduced glutathione activity in mice feeding on high fat.

Table. 4.1.9: lipid peroxidation activity in mice feeding on high fat diet (Mean ± S.D.).

Group / Parameter	Control N=5	HFD N=5
LPO (µM/g fresh tissue)	110±13.4	214±**8.3***

N: Number of animals
* denotes significance at p≤0.05 when compared to control

LPO : lipid peroxidation

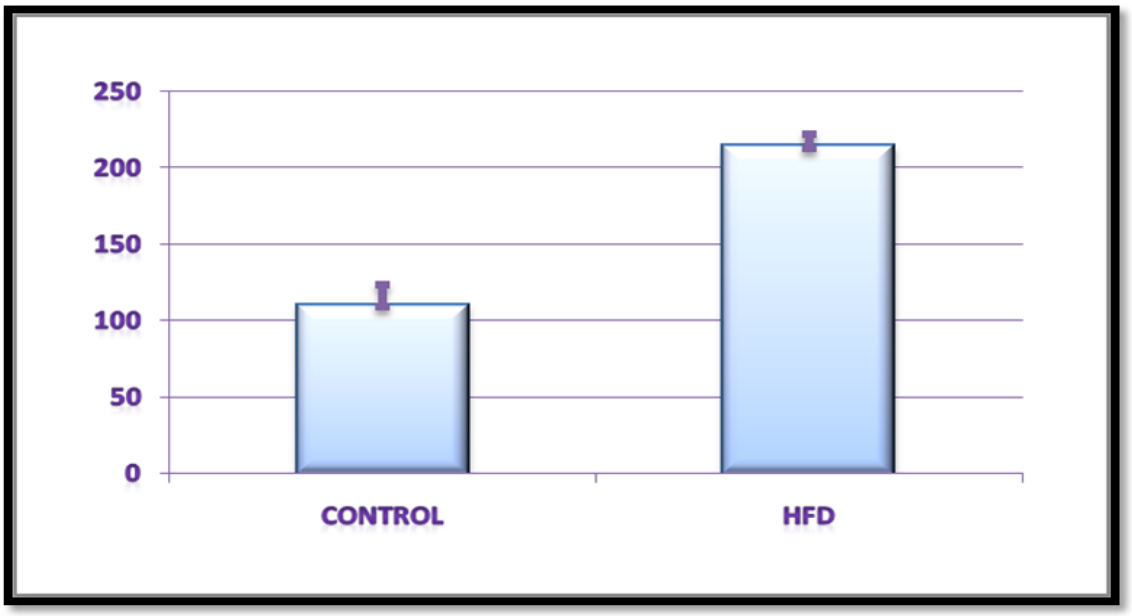


Fig .4.1.9:Histogram of Hepatic lipid peroxidation activity in mice feeding on high fat diet .

Table.4.1.10: Hepatic lactate dehydrogenase activity and phosphate content in mice feeding on high fat diet (Mean ± S.D.).

Group / Parameter	Control N=5	HFD N=5
LDH (IU/mg protein)	1087 ±472	824 ±**30**
PO4 (mg/g fresh tissue)	24.8 ±**13.6**	22 ±**7.5**

N: Number of animals

* denotes significance at p≤0.05 when compared to control

LDH : lactate dehydrogenase.

PO4: Inorganic phosphate.

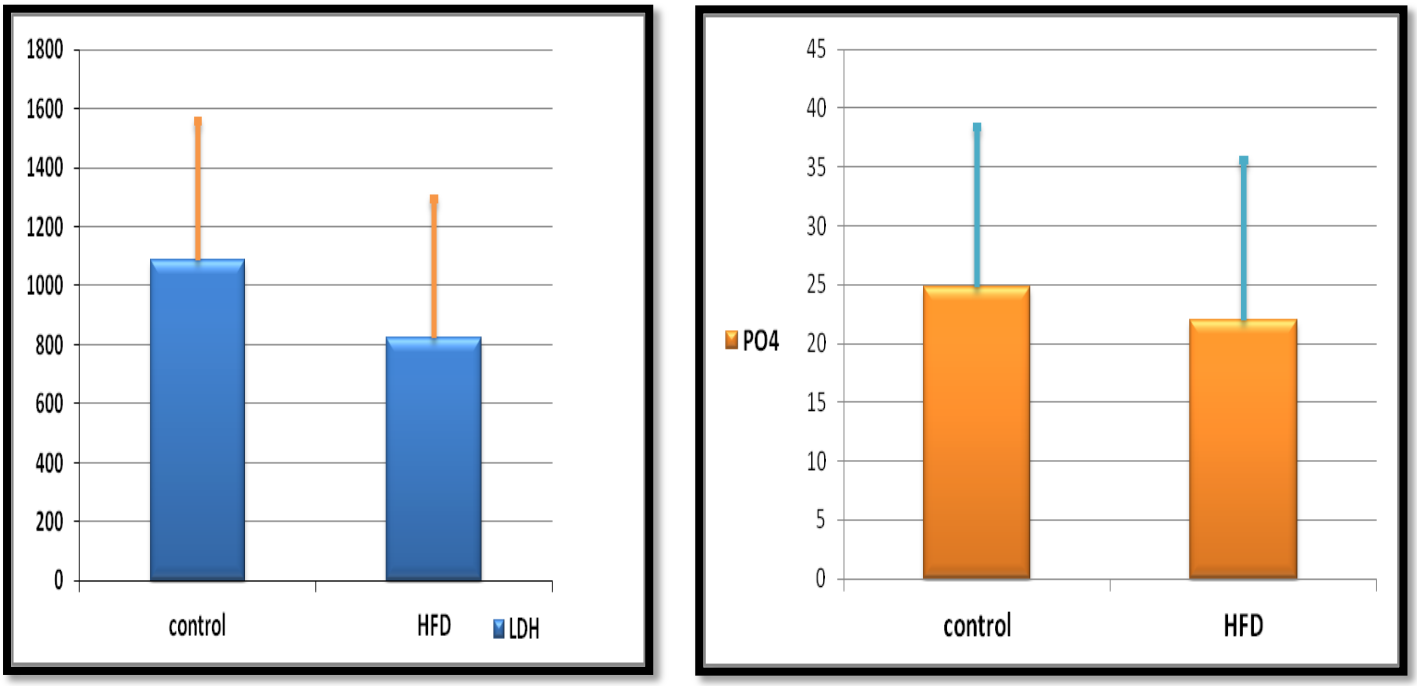


Fig .4.1.10: Histogram of Hepatic Hepatic lactate dehydrogenase activity and phosphate content in mice feeding on high fat diet.

Table. 4.1.11: Body weight in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.).

Group / Parameter	HFD1	HFDC1	HFD2	HFDC2	HFD3	HFDC3
weight (gm)	31.37±2.7	32.34± 2.6	31.28± 1.89	32.65± 1.52	31.22± 0.71	31.78± 2.61

N: Number of animals

* denotes significance at $p \leq 0.05$ when compared to high fat diet

HFD1= weight of of high fat diet group mice at first day of feeding

HFD2= weight of high fat diet group after two weeks of feeding high fat diet

HFD3= weight of high fat diet group after five weeks of feeding high fat diet

HFDC1= weight of of high fat diet and cold stress group mice at first day of feeding

HFDC= weight of high fat diet cold stress group after two weeks of feeding high fat diet

HFDC3= weight of high fat diet cold stress group after five weeks of feeding high fat diet

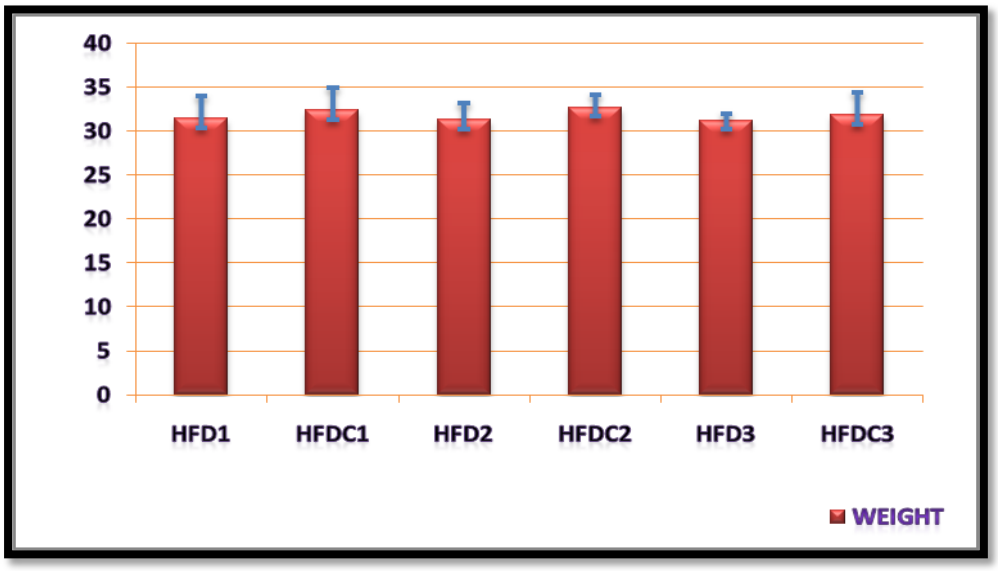


Fig .4.1.11:Histogram of Body weight in mice feeding on high fat diet and on cold stress.

Table. 4.1.12: Fasting serum glucose concentration in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.).

Group / Parameter	HFD1	HFDC1	HFD2	HFDC2	HFD3	HFDC3
FBS **(mg/dl)**	196±38	206±18	249±**47**	205±51	322±**63**	232±27*

N: Number of animals

* denotes significance at p≤0.05 when compared to high fat diet group

HFD1= fasting serum glucose of of high fat diet group mice at first day of feeding

HFD2= fasting serum glucose of high fat diet group after two weeks of feeding high fat diet

HFD3= fasting serum glucose of high fat diet group after five weeks of feeding high fat diet

HFDC1= fasting serum glucose of of high fat diet and cold stress group mice at first day of feeding

HFDC2= fasting serum glucose of high fat diet cold stress group after two weeks of feeding high fat diet

HFDC3= fasting serum glucose of high fat diet cold stress group after five weeks of feeding high fat diet

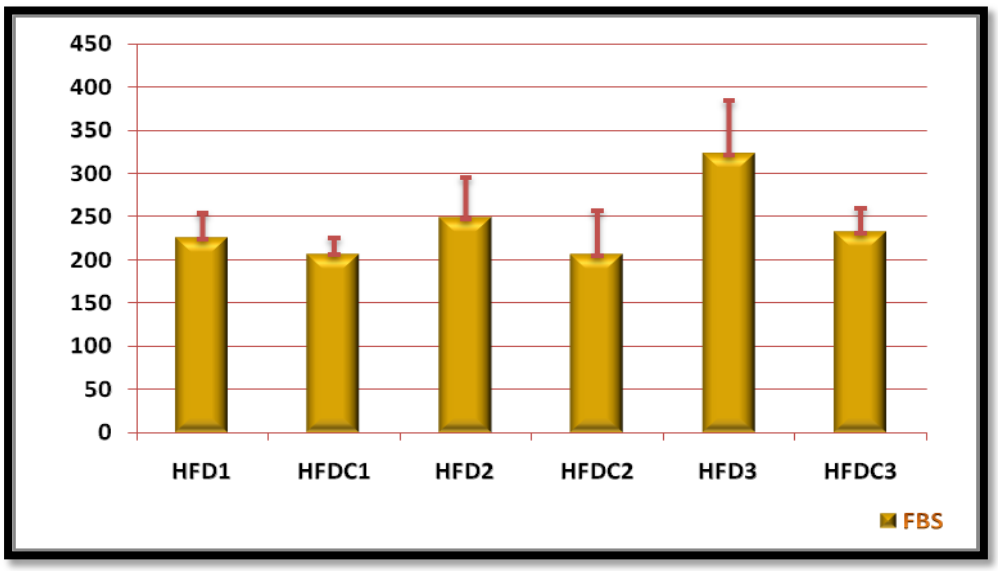


Fig .4.1.12: Histogram of glucose concentration in mice feeding on high fat diet and on cold stress.

Table. 4.1.13: Adiponectin concentration in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.).

Group / Parameter	HFD 2wk	HFDC 5wk	HFD 2wk	HFDC 5wk
Adiponectin (μg/ml)	3.59±0.33	2.86±0.30*	3.45±0.55	3.11±0.21

N: Number of animals
* denotes significance at p≤0.05 when compared to high fat diet group.

HFD2wks=mean adiopnectin concentration after two weeks in high fat diet group.

HFD 5wks= mean adiopnectin concentration after five weeks in high fat diet group.

HFDC2wks= mean adiopnectin concentration after two weeks in high fat diet and cold group.

HFDC 5wks= mean adiopnectin concentration after five weeks in high fat diet and cold group.

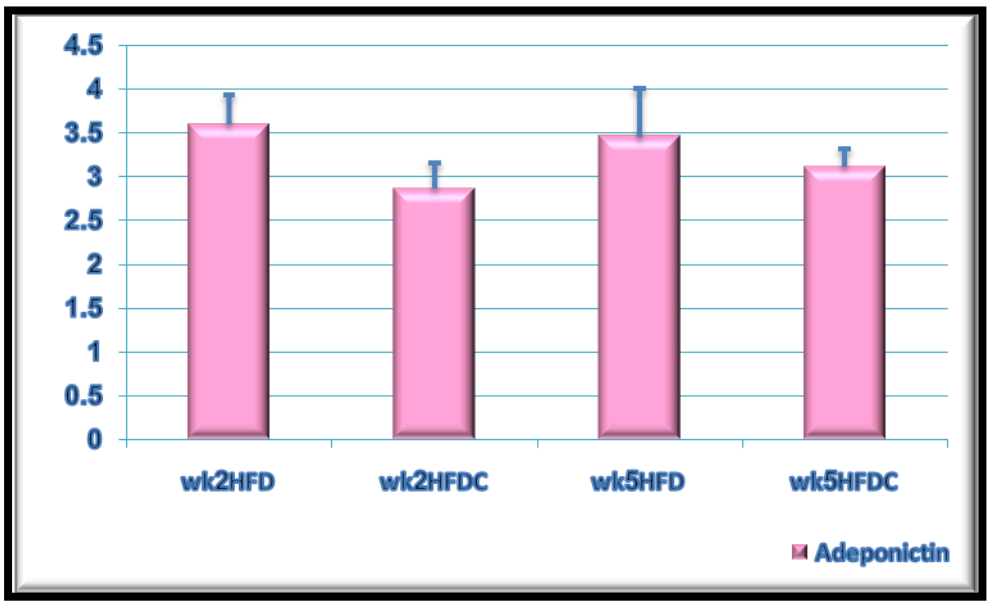


Fig .4.1.13: Histogram of adiponectin concentration in mice feeding on high fat and on cold stress.

Table. 4.1.14: Hepatic Glutamic-Pyruvic Transaminase (GPT)ctivity in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.).

Parameter \ Group	HFD N=5	HFDC N=5
GPT(IU/mg protein)	8.9±1.75	4.3±**0.727***

N: Number of animals

* denotes significance at p≤0.05 when compared to high fat diet group

GPT: Glutamic-Pyruvic Transaminase

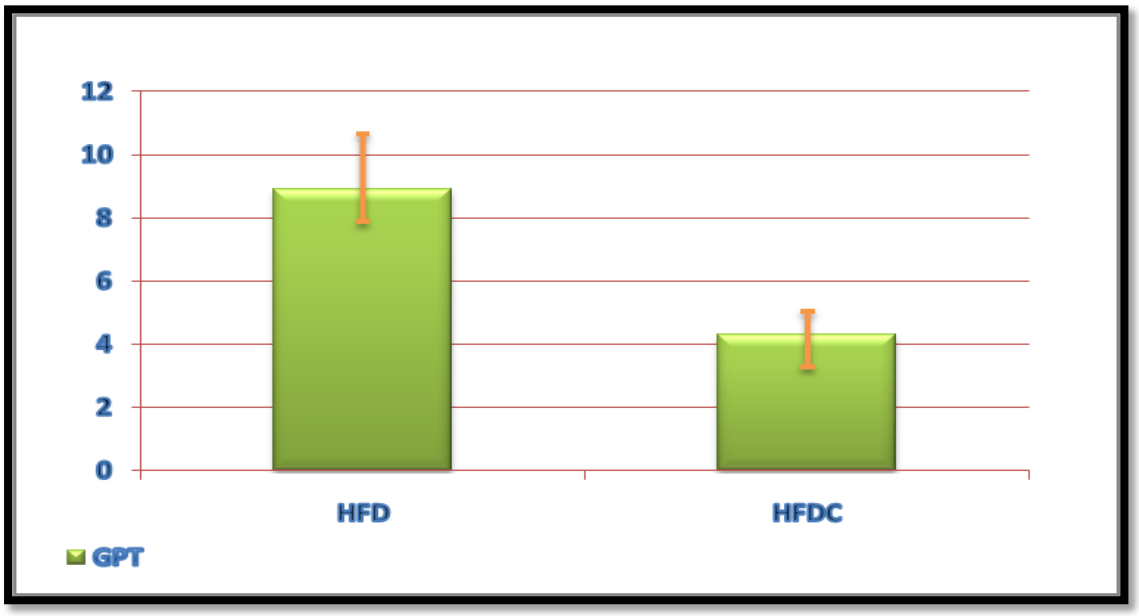


Fig .4.1.14: Histogram of Hepatic GPT activity in mice feeding on high fat and on cold stress .

Table.4.1.1 5 : Hepatic Glutamate-oxaloacetate transaminase (GOT) enzyme activity in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.).

Group / Parameter	HFD N=5	HFDC N=5
GOT (IU/mg protein)	7.8±2.5	1.6±0.9*

N: Number of animals.
* denotes significance at p≤0.05 when compared to high fat diet group.

GOT: Glutamate-oxaloacetate transaminase.

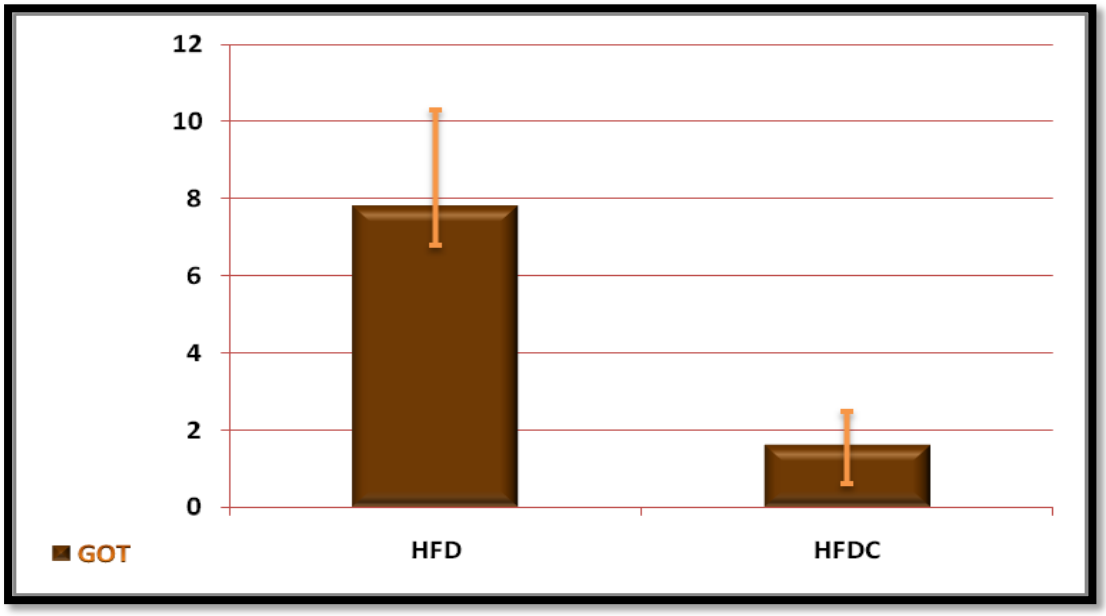


Fig 4.1.15:Histogram of Hepatic GOT activity in mice feeding on high fat diet and on cold stress.

Table. 4.1.16: Hepatic alkaline phosphatase activity in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.)

Group / Parameter	HFD N=5	HFDC N=5
ALKP(IU/mg protein)	1.65±**0.10**	6.95±**1.47***

N: Number of animals

* denotes significance at p≤0.05 when compared to high fat diet group

ALKP: alkaline phosphatase

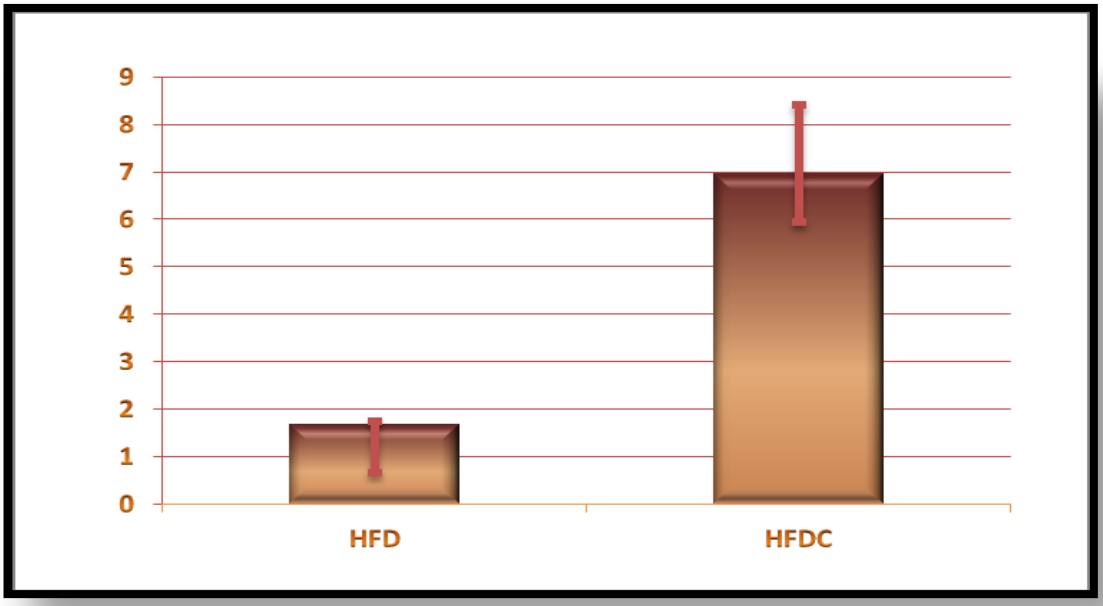


Fig .4.1.16: Histogram of Hepatic Liver alkaline phosph - atase activity in mice feeding on high fat and on cold stress.

Table.4.1.17: Hepatic glucose 6-phosphate dehydrogenase activity in mice feeding on high fat diet and on cold stress (HFDC) (Mean ± S.D.).

Group / Parameter	HFD N=5	HFDC N=5
G6PDH(IU/mg protein)	285±**10**	591±**6***

N: Number of animals .
* denotes significance at p≤0.05 when compared to high fat diet group.

G6PDH: glucose 6-phosphate dehydrogenase.

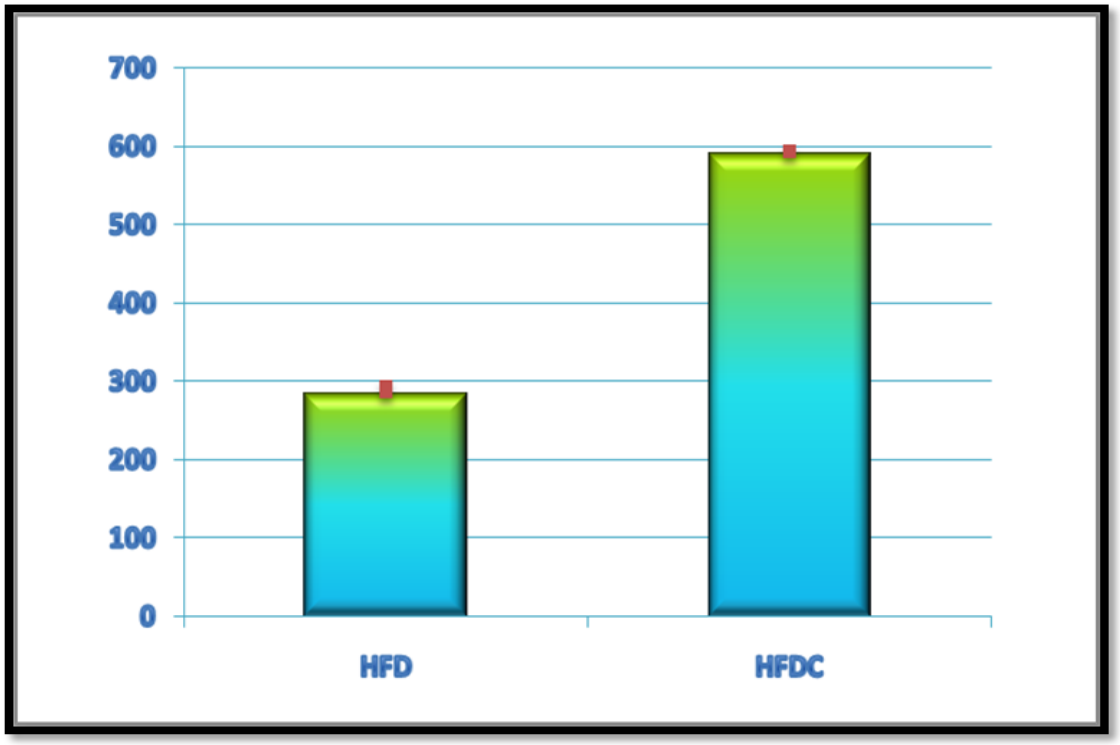


Fig .4.1.17: Histogram of Hepatic glucose 6-phosphate dehydrogenase in mice feeding on high fat and on cold stress.

Table.4.1. 18: reduced glutathione activity in mice feeding on high fat diet and on cold stress (Mean ± S.D.).

Group / Parameter	HFD N=5	HFDC N=5
GSH (mg/ mg protein)	21±3.3	29±12*

N: Number of animals

* denotes significance at p≤0.05 when compared to high fat diet group

GSH : reduced glutathione

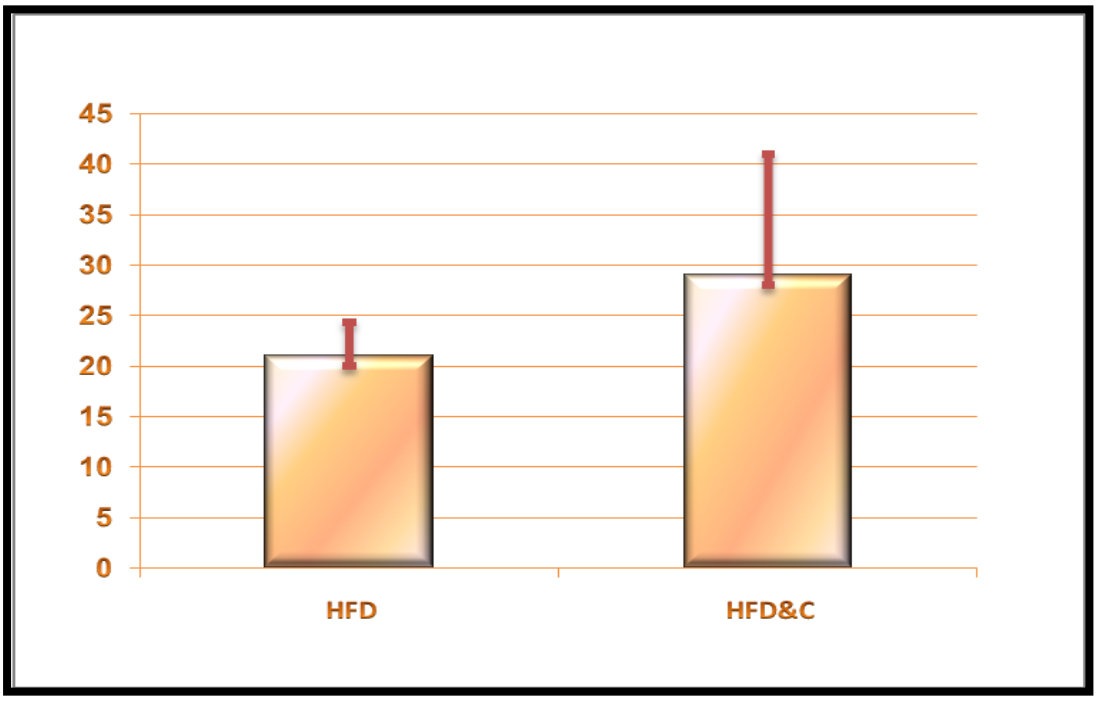


Fig .4.1.18: Histogram of Hepatic reduced glutathione activity in mice feeding on high fat and on cold stress .

Table.4.1. 19: lipid peroxidation activity in mice feeding on high fat diet and on cold stress (Mean ± S.D.).

Group / parameter	HFD N=5	HFDC N=5
LPO (µM/g fresh tissue)	214±8.3	90±5.2*

N: Number of animals.
* denotes significance at p≤0.05 when compared to high fat diet group.

LPO : lipid peroxidation.

Fig .4.1.19:Histogram of Hepatic lipid peroxidation activity in mice feeding on high fat and on cold stress.

Table.4.1.20: Hepatic lactate dehydrogenase activity and phosphate content in mice feeding on high fat diet and on cold stress (HFD&C) (Mean ± S.D.).

Group / Parameter	HFD N=5	HFD&C N=5
LDH (IU/mg protein)	824±30	1793±19*
PO4 (mg/g fresh tissue)	22±8	38±19

N: Number of animals
* denotes significance at p≤0.05 when compared to high fat diet group

LDH : lactate dehydrogenase.

PO4 : Inorganic phosphate.

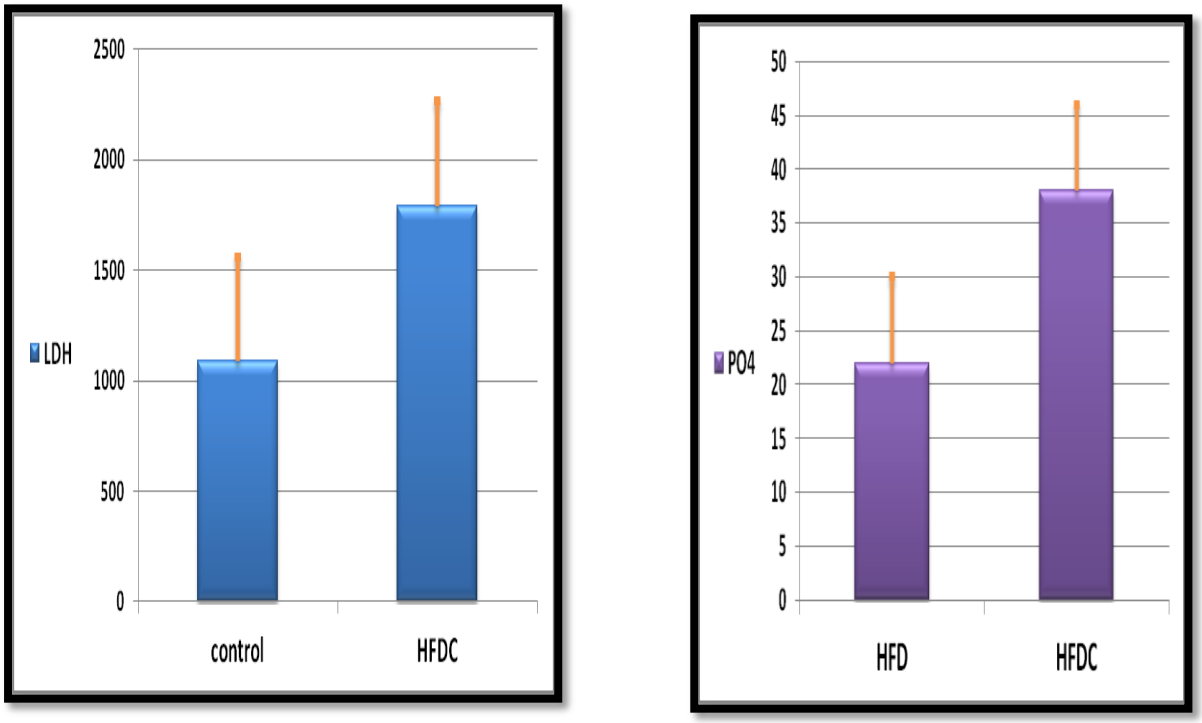


Fig .4.1.20: Histogram of Hepatic Hepatic lactate dehydroge -nase activity

Table .4.1.21: Body weight in mice feeding on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / Parameter	Control N=5	NDC1 N=5	NDC2 N=5	NDC3 N=5
Weight (gm)	32.5± 0.9	31.9± 2.4	24.60±1.97*	24.55±**3.63***

N: Number of animals

* denotes significance at p≤0.05 when compared to control

NDC1= weight of normal diet and cold stress group at first day of feeding.

NDC2= weight of normal diet and cold stress group after two weeks of feeding.

NDC3= weight of normal diet and cold stress group after five weeks of feeding.

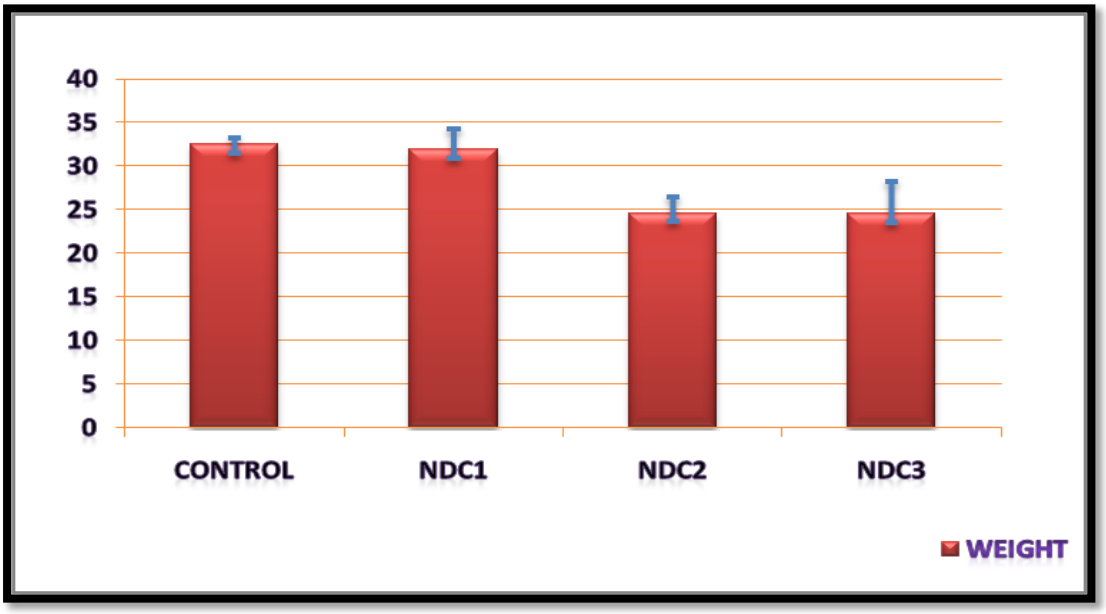


Fig :4.1.21.Histogram of Body weight in mice feeding on normal diet and mice feeding normal diet and on cold stress.

Table.4.1.22: Fasting serum glucose concentration in mice feeding on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / Parameter	Control N=5	NDC1 N=5	NDC2 N=5	NDC3 N=5
FBS**(mg/dl)**	196±38	171±**33**	209±55	211±20

N: Number of animals
* denotes significance at p≤0.05 when compared to control.

FBS: Fasting serum glucose concentration.

NDC1= FBS of normal diet and cold stress group at first day of feeding.

NDC2= FBS of normal diet and cold stress group after two weeks of feeding.

NDC3= FBS of normal diet and cold stress group after five weeks of feeding.

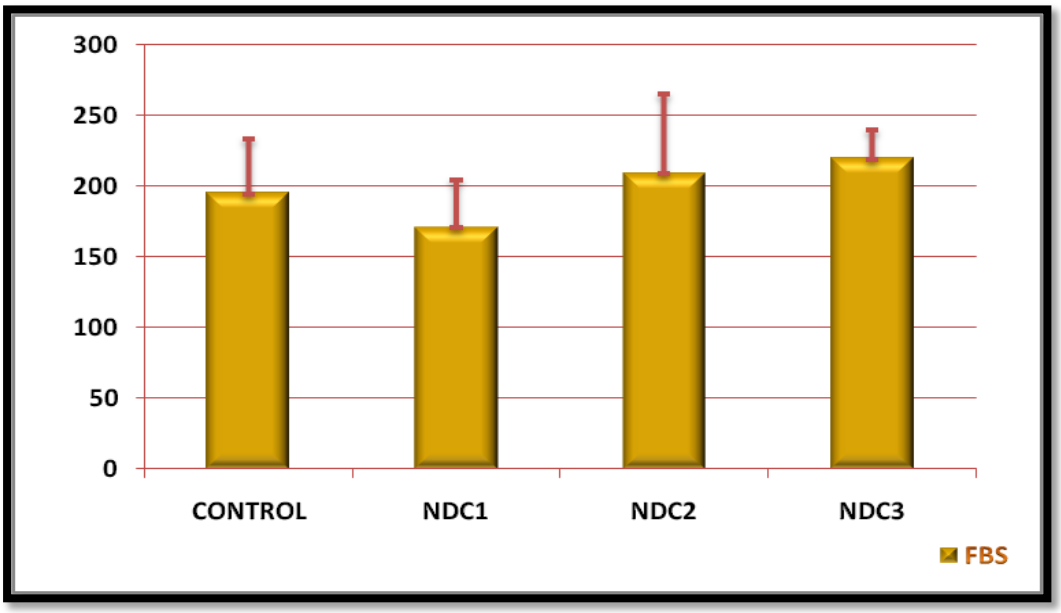


Fig .4.1.22: Histogram of glucose concentration in mice feeding on normal diet and on cold stress .

Table. 4.1.23: Adiponectin concentration in mice feeding on normal diet and on cold stress (Mean ± S.D.).

Group / Parameter	CONTROL 2WKS N=5	CONTROL 5WKS N=5	NDC 2WKS N=5	NDC 5WKS N=5
Adiponectin (μg/ml)	2.70±0.7	2.60±0.9	3.04±0.15	3.12±0.28

N: Number of animals
* denotes significance at p≤0.05 when compared to control

Control 2wks=mean adiopnectin concentration after two weeks in control group

Control 5wks= mean adiopnectin concentration after five weeks in control group

NDC 2wks= mean adiopnectin concentration after two weeks in chow diet and cold stress group.

NDC 5wks= mean adiopnectin concentration after five weeks in chow diet and cold stress group.

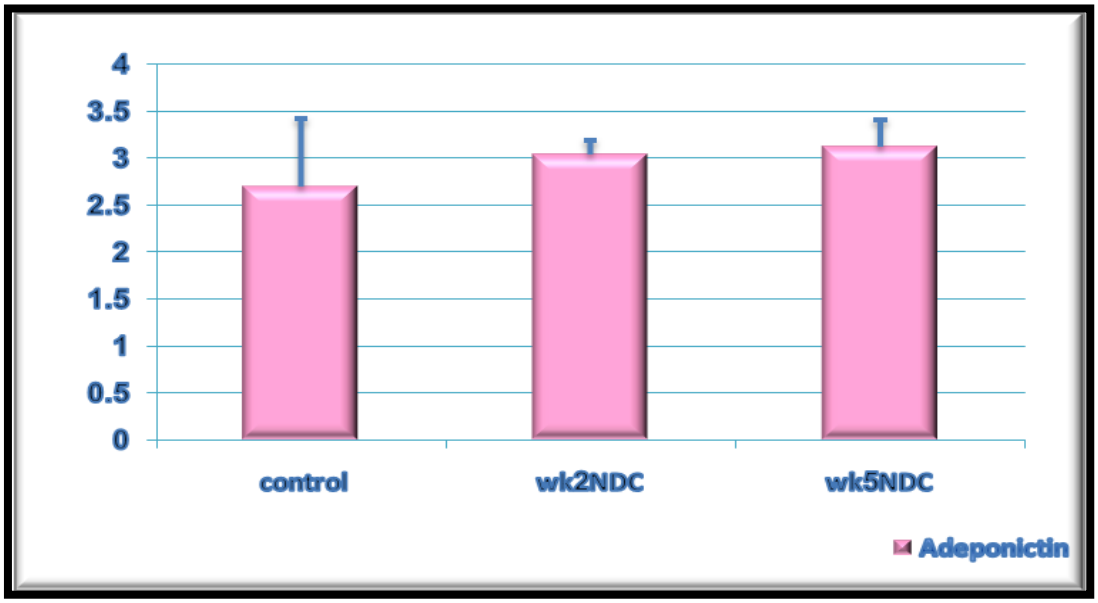


Fig .4.1.23:Histogram of adiponectin concentration in mice feeding on normal diet and on cold stress.

Table.4.1.24: Hepatic Glutamic-Pyruvic Transaminase (GPT)ctivity in mice feeding on on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
GPT (IU/mg protein)	2.6±1.9	7.95±1.7*

N: Number of animals
* denotes significance at p≤0.05 when compared to control

GPT : Glutamic-Pyruvic Transaminase

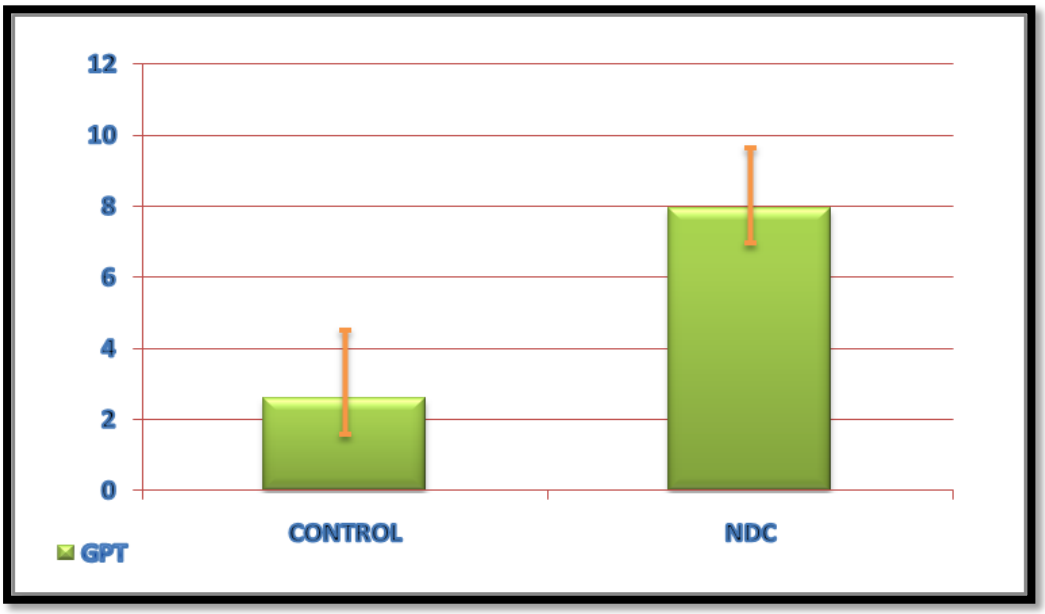


Fig .4.1.24: Histogram of Hepatic GPT activity in mice feeding on on normal diet and mice feeding normal diet and on cold stress.

Table. 4.1.25: Hepatic Glutamate-oxaloacetate transaminase (GOT) enzyme activity in mice feeding on on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
GOT (IU/mg protein)	4.7±1.3	8.7±2.0*

N: Number of animals

* denotes significance at p≤0.05 when compared to control

GOT : Glutamate-oxaloacetate transaminase

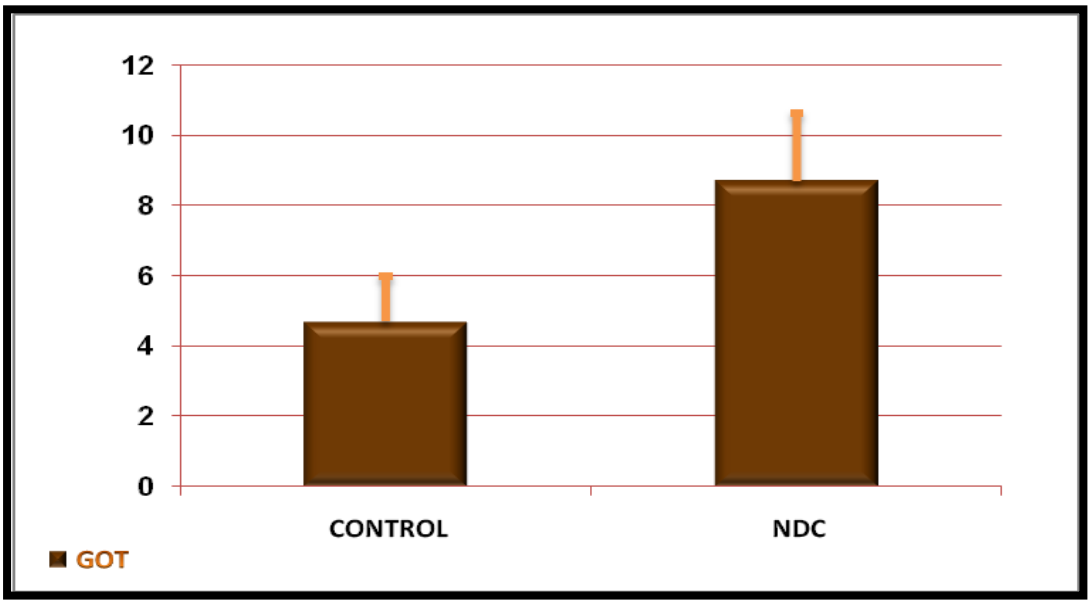


Fig .4.1.25: Histogram of Hepatic GOT activity in mice feeding on on normal diet and mice feeding normal diet and on cold stress .

Table. 4.1.26: Hepatic alkaline phosphatase activity in mice feeding on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
ALKP(IU/mg protein)	3.90±**0.98**	2.50±**0.70**

N: Number of animals
* denotes significance at p≤0.05 when compared to control

ALKP: alkaline phosphatase

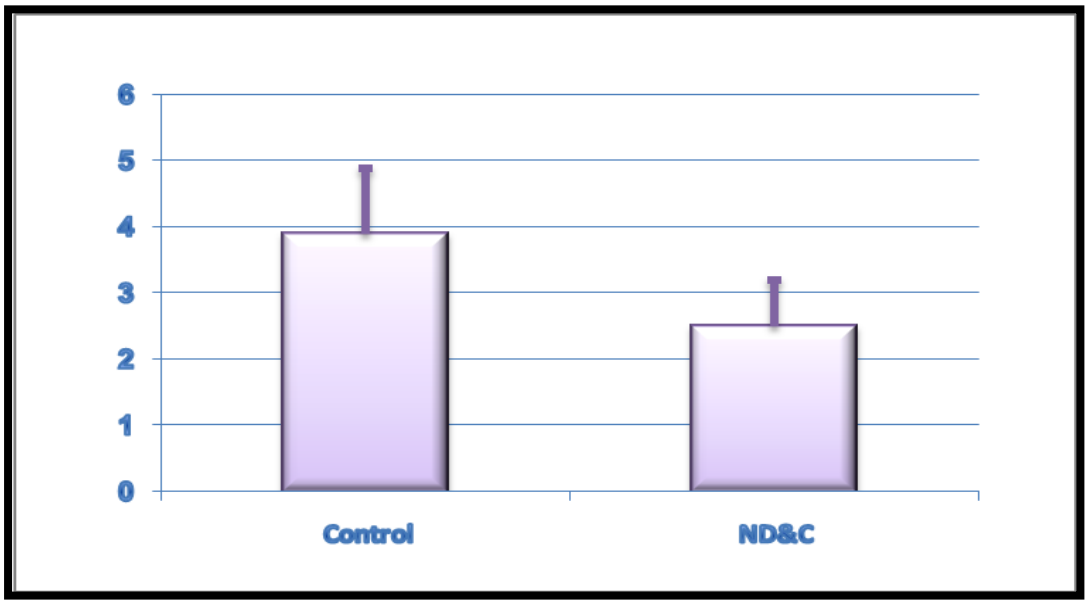


Fig .4.1.26: Histogram of Hepatic Liver alkaline phosphatase activity in mice feeding on normal diet and mice feeding normal diet and on cold stress

Table. 4.1.27: Hepatic glucose 6-phosphate dehydrogenase activity in mice feeding on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
G6PDH(IU/mg protein)	519 ±31.7	337±6.3*

N: Number of animals
* denotes significance at p≤0.05 when compared to control

G6PDH : glucose 6-phosphate dehydrogenase

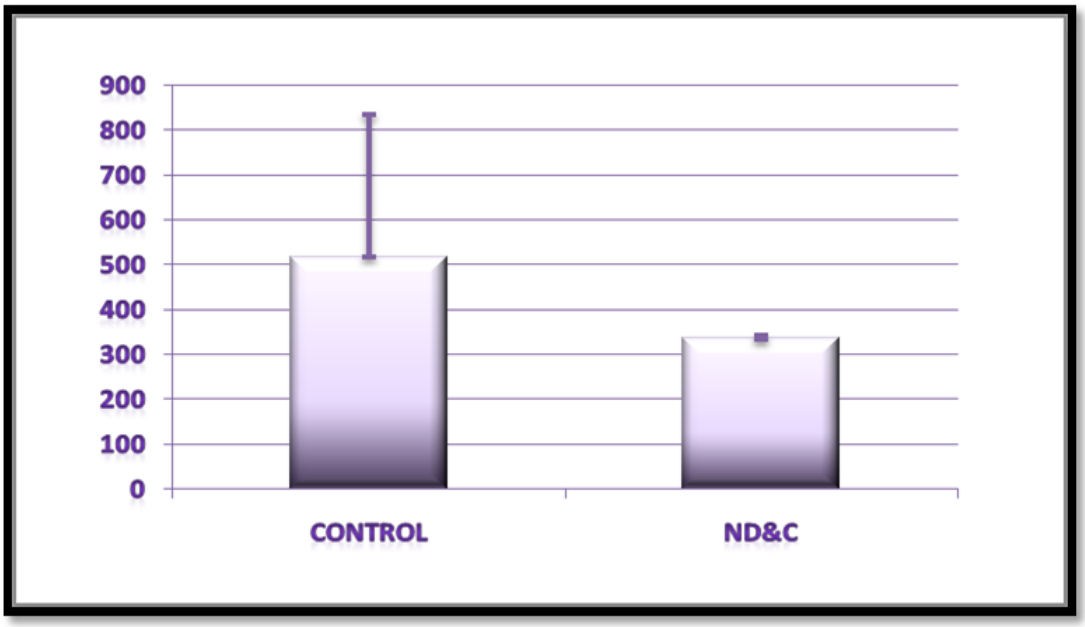


Fig .4.1.27: Histogram of Hepatic glucose 6-phosphate dehydrogenase in mice feeding on normal diet and mice feeding normal diet and on cold stress.

Table.4.1. 28: reduced glutathione activity in mice feeding on (M normal diet and mice feeding normal diet and on cold stress mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
GSH (mg/ mg protein)	24.9±**4.4**	69±**4.0***

N: Number of animals

* denotes significance at p≤0.05 when compared to control

GSH : reduced glutathione

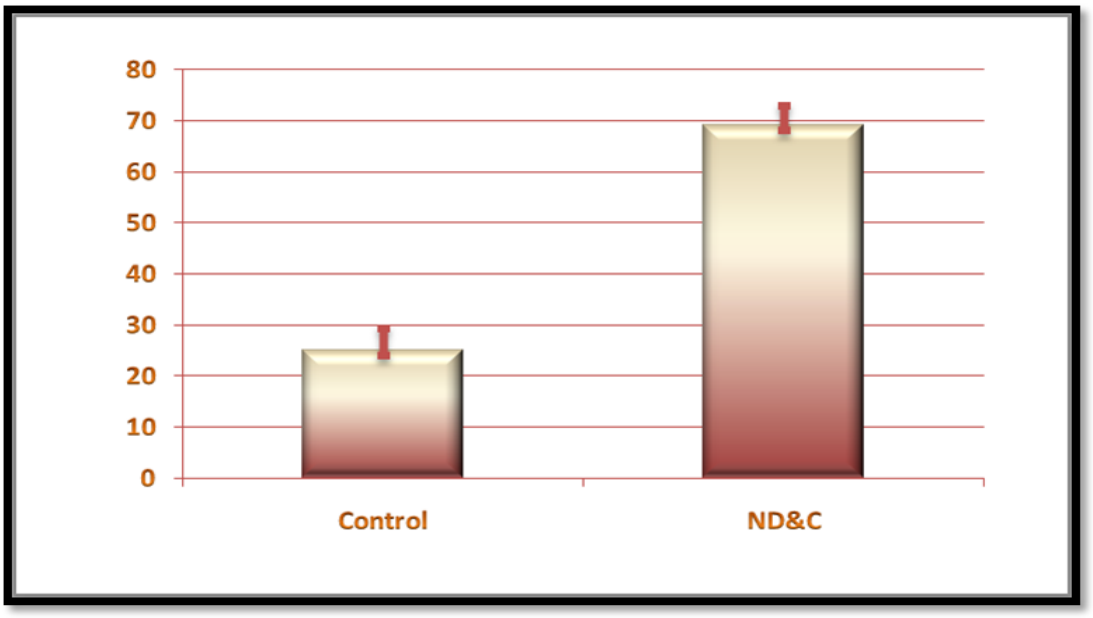


Fig .4.1.28: Histogram of Hepatic reduced glutathione activity in mice feeding on normal diet and mice feeding normal diet and on cold stress.

Table. 4.1.29: lipid peroxidation activity in mice feeding on normal diet and mice feeding normal diet and on cold stress (Mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
LPO (µM/g fresh tissue)	110±13.4	236±**2.5***

N: Number of animals .
* denotes significance at p≤0.05 when compared to control.

LPO : lipid peroxidation.

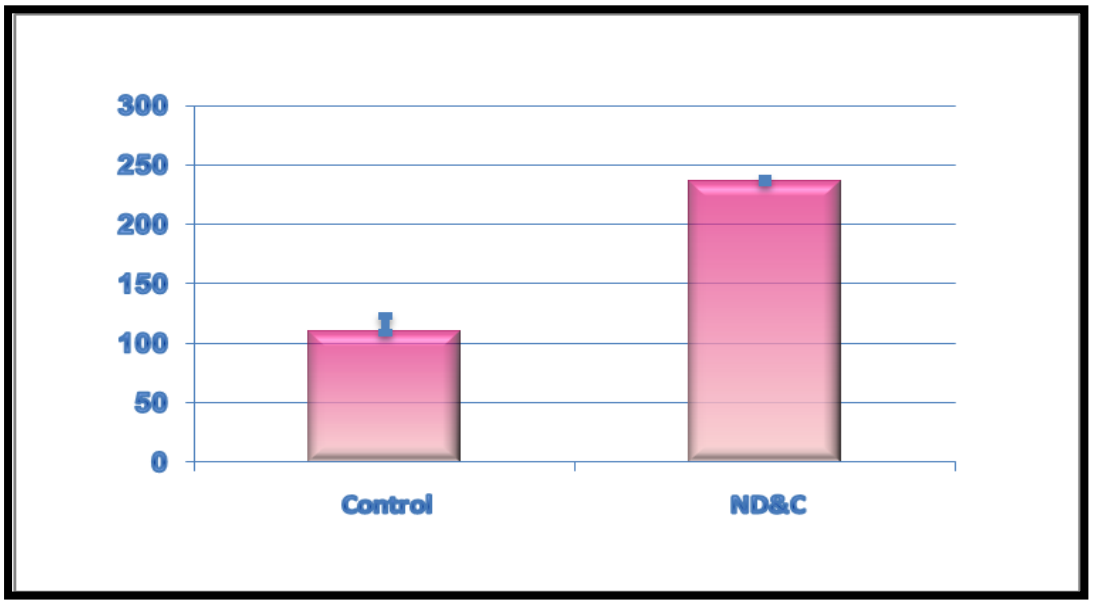


Fig .4.1.29: Histogram of Hepatic lipid peroxidation activity in mice feeding on normal diet and on cold stress.

Table.4.1. 30: Hepatic lactate dehydrogenase activity and phosphate content in mice feeding on (normal diet and mice feeding normal diet and on cold stress Mean ± S.D.).

Group / parameter	Control N=5	NDC N=5
LDH (IU/mg protein)	1087 ±472	752.9±**21**
PO4 (mg/g fresh tissue)	24.8 ±**13.6**	29.42±**4.6**

N: Number of animals
* denotes significance at p≤0.05 when compared to control

LDH : lactate dehydrogenase

PO4 : phosphate

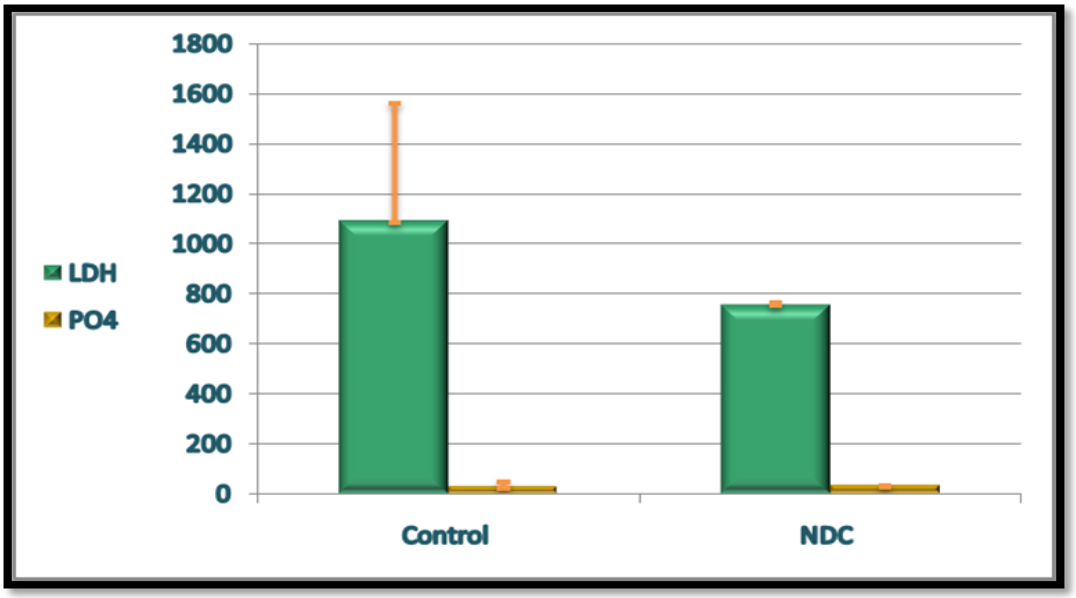


Fig .4.1.30: Histogram of Hepatic Hepatic lactate dehydrogen -ase activity and phosphate content in mice feeding on normal diet and mice feeding normal diet and on

Table . 4.1.31: Body weight in mice feeding on normal diet and mice feeding normal diet and on dark stress (NDD) (Mean ± S.D).

group / Parameter	Control	NDD1	NDD2	NDD3
Weight (gm)	25.82±1.83*	24.65±1.29*	32.10±2.43	32.47±0.79

N: Number of animals

* denotes significance at p≤0.05 when compared to control

NDD1= weight of of dark group mice at first day of feeding.

NDD2= weight of dark group after two weeks of feeding.

NDD3= weight ofdark group after five weeks of feeding.

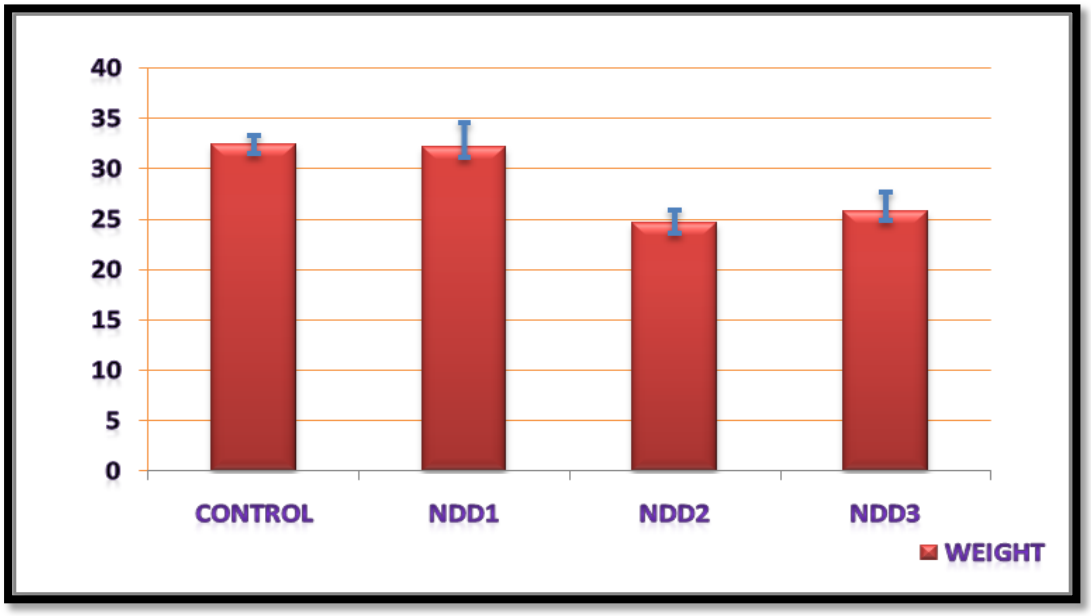


Fig .4.1.31: Histogram of Body weight in mice feeding on normal diet and mice feeding normal diet and on dark. stress

Table.4.1.32: Fasting serum glucose concentration in mice feeding on normal diet and mice feeding normal diet and on dark stress(NDD) (Mean ± S.D.)

Group / Parameter	Control	NDD1	NDD2	NDD3
FBS	170±37.96	170±41.68	203±55.47	302±68.70*

N: Number of animals
* denotes significance at p≤0.05 when compared to control.

NDD1= fasting serum glucose concentration of dark group mice at first day of feeding

NDD2= fasting serum glucose concentration of dark group after two weeks of feeding

NDD3= fasting serum glucose concentration of dark group after five weeks of feeding

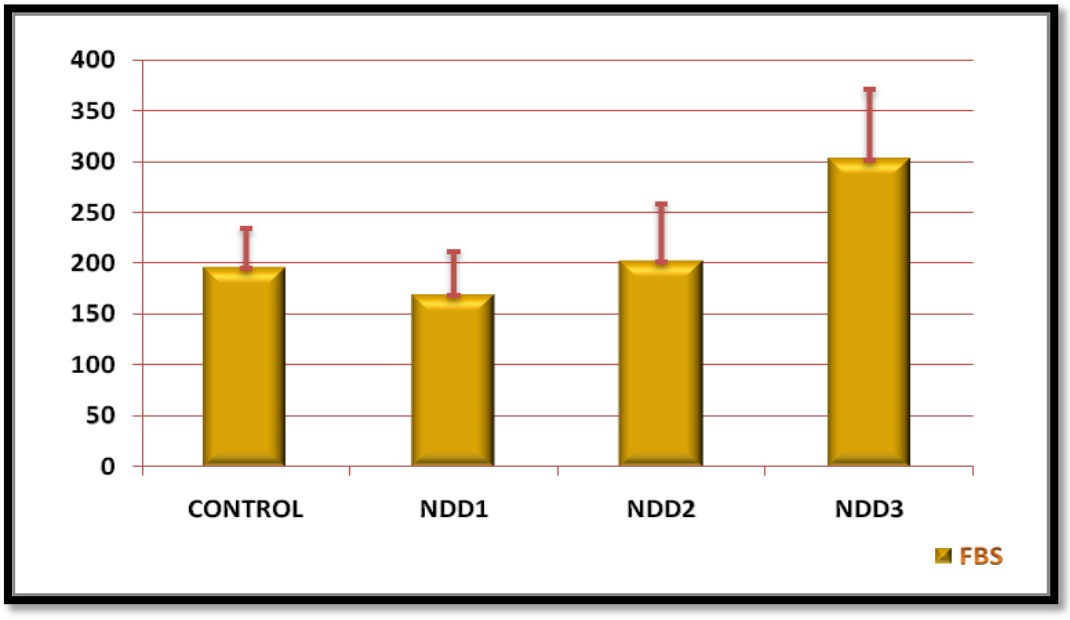


Fig .4.1.32 : Histogram of glucose concentration in mice feeding on normal diet and mice feeding normal diet and on dark stress .

Table. 4.1.33: Adiponectin concentration in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.)

Group / Parameter	CONTROL 2WKS	CONTROL 5WKS	NDD 2WKS	NDD 5WKS
Adiponectin (μg/ml)	2.70±0.72	2.60±0.90	2.76±0.36	2.98±0.31

N: Number of animals

* denotes significance at p≤0.05 when compared to control

Control 2wks=mean adiopnectin concentration after two weeks in control group

Control 5wks= mean adiopnectin concentration after five weeks in control group

NDD 2wks= mean adiopnectin concentration after two weeks in chow diet and dark stress group.

NDD 5wks= mean adiopnectin concentration after five weeks in chow diet and dark stress group.

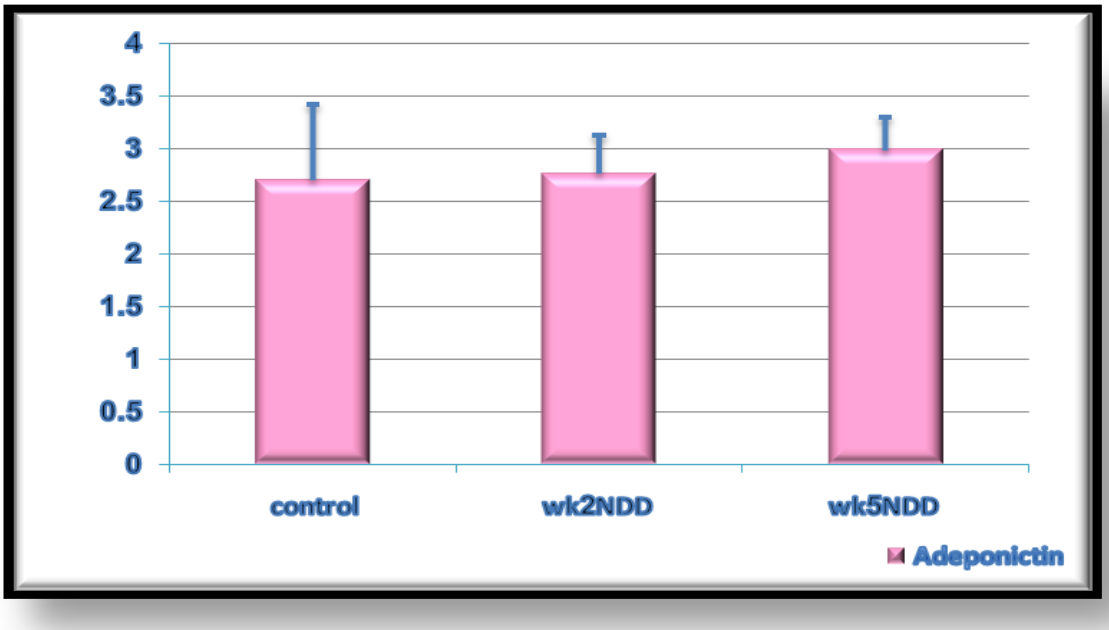


Fig .4.1.33:Histogram of adiponectin concentration in mice feeding on normal diet and mice feeding normal diet and on

Table. 4.1.34: Hepatic Glutamic-Pyruvic Transaminase (GPT) activity in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.).

Group / parameter	Control	NDD
GPT IU/mg protein	2.6±1.9	8.4±1.9*

N: Number of animals.
* denotes significance at p≤0.05 when compared to control.

GPT= Glutamic-Pyruvic Transaminase

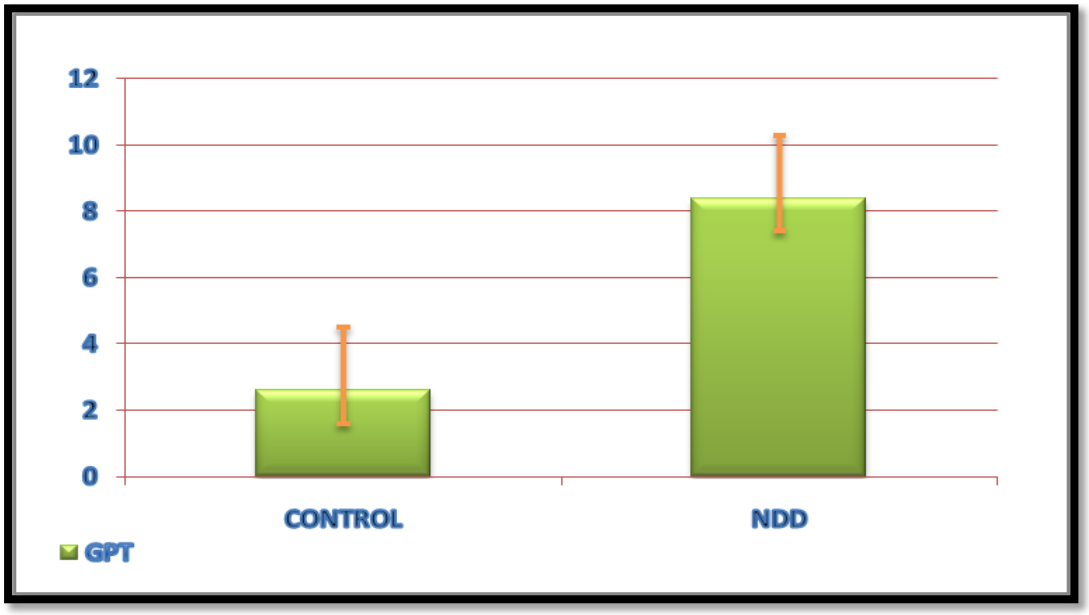


Fig .4.1.34: Histogram of Hepatic GPT activity in mice feeding on normal diet and mice feeding normal diet. and on dark stress

Table . 4.1.35 : Hepatic Glutamate-oxaloacetate transaminase (GOT) enzyme activity in mice feeding on normal diet and mice feeding normal diet and on dark stress(NDD) (Mean ± S.D.)

Group / Parameter	CONTROL	NDD
GOT IU/mgprotein	4.7±**1.3**	5.22±1.28

N: Number of animals.
* denotes significance at p≤0.05 when compared to control.

GOT : Glutamate-oxaloacetate transaminase .

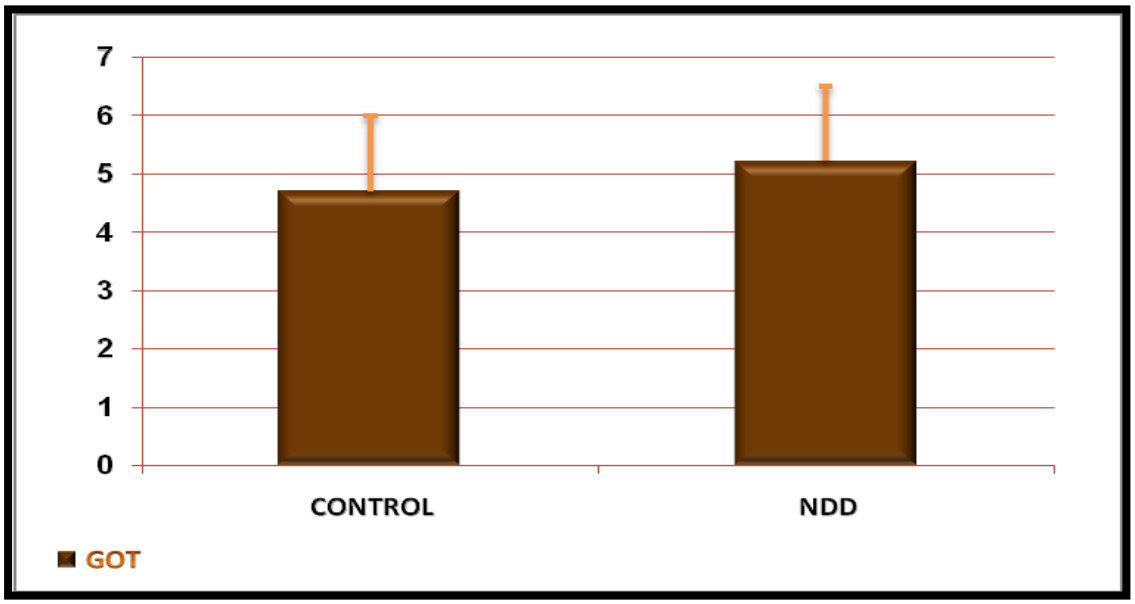


Fig 4.1.35: Histogram of Hepatic GOT activity in mice feeding on normal diet and mice feeding normal diet and on dark stress.

Table.4.1.36: Hepatic alkaline phosphatase activity in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.).

Group / Parameter	Control N=5	NDD N=5
ALKP IU/mg protein	3.90±**0.98**	4.3±1.2

N: Number of animals

* denotes significance at p≤0.05 when compared to control

ALKP= alkaline phosphatase

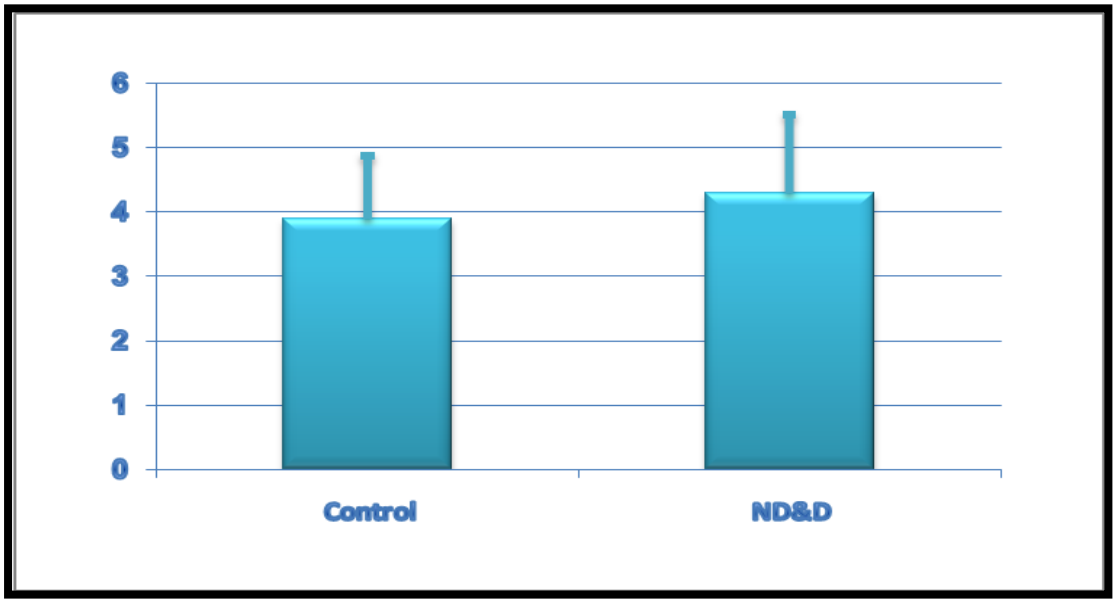


Fig .4.1.36: Histogram of Hepatic Liver alkaline phosphatase activity in mice feeding on normal diet and mice feeding normal diet and on dark stress.

Table.4. 1.37: Hepatic glucose 6-phosphate dehydrogenase activity in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.)

Group / Parameter	Control N=5	NDD N=5
G6PDH IU/mg protein	519 ±31.7	227.6±10.6*

N: Number of animals .
* denotes significance at p≤0.05 when compared to control.

G6PDH : glucose 6-phosphate dehydrogenase.

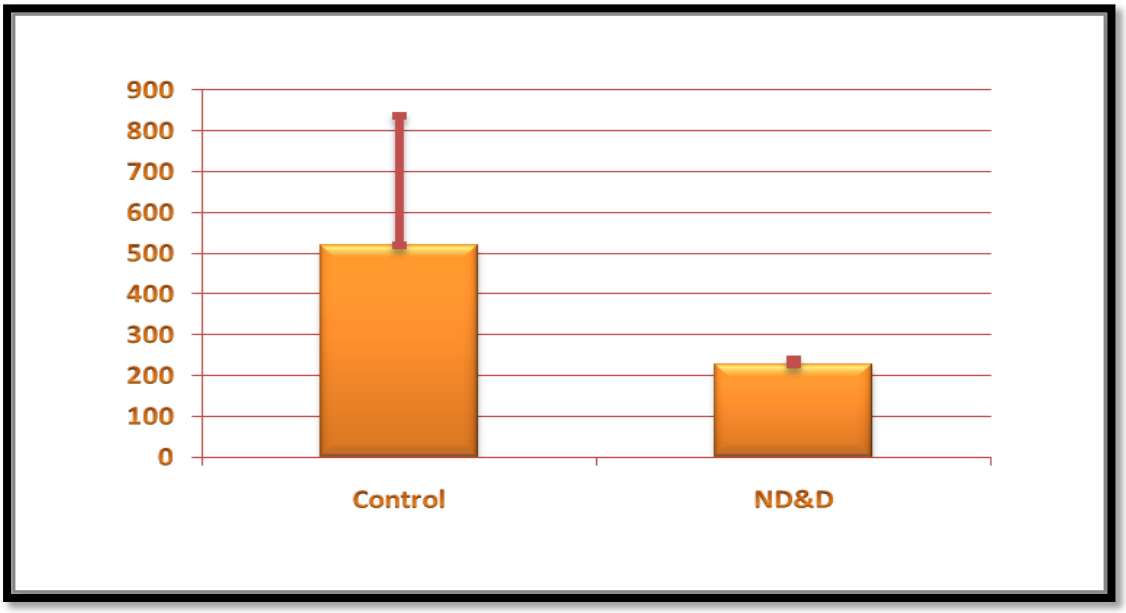


Fig .4.1.37: Histogram of Hepatic glucose 6-phosphate dehydrogenase in mice feeding on normal diet and mice feeding normal diet and on dark stress.

Table. 4.1.38: reduced glutathione activity in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.).

Group / Parameter	Control N=5	NDD N=5
GSH mg/ mg protein	24.9±**4.4**	68±**5.6***

N: Number of animals.
* denotes significance at p≤0.05 when compared to control.

GSH : reduced glutathione.

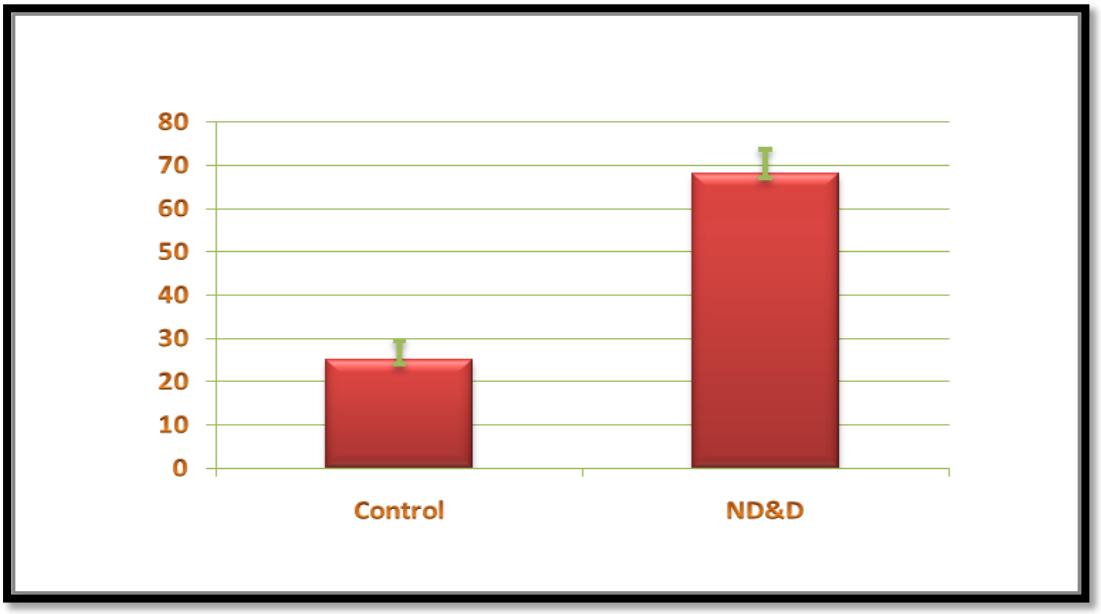


Fig .4.1.38.Histogram of Hepatic reduced glutathione activity in mice feeding on normal diet and mice feeding normal diet and on dark stress

Table.4.1.39: lipid peroxidation activity in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.)

Group / Parameter	Control N=5	NDD N=5
LPO µM/g fresh tissue	110±13.4	360±11.4*

N: Number of animals

* denotes significance at p≤0.05 when compared to control

LPO : lipid peroxidation

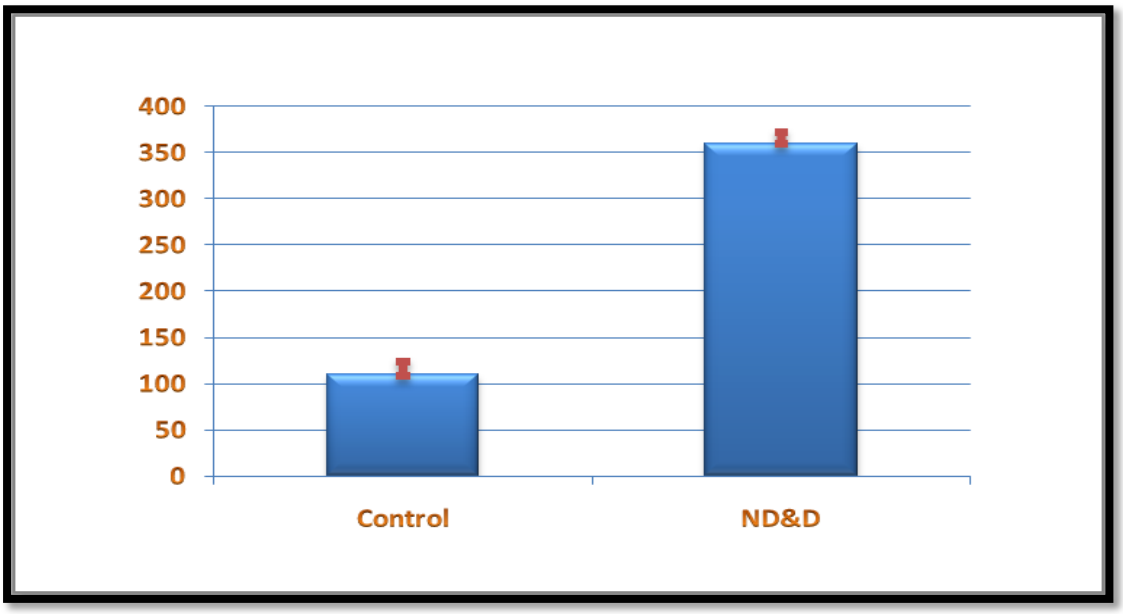


Fig 4.1.3.9: Histogram of Hepatic lipid peroxidation activity in mice feeding on normal diet and mice feeding normal diet and on dark stress.

Table.4.1.40: Hepatic lactate dehydrogenase activity and phosphate content in mice feeding on normal diet and mice feeding normal diet and on dark stress (Mean ± S.D.).

Group / Parameter	Control N=5	NDD N=5
LDH IU/mg protein	1087±472	3297±332*
PO4 mg/g fresh tissue	24.8±8.6	29.4±9.9

N: Number of animals

* denotes significance at p≤0.05 when compared to control

LDH lactate dehydrogenase

PO4 phosphate

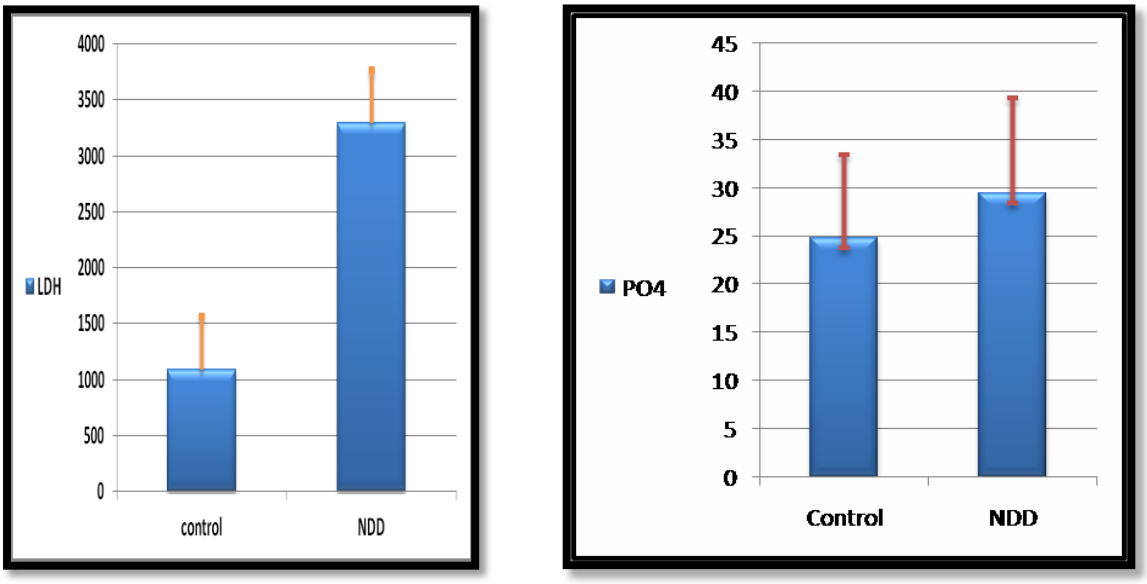


Fig .4.1.40:Histogram of Hepatic Hepatic lactate dehydrogenase activity and phosphate content in mice feeding on normal diet and mice feeding normal diet and on dark stress

4.2.Histological Results:

Figure .4.2.1: A Photomicrograph for control mice liver

Normal liver

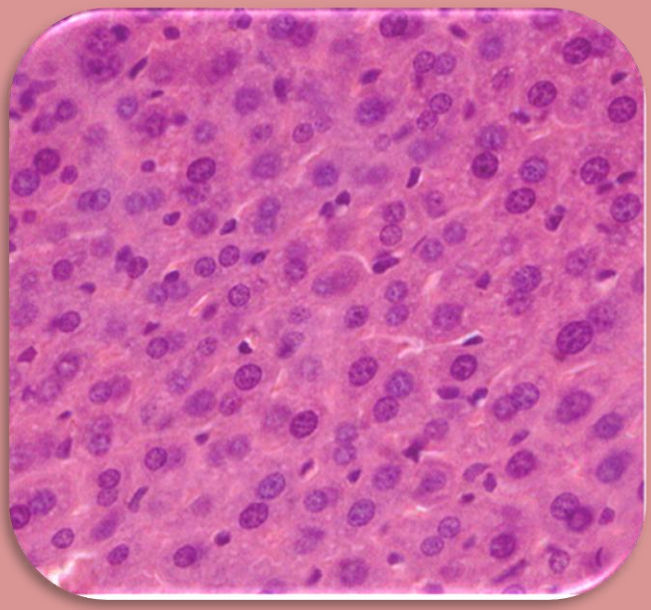


Figure .4.2.2.a: A photomicrograph in the liver of high fat diet

A photomicrograph in the liver of **high fat diet** mice , necrotic hepatocytes arround the central veins with small darkly stained nuclei (arrows). (H &E X400).

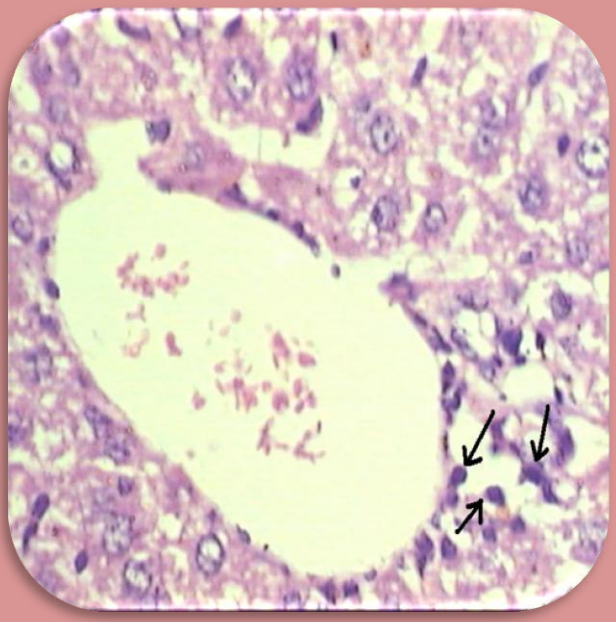


Figure .4.2.2.b: A photomicrograph in the liver of high fat diet

A parrafin section in the liver of **high fat diet** animals showing a wide area in the liver paren-chyma showing disturbed architecture with necrotic cells (arrows) and pyknotic nuclei (arrow heads) (H &E X400).

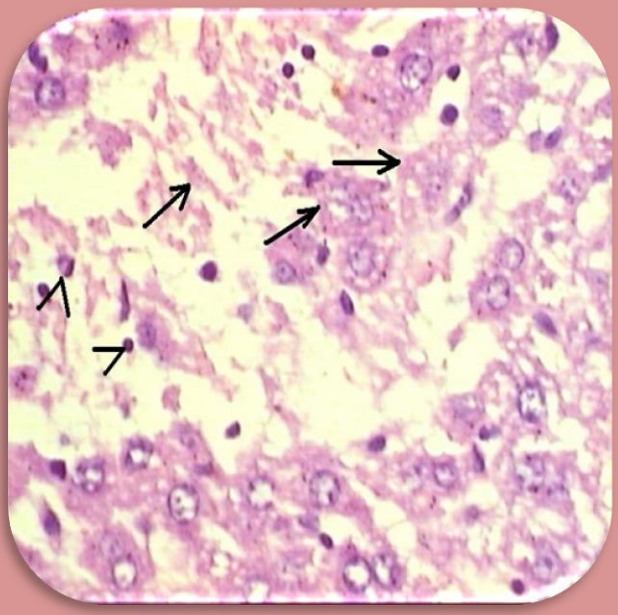


Figure .4.2.3.a: A photomicrograph in the liver of high fat diet and cold animal

A parrafin section in the liver of **high fat diet& cold animals** showing a disorganized area of the classical lobule with heavy infiltration with inflammatory cells (arrows). An apoptic body (arrow head) with small darkly stained nuclei & acidophilic cytoplasm is seen. (H &E X400).

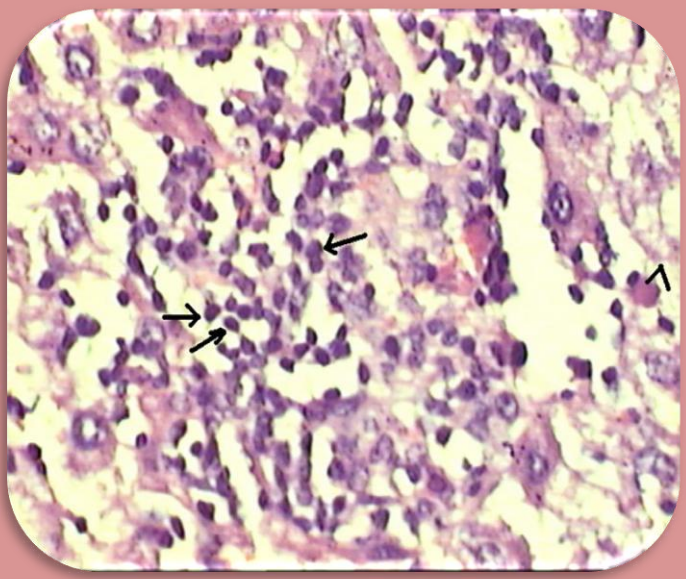


Figure .4.2.3.b: A photomicrograph in the liver of high fat diet and cold animal

A parrafin section in the centre of the classical hepatic lobule of **high fat diet & cold animals** showing the central vein (cv) surrounded with inflammatory cells (arrows) (H &E X400).

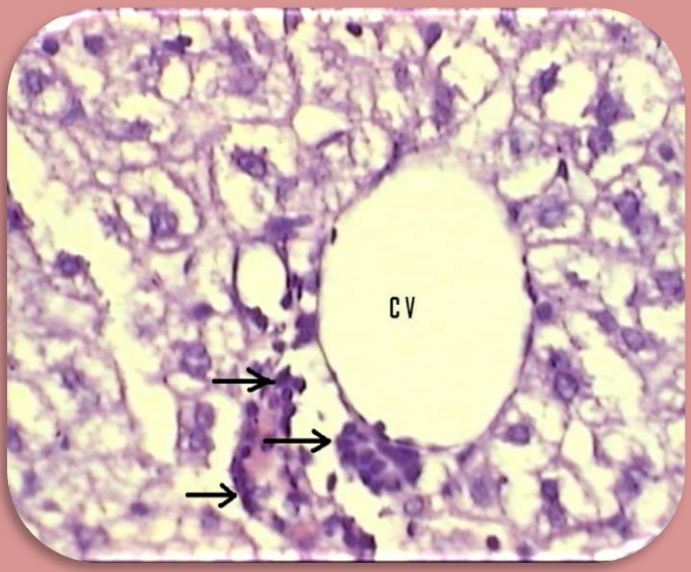


Figure.4.2.4 : A photomicrograph in the liver of normal diet and cold animal

A parrafin section of **normal & cold liver** showing hepatocytes with vacuolated cytoplasm (arrows) and rounded open face nuclei (arrow heads).(H &E X400).

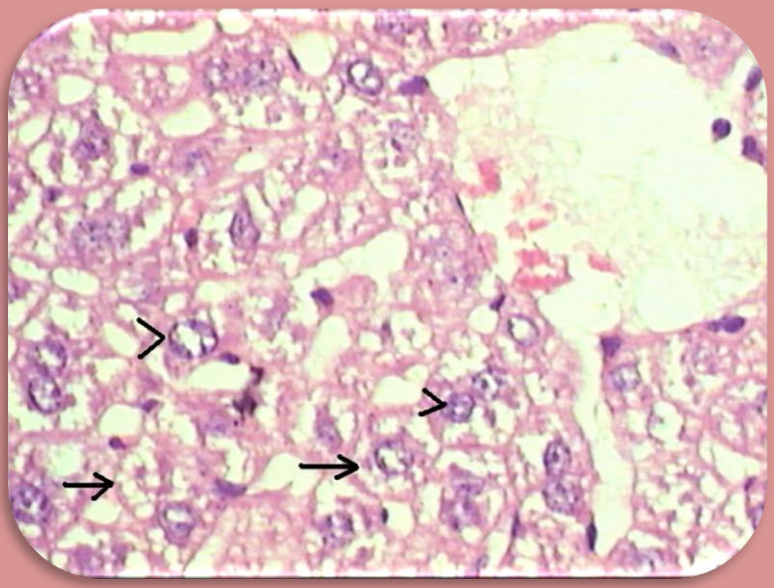
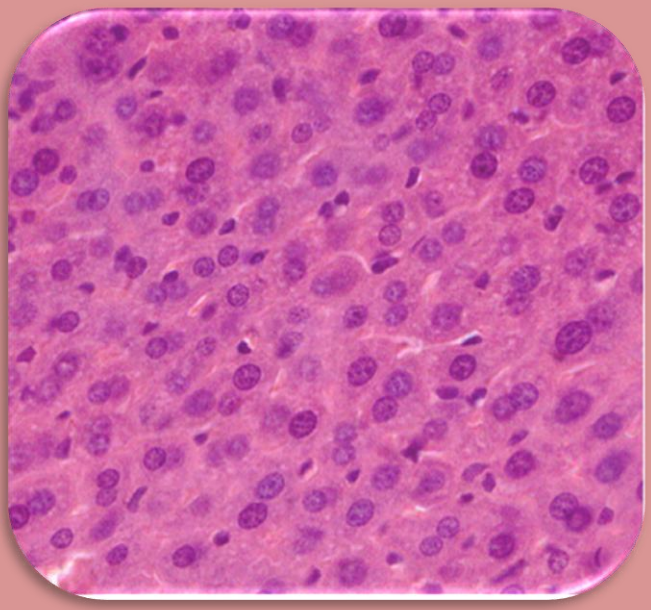


Figure .4.2.5: A photomicrograph in the liver of normal diet and dark animal

No changes.



CHAPTER 5

DISCUSSION

5.0.DISCUSSION

Stress has been thought to take part in the pathogenesis of many diseases, though its mechanisms of action and role as a risk factor remain be fully understood. It is known that stress exerts its actions through the stimulation of the sympatho-adrenomedullary activity that is responsible for the fight and flight responses. It is the body's main mechanism of weight loss, which is mediated through the β-adrenoceptor–mediated lipolysis and inhibition of adipocyte proliferation (Bowers, R.R.,2004) in white adipose tissue (WAT), and stimulation of thermogenesis in brown adipose tissue (BAT) (Bachman, E.S. ,2002). The increase in sympathetic activity in obese humans due to stress (Turtzo, L.C. ,2002) indicates that β-adrenergic activity might compensate for other factors that promote weight gain. These factors include neuropeptide Y (NPY), a peptide derived from the brain and sympathetic nerves that has potent orexigenic activity, favoring the intake of carbohydrate-rich foods (Kalra, S.P. 2004).

Insulin resistance, impaired glucose metabolism and type 2 non-insulin dependent diabetes (NIDDM) are at least in part caused by high fat diet (American Diabetes Association.1987- Glueck, C. J.,1969- Himsworth, H. P.1935- Kolterman, O. G.,1979 - Reaven .,1967- Storlien.,1968). These, in addition to the direct effect of high fat diet could eventually cause coronary artery disease and stroke (Lipid Research Clinics Program. ,1984). The molecular changes underlying these pathological changes are still partly obscure and a matter of intensive investigation. However, it is believed that decreased glucose uptake into skeletal muscle and adipose tissue, and the inhibition of glycolysis and glycogen

synthesis are caused by the impact of high fat diet on the representing insulin receptors in these organs (Anderson., 1982- Anderson., 1985). As a result glycogen storage and glucose oxidation will be affected, and on the contrary gluconeogenesis will be increased. These complications are common in many diabetic patients

High fat diet may lead to increased synthesis of cholesterol, which would associat with elevation of low density lipoproteins (LDL) in addition to the elevation of the very low density lipoproteins (VLDL). Both are lipid species known for their association with arteriosclerosis.
Increased uptake of high fat diet challenges the tissue entry of glucose, and enhances glucose production increasing blood glucose and initiating events leading to insulin resistance (Glueck., 1969- Anderson., 1985- Reaven.,1967).

Glucocorticoids enhance the secretion of VLDL from the liver thus increasing tissue delivery of triglycerides. Glucocorticoids levels are elevated through the hypothalamic-pituitary axis. It is believed that this axis is influenced by high fat diet (Brindley., 1981-Hulsmann.,1978- 35). This finding was demonstrated by an experiment using high fat diet in perfused liver and monolayer cultures of hepatocytes (Bartlett, 1988- Mangiapane., 1985). This is exaggerated by the decrease in the hydrolysis of VLDL as a result of the inhibition of lipoprotein lipase by the glucocorticoid hormones (Taylor.,1988). Pertinent to this is the weak degradation of LDL, which is proven to be experimentally caused by dexamethasone, the synthetic glucocoricoid, and explained by the lowered binding of LDL to hepatic receptors (Goldstein., 1977). Furthermore, glucocorticoids increase the breakdown of triglycerides, glycogen and proteins, thus antagonizing the actions of insulin. Amino

acids released through this process could participate in the gluconeogenesis process. All these effects are shared by glucocorticoids and high fat diet, therefore the effects seen because of high fat diet could be mediated through the release of glucocorticoids.

It can be concluded that high fat diet appear to play the role of chronic stressor. Chronic stress facilitates glucocorticoid release caused by acute stress (Akana .,1992).

Recently, several studies suggested the important role played by adipocytokines such as adiponectin in the regulation of energy expenditure and mobilization. In addition significant relationships have been drawn from related experiments linking adiponectin to sugar and lipid metabolism through its effect on insulin tolerance.

To date the mechanism through which adiponectin affects the regulation of metabolism is not completely understood. This was the motive for us to design and run current study, in which we tested the effect of HFD, on its own or combined with brief stress on mice.

This study was concerned with the effect of various forms of stress on intermediary metabolism. The body's handling of food energy content is subject to a complicated network of stimulating and inhibiting signals. The mouse was chosen in this study as a model to show the effect of a high fat diet and cold stress on the secretion and metabolic effects of adiponectin hormone.

Several lines of evidence have recently suggested that adiponectin is involved in regulating carbohydrate and lipid metabolism.

In mice, systemic adiponectin treatment resulted in increased fatty acid oxidation in muscle (Fruebis J, 2001), decreased plasma glucose level by enhancing the ability of insulin to suppress hepatic gluconeogenesis (Berg AH, 2001), and improved insulin sensitivity in insulin resistant models (Yamauchi T2001). In addition, loss of the adiponectin gene in mice reportedly resulted in decreased insulin responsiveness and enhanced atherogenesis (Maeda N., 2002, Kubota N., 2002).

Furthermore, a central action of adiponectin was shown by a decrease in body weight due to stimulation of energy expenditure (Qi Y., 2004).

Together, these data strongly suggest that adiponectin plays an important role in preventing the development of the metabolic syndrome.

Despite the knowledge on the physiological effects of adiponectin, the mechanism regulating its release and activity is yet to be completely understood.

In the present study, cold exposure of mice on normal diet resulted in a non significant increase in the serum adiponectin concentration.

In a second experiment, exposure of mice on high fat diet to cold inhibited the rise in serum adiponectin levels. Adiponectin levels were seen to rise by high fat diet in comparison to chow diet group.

There is considerable evidence that adiponectin levels correlate negatively with body weight (Arita., 1999 – Weyer., 2001). In addition, adiponectin expression is reportedly decreased in enlarged adipocytes (Yang., 2004). However, in the present study it is noteworthy that the body weight significantly decreased with cold exposure.

Going with our study, Puerta et al. (Puerta M, 2002) reported that in rats, acute cold exposure did not significantly change adiponectin expression in WAT or serum.

In contrast to our study, (Imai et al., 2006) reported that, mice were exposed to 4 °C for 24 hours, which suppressed serum adiponectin levels. The reasons for this discrepancy may be due to differences in the magnitude and periods of cold exposure.

In our study, mice were exposed to 4°C for 1 hour every day for five weeks, whereas in the previous report, rats were subjected to milder cold stimulation, i.e., 6°C for 18 hours. In a study by Imai and co-workers in 2006, mice were exposed to 4 °C for 24 hours. Alternatively, mice might be more sensitive to cold stimulation than rats.

Not so long ago, Haman and coworkers (2004) explored the effect of cold exposure on adiponectin levels in humans. Plasma levels of this protein were analyzed in the samples from glycogen-depleted and glycogen-loaded men exposed to a temperature of 10^{o}C for 2 h. Their results showed that (i) adiponectin levels increased significantly after 90 min of cold exposure in both glycogen-depleted and glycogen-loaded men and (ii) changes in circulating adiponectin tended to be associated with changes in plasma glucose oxidation rates but not with changes in lipid oxidation rates during cold exposure (Imbeault et al. 2004a). Ongoing studies by Haman and coworkers suggested that the observed increase in adiponectin levels is not explained by the decrease in plasma volume occurring during cold exposure. These results suggest that cold exposure is one of the few stimuli identified so far that affect adiponectin levels in

a short period of time in humans. However, the mechanisms underlying the rise in adiponectin levels during cold exposure are still to be identified. Cold exposure may therefore help to shed some light on the acute modulation and biological actions of this adipose-derived protein in humans.

Another interesting finding in this study is the markedly different degrees of responsiveness to cold exposure among antioxidant/oxidant system.
Liver was affected most in cold stress model, which produces oxidative stress (more than kidney and heart) (Emel Şahın, 2007)

It is well known that cold exposure may be reflected in an elevated metabolic rate and also increased production of reactive oxygen species (ROS) (Şahin, 2004). When ROS production exceeds the capacities of protection and repair mechanisms oxidative stress occurs, resulting in damage to proteins and lipids (Selman, 2002).
It has been demonstrated in some forms of stress, such as exercise, starvation, cold and water-immersion restraint stress increasing the free radical generation and lipid peroxidation, which is one of the most important damaging effects of free radicals on tissues (Seçkin, 1997).

Acute cold stress significantly decreased blood GSH levels and perturbation of GSH metabolism in several visceral organs (Tremoto, 1998).

The physiological components of stress response to cold covers metabolic, circulatory and hormonal processes however, the cellular and molecular mechanisms mediating these responses remain to be elucidated. Immobilization and acute cold stress are widely used

experimental models accompanied by considerable decrease in antioxidative capacity in animals (Simmons, 1991- Akhalaya, 2006).

Lipid peroxidation has been implicated in a number of deleterious effects and increase in the levels of TBARS indicate the enhanced lipid peroxidation leading to tissue injury and failure of antioxidant defense mechanisms to prevent the formation of excess free radicals (Valko M., 2007).

In the present study liver and plasma MDA levels were found to be significantly increased in the mice exposed to cold stress when compared to the controls.
These results are in agreement with the previous findings, which are related to stress-induced lipid peroxidation in plasma and liver of the animals (Akhalaya, 2006, Şahin, 2007, Çiğdem Özer, 2009).

GSH is an important endogenous defense substance against the reactive oxygen species (ROS) and the tissue GSH concentration reflects a potential detoxification marker. It has been previously reported that cold stress reduced the GSH levels in liver and in the other tissues in mice (Simmons, 1991) and rats (Shustanova et al., 2004).

Shustanova et al., (2004) reported that cold stress caused an inhibition of antioxidant enzyme activities such as glutathione reductase in brain, liver and erythrocytes (Rauhala, 2005). However, in our study GSH level was increased in cold stress group compared with the control group, Nonetheless, the increase in GSH level might be due to the period of cold exposure (one hour daily for five weeks). GSH reach highest level to counteract oxidative stress.

In our study feeding mice high saturated-fat diet for two weeks resulted in a significant increase in fasting blood sugar (FBS). Our mice were fed diet containing 20 % saturated fat that was reflected in a significant increase in adiponectin. However, this increase did not lead to a decrease in FBS.

This could be explained by two facts, firstly, contrary to the parentral use of adiponectin in laboratory animals, its increase through the use of HFD is expected to be gradual and its effects are compensated by:

1. Glucose provides substrates for fatty acids and glycerol synthesis and since the diet taken is rich in fat the responsible pathway is inhibited along with glycolysis .

2. Increased levels of circulatory free fatty acids have a negative effect on liver clearance of insulin and insulin mediated suppression of glucose production, in addition to a positive effect on gluconeogenesis.

3. Free fatty acids have a negative effect on insulin stimulated glucose utilization through their inhibitory effect on skeletal muscle sensitivity to insulin.

   (Avram et al., 2005)

It has been reported that adiponectin administered systemically to mice cause a decrease in FBG, which was previously explained by Berg (2001) by the enhancing effect of adiponectin on insulin sensitivity.

Therefore, in our study the apparent increase in circulating free fatty acids released form high fat diet overwhelms the effect of adiponectin on blood glucose, and most likely a matter of correlation between effect and concentration.

In the present study cold exposure compensated against the effect of HFD and caused a reduction in adiponectin to none significant levels. This is in agreement with the results reported by Imai et al. (2006) when they exposed mice to 4 C cold climates for 24 hours and concluded that cold stress suppresses serum adiponectin levels.

On the other hand cold exposure has been shown to improve glucose tolerance test (GTT). Therefore, the FBS increase seen because of high fat diet was reversed when animals were exposed to cold stress, which has been suggested to enhance tissue sensitivity to insulin.

In addition, discrepancies between results and conclusion reported by different research teams could be attributed to the duration and magnitude of cold exposure added to the experimental animal type.

Our results are partly in agreement with those of Junta and co-workers (2006). They concluded the cold exposure did not affect body weight, but did significantly lower adiponectin concentration. They postulated that the sympathetic nervous system physiologically regulates the effects of cold stress. This conclusion was reached since cold stress caused the elevation of the uncoupler protein 1 (UCP1).

It is well known that cold exposure may be reflected in an elevated metabolic rate and in increased reactive oxygen species production (Sahin, 2004).

Although the liver contributed to increased oxygen uptake during cold stress (31, 32), in the present study the increase in lipid peroxides measured as malondialdehyde was only obviously seen in animals kept on high fat diet, but was diminished when HFD was combined with cold stress.

Milagro et al., (2006) presented a correlation, based on an experimental trial on rats, showing that liver MDA as a good estimate of lipid peroxidation increases concomitantly with increasing other variable such as insulin, when animals were fed cafeteria diets. This suggests that HFD increases oxidative stress in plasma as well as in white adipose tissue and liver. On the other hand this may offer explanations for the histological damage seen in the liver of animals kept on high fat diet.

When considering the molecular action of adiponectin, Kadowaki's team (2005) demonstrated that full length adiponectin stimulates AMP-activated protein kinase in the liver, while globular adiponectin affects both muscle and liver causing increased fatty acid combustion (Capeau, 2007). This could offer an explanation for the effect of moderate HFD (20 %) used in the current study, which resulted in an increase in FBS with no increase in body weight, i.e. increased catabolism of fatty acids was sufficient to stop the weight increase induced by the full length adiponectin.

Several lines of evidence have recently suggested that adiponectin is involved in glucose and lipid metabolism.

In mice, systemic adiponectin treatment increased fatty acid oxidation in muscle (Fruebis J,2001), decreased plasma glucose by enhancing the ability of insulin to suppress hepatic glucose production (Berg AH, 2001), and improved insulin sensitivity in insulin resistant models (Yamauchi T2001). In addition, loss of the adiponectin gene in mice reportedly decreased insulin responsiveness and enhanced atherogenesis (Maeda, 2002; Kubota, 2002).

Stress on its own whether cold exposure or dark containment caused significant decrease in body weight. On the other hand animals exposed to cold stress and kept on high fat diet did not show an increase in FBS, possibly due to enhanced sensitivity of peripheral tissue to insulin. Furthermore dark containment did cause an increase in FBS. Therefore, stress seemed to have an effect on FBS, but this effect is specific for each type of stress. This is clearly reflected in the increased level of FBS in animals exposed to dark containment stress. In these animals, apparently, the role played by stress hormones is obvious, and darkness apparently does not influence mechanisms similar to those influenced by cold stress to improve insulin sensitivity.

Cold exposure increases brown adipose tissue (BAT) mass and total cytochrome oxidase activity, in addition to increasing peripheral tissue sensitivity to insulin. BAT and cytochrome oxidase both specifically enhance the burning of FAT, therefore reduce the impact of HFD damage on liver cells.

The aminotransferases are excellent indicators of liver damage when both ALT and AST are elevated. Our results showed significant increases in both enzymes following 5 weeks of HFD feeding. Although liver parenchymal damage at the histological level is more clearly demarked when cold stress was combined with HFD feeding, the increase in these enzymes was diminished.

Elevation of GPT is a marker of liver tissue damage and a surrogate for fatty liver disease. Its increase even within the normal level is associated with deterioration in insulin sensitivity and glucose tolerance, in addition to increased FFA and triglycerol level (Bergert et al., 2006).

For GPT, its increase in animals sustained on normal chow exposed to cold or dark stress could result from the effect of stress on muscles and other organs.

GOTis expressed by myocardial and muscle cells more than GPT, and this enzyme significantly increase due to cold stress, but not dark exposure.

In the present study it was noticed that ALP was significantly increased when mice fed high fat diet and were made to bare brief cold stress. The ALP enzyme is considered as an indicator of hepatocellular damage (Yang and Chen, 2003; Vozarova et al., 2002). It is a common knowledge that NAFLD can be caused by the accumulation of triglycerides in the hepatocytes. Furthermore, it has been reported that ALP is a marker for both plasma membrane and endoplasmic reticulum. In addition, it has been reported that oxidative stress is an imperative factor in the development of non-alcoholic fatty liver disease and cold stress has been shown to induce oxidative stress, hence membrane changes and increased ALP release.

Cold stress in mice (0-1 C) decreases liver content of glutathione. In addition repeated exposure for 3 times per week for 1 month further caused a fall in glutathione (Simmons et al., 1990, 1991; Yamamoto et al., 1995).

CHAPTER 6

SUMMARY AND CONCLUSION

we conclude that five weeks of a high-fat diet containing 20% fat was not enough to proeduce obese mice. Therefore, we can be sure that our results were restricted to the effects of the diet and were not due to obesity- induced metabolic changes.

The decrease in serum adiponectin levels, suggesting that there is a compensatory effect of the other fat depots on serum adiponectin levels.

Further studies will elucidate whether this is a transitory compensatory mechanism or a sign of target organ resistance to adiponectin.

High fat diet can disrupt the balance in an oxidant/antioxidant system and cause oxidative damage to liver tissues by altering the enzymatic and non-enzymatic antioxidant status, and lipid peroxidation.

Further investigation into the mechanisms of this model may lead to new targets for drug therapy as well as new insights into the role of diet composition in energy balance.

According to observation made on Cold stress coupled to a High-fat diet group it could be seen as expression of antisynergistic effect .

The obesogenic effect of a high-fat (HF) diet is counterbalanced by stimulation of energy expenditure and lipid oxidation in response to cold stress .

Cold stress can affect liver tissue metabolism in different ways:

1. By disrupting the balance in an oxidant/antioxidant system and causing oxidative damage to liver tissues.

2 .By Changing levels of liver LDH as compensatory mechanism ,hepatic energy demands not by alternate pathways .switching to an

aerobic metabolism was affected by oxidative stress. This can be viewed as functional or physiological adaptation of the liver.

3. Enhancement the activities of transaminases ALT and T

The degree and duration of Cold stress in our experiment can also effect adipocyte which is reflected by high level of serum adiponectin which explained by the compensatory mechanism.

-Disturbing the lighting for five weeks caused metabolic changes which indicates stress.

Dark stress can also effect liver tissue and secretion of adiponectin in different ways same as cold stress .

Finally, this study showed many interesting results and the study attempted to explain them. This may lead to conduction of more researches based on these findings.

CHAPTER 7

الملخص العربي

7.0.الملخص العربي

هذه الدراسة أجريت على فئران المختبرات للتوصل أولا لمحاكاة التغيرات البيولوجية المصاحبة لتعرض الإنسان لأنواع مختلفة من الإجهاد الناتج ارتفاع نسبة الدهون في الغذاء، والتعرض إلى درجة حرارة منخفضة تصل إلى 4 درجات مئوية بالإضافة الى تأثير الأجهاد الناتج من التواجد في أماكن مظلمة .

وقد أجريت هذه الدراسة على نموذج حيواني متمثل في فئران التجارب من خلال تعريضها لأنواع مختلفة من الإجهاد كما سلف ذكره، مثل هذا النموذج قد يكون مفيدا في الحصول على نظرة ثاقبة لتعرض الجسم لأنواع مختلفة من الإجهاد من خلال التغيرات في الأنسجة و الاضطرابات الكيميائية الحيوية.

أهداف ألبحث:

1. تحفيز الفئران بأشكال متعددة من الإجهاد المزمن لمدة خمس اسابيع، إما بمفرده أو بالاشتراك مع نظام غذائي مكون من نسبة عالية من الدهون.

2. دراسة تأثير ارتفاع نسبة الدهون في الغذاء ، وإلاجهاد الناتج من انخفاض درجات الحرارة على الكبد ومستوى الاديبونيكتين في الدم ، و نسبة السكر في الدم كل على حدا او معا.

3. ايجاد علاقة بين الإجهاد وارتفاع نسبة الدهون وتأثير ذلك على الوزن، حيث وجد ان هناك اختلاف بين الناس من حيث الأستجابة للأجهاد ,تغير في الوزن .

تم تقسيم الفئران إلي خمس مجموعات , الأولي كدليل تحكم,الثانية أعطيت غذاء متكون من نسبه مرتفعه من الدهون ,الثالثة أعطيت لها حمية عالية الدهون بالأضافه إلي الإجهاد البارد (كل يوم في الساعة 9:30 صباحا توضع الفئران في درجة حراره 4 C^{o} لساعة واحدة) ,الرابعة تحت تأثير الإجهاد البارد فقط والخامسة تحت تأثير إجهاد الظلام(كل يوم في الساعة 9:30 صباحا الفئران توضع في غرفه مظلمة لساعة واحدة) .

مدة التجربة خمس أسابيع وفى نهاية الزمن المحدد تم قتل الحيوانات تحت التخدير بعد سحب الدم لأجل الاختبارات وأخذت الكبد لدراسة الأنسجة مجهريا ولإجراء القياسات الحيوي.

ملخص النتائج كالأتي :

المجموعة الثانية:

الخلاصة أن خمسة أسابيع من اتباع نظام غذائي غني بالدهون (بنسبة 20٪) لم يكن كافيا لأنتاج فئران سمينة. ولذلك، يمكننا أن نكون على يقين من أن النتائج التي توصلنا اليها علی الآثار المترتبة على النظام الغذائي ولم تكن نتيجة للتغيرات التمثيل الغذائي الناجمة عن البدانة.

انخفاض في مستويات اديبونيكتين ، مما يدل على أن هناك تأثير تعويضي من مستودعات الدهون الأخرى على مستويات اديبونيكتين في الدم .

ظهرت الحاجه لإجراء المزيد من الدراسات لتوضيح ما إذا كانت هذه عباره عن آلية تعويضية انتقالية أو علامة على مقاومة الجهاز المستهدف الاديبونيكتين.

اتباع نظام غذائي عالي الدهون يخل بالتوازن في نظام الأكسدة / المضادة للاكسدة وتسبب في الضرر التأكسدي لأنسجة الكبد عن طريق تغيير الوضع مضادات الأكسدة الأنزيمية وغير الأنزيمية، وبيروكسيد الدهون.

المجموعة الثالثة:

أن الجمع بين الإجهاد الناتج عن البرد مع اتباع نظام غذائي عالي الدهون له أثر مضاد .

حيث إن أضافه الإجهاد الناتج عن البرد يؤدي الي تحفيز استهلاك الطاقة وزيادة أكسدة الدهون فبذلك يمنع تأثير الدهون الناتج من النظام الغذائي .

المجموعة الرابعة:

- الإجهاد البارد يمكن أن يؤثر في أنسجة الكبد بطرق مختلفة:

1. عن طريق تعطيل التوازن في نظام الأكسدة / المضادة للأكسدة والتسبب في الضرر التأكسدي للأنسجة الكبد.

2. عن طريق تغيير مستويات LDH كآلية تعويضية من أنسجة الكبد لتلبية الطلب على الطاقة من خلال مسارات بديلة.

3. بواسطة تعزيز أنشطة ALT & AST

-و أيضا جد ان درجة ومدة إجهاد البرودة في تجربتنا اثر في الخلايا الشحميه الأمر الذي انعكس علي ارتفاع مستوى اديبونيكتين في الدم و الذي يفسر كآلية تعويضية.

المجموعة الخامسة:

تعكير الإضاءة لمدة خمسة أسابيع يسبب التغيرات الاستقلابية مما يدل على الضغط النفسي.

الإجهاد المظلم يمكن أن يؤثر أيضا في أنسجة الكبد وإفراز اديبونيكتين .

أخيرا، أظهرت هذه الدراسة العديد من النتائج المثيرة للاهتمام . وهذا قد يؤدي إلي إجراء المزيد من البحوث في المستقبل.

CHAPTER 8

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