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Evaluation of some immunological aspects in patients with diabetes type II

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Dedication

To my father.....

For his unlimited care, guidance and Sacrifices

To my lovely mother .....

For her unlimited love and Kindness

To my dearest sisters and brothers.....

I dedicate this work

In gratitude for your

Support

Patience

Understanding

Alaa

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Summary

Type 2 diabetes mellitus (T2DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of fat and protein metabolism resulting from defects in insulin secretion , insulin action or both, and mediated in large part by the alteration in adaptive immunity and cytokines. The present study was planned to determine the lymphocyte phenotyping by using monoclonal antibodies against CD4+ T cells and CD8+ T cells, serum levels of tumor necrosis factor-alpha (TNF)-α and interleukins- 12(IL-12), proinsulin hormone ,as well as study the DNA fragmentation.

Fifty T2DM patients attending the National Diabetes Center for Treatment and Research at Al-Mustansiriya University between December 2012- January 2013 were recruited for this study. For the purpose of comparison, 30 control subjects matched for age, gender and ethnic background were also included. The patients were characterized by family history of diabetes. The patients were also assessed for the duration of disease; The body mass index (BMI), waist-to-hip (W-H) ratio were measured, also the fasting plasma glucose (FPG), total cholesterol (TC), HDL-cholestrol, LDL-cholesterol, Triglyceride(TG), glycosylated hemoglobin (HbA_{1c}) and proinsulin hormone were measured . The percentage mean of (CD4 and CD8) T cells were measured by Apogee flow cytometry by adding CD4 and CD8 markers, both CD4+T cells (17.19% *vs*. 66.25%) and CD8+T cells (18.14% *vs*. 34.92%) was significantly lower in T2DM patients as compared to healthy control ($p \leq 0.001$). The levels of cytokines (TNF-α and IL-12) were determined by ELISA kits, the TNF-α showed a highly significant increased ($p \leq 0.001$) in patients compared controls (117.6 *vs*. 97.06 pg/ml) .Also the IL-12 levels demonstrated a highly

Summary

significant increase ($p \leq 0.001$) in T2DM patients as compared to controls. (58.02 vs. 28.05 pg/ml). Also 20% of patients revealed DNA damage by analyzed the DNA fragment by gel electrophoresis on 1.8% agarose gel at 24 voltage for 4 hour, this damage might be due to reactive free radicals and may depend on severity and chronicity of the case. In conclusion this study suggests that aberrant glucose concentrations, hyperinsulinaemia, infections or underlying disease can exert enough stress to affect the immune cell population and influence adversely the outcome of disease in patients. This may be reflected in a lowered immunocompetence and metabolic dysfunction.

List of Contents

List of Tables

List of Figures

Abbreviations

AGE	Advanced Glycation End-product
AGEs	Advanced Glycation End-products
AR	Aldose Reductase
ADA	American Diabetes Association
Bcl-2	B-cell leukemia/lymphoma2
BMI	Body Mass Index
bp	Base pair
CAD	Cardiovascular Disease
CD	Cluster of Differentiation
CTLs	Cytotoxic T-Lymphocyte
DM	Diabetes mellitus
CMI	Cell Mediated Immunity
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme linked immunosorbant assay
FBG	Fasting Blood Glucose
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FPG	Fasting plasma glucose
HbA1c	Glycosylated haemoglobin
HDL	High Density Lipoprotien
HRP	Horseradish peroxidase
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IFN-γ	Interferon – gamma
IGT	Impaired glucose tolerance
IL	Interleukin
IR	Insulin resistance

LDL	Low Density Lipoprotien
LSD	Least Significant Difference
MHC	Major Histocompatibility Complex
MMP	Mitochondrial Membrane Permeability
MMPs	Matrix metalloproteinase
MODY	Maturity onset diabetes of the young
MP	Mononuclear phagocyte
MPO	Myeloperoxidase
NIDDM	Non-insulin dependent diabetes mellitus
NADPH	Nicotinamide Adenine Dinucleotide
NK	Natural Killer
O.D	Optical Density
P	Probability
PBMC	Peripheral Blood mononuclear cell
PBS	Phosphate Buffer Saline
PMNs	Polymorphonuclear leukocyte
R2	Determination coefficient
RBC	Red Blood Cell
RNS	Reactive Nitrogen Species
ROC	Reactive Operator Curve
ROS	Reactive Oxygen Species
SD	Standard Deviation
SMC	Smooth Muscle Cell
SSC	Side scatter
T1DM	Type 1 Diabetes Mellitus
T2DM	Type2 Diabetes Mellitus
TC	Cytotoxic T Cell
TCR	T cell receptor
TBE	Tris_boric_EDTA
TG	Triglyceride
TGF-β	Transforming Growth Factor-Beta
Th1	T helper-1 lymphocyte

Th2	T helper-2 lymphocyte
TNF-a	Tumor Necrosis Factor-alpha
W-H	Waist –to- Hip
WHO	Word Health Organization

1.1 Introduction

The term diabetes mellitus (DM) describes a metabolic disorder of multiple etiologies characterized by disturbances of carbohydrate ,fat and protein metabolism resulting from defects in insulin secretion , insulin action or both (WHO, 1999). It is a major worldwide health problem predisposing to markedly increased cardiovascular mortality and serious morbidity and mortality related to the development of nephropathy , neuropathy and retinopathy (WHO, 2005).

Three principal types of DM are recognized ; Type 1 DM (T1DM), Type 2DM (T2DM) and gestational diabetes. Type 1 diabetes mellitus is characterized by loss of the insulin- producing beta cells of the islets of Langerhans in the pancreas leading to a deficiency of insulin, while T2DM is characterized differently and is due to insulin resistance or reduced insulin sensitivity , combined with relatively reduced insulin secretion which in some cases becomes absolute (American Diabetes Association, 2012). Due to increasing obesity, sedentary life style and dietary habits in both Western and developing countries, the prevalence of T2DM is growing at an exponential rate . The increase in T2 DM is also seen in younger people and in developing countries , and estimates in the Middle East and Africa revealed that the prevalence is high and set to increase dramatically during the next 18 years (WHO, 2008; Li *et al*., 2009).

The aetiology of T2DM is not well-understood , although associated health risk factors are recognized ; for instance, a family history of diabetes age over 45 years , race or ethnic background, metabolic

syndrome (also called insulin resistance syndrome), obesity, hypertension, and history of vascular disease such as stroke, abnormal cholesterol levels and history of gestational diabetes (American Diabetes Association , 2012) .

1.2 Aims of Study

The present study was planned to investigate the following immunological markers, biochemical and genetic parameters in Diabetic Type 2 patients:

a) Determining the blood levels of glucose, cholesterol, HDL cholesterol, LDL cholesterol , triglyceride , glycosylated hemoglobin A_{1C} and Proinsulin Hormone .

b) Determining the phenotyping by using monoclonal antibodies against (CD4 and CD8)T cells.

c) Determination the role of pro_inflammatry cytokines by measuring TNF_a and IL-12.

d) Assessing the amount of DNA damage in diabetic patient.

1.2 Literature Review

1.2.1 Diabetes mellitus

1.2.1.1 Definition and Classification

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage , dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels, that impose a tremendous burden on the individual with diabetes and on the health care system (American Diabetes Association , 2013).

It is classified on the basis of pathogenic process that leads to hyperglycemia. The two broad categories of DM are designated TIDM and T2DM. Other forms of DM are also categorized separately from these two types, and examples include gestational diabetes, congenital diabetes due to genetic defects of insulin secretion, cystic fibrosis-related diabetes, steroid diabetes induced by high doses of glucocorticoids, and several forms of monogenic diabetes (Craig *et al*., 2009).

1.2.1.2 Historical Background

Diabetes was first recorded in England, in a medical text written around 1425. In 1675, Thomas Willis added the word mellitus from the Latin meaning "honey"; a reference to the sweet taste of the urine. This sweet taste had been noticed in urine by the ancient Greeks, Chinese, Egyptians, Indians, and Persians (Dwivedi and Dwivedi , 2007) .

The discovery of a role for the pancreas in diabetes is generally ascribed to Joseph von Mering and Oskar Minkowski, who in 1889 found that dogs whose pancreas was removed developed all the signs and symptoms of diabetes and died shortly afterwards. In 1910, Sir Edward Albert Sharpey-Schafer suggested that people with diabetes were deficient in a single chemical that was normally produced by the pancreas; he proposed calling this substance insulin, from the Latin insula, meaning island, in reference to the insulin-producing islets of Langerhans in the pancreas.(Leonid, 2009).

The endocrine role of pancreas in metabolism, and indeed the existence of insulin , was not further clarified until 1921, when Sir Frederick Grant Banting and Charles Herbert Best repeated the work of Von Mering and Minkowski, and went further to demonstrate that they could reverse induced diabetes in dogs by giving them an extract from the pancreatic islets of Langerhans of healthy dogs. Banting and laboratory director MacLeod received the Nobel Prize in Physiology and Medicine in 1923 (Patlak, 2002).

1.2.1.3 Diagnosis

The National Diabetes Data Group and the World Health Organization have issued DM diagnostic criteria, which are based on the following two principles (American Diabetes Association, 2012):

1.The spectrum of fasting plasma glucose (FPG) and the response to an oral glucose load varies among normal individuals.

2. The level of glycemia at which diabetes-specific complications occur rather than on deviations from a population-based mean.

These criteria are:

i. Symptoms of diabetes plus random blood glucose concentration ≥ 11.1 mmol/L (200 mg/dL): Random is defined as without regard to time since the last meal.

ii. Fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL): Fasting is defined as no caloric intake for at least eight hours.

iii. Two-hour plasma glucose ≥ 11.1 mmol/L (200 mg/dL) during an oral glucose tolerance test: The test should be performed using a glucose load containing the equivalent of 75 grams anhydrous glucose dissolved in water.

1.2.2 Type2 Diabetes mellitus

Type 2 Diabetes Mellitus (T2DM) is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion, and increased glucose production. Distinct genetic and metabolic defects in insulin action and secretion give rise to the common phenotype of hyperglycemia in T2DM (Ripoll and Leutholtz , 2011). Distinct pathogenic processes have important potential therapeutic implications, as pharmacological agents that target specific metabolic derangements have become available. It is preceded by a period of abnormal glucose homeostasis classified as Impaired Fasting Glucose (IFG) or Impaired Glucose Tolerance (IGT) (Powers, 2006).

1.2.2.1 Epidemiology

The worldwide prevalence of DM has risen dramatically over the past two decades. Likewise, prevalence rates of IFG are also increasing. Recent estimates indicate that there were 371 million people in the year 2012 had diabetes, with type 2 making up about 90% of the cases. Its incidence is increasing rapidly, and by 2030, 552 million people will have the disease (American Diabetes Assocciation, 2012). Although the prevalence of both T1DM and T2DM is increasing worldwide, the prevalence of T2DM is expected to rise more rapidly in the future because of increasing obesity and reduced activity levels (Stumvoll, 2005). The Global data from the International Diabetes Federation (IDF) show that the prevalence of diabetes in adults (age > 20 years) in the Middle East and Africa is high and set to increase dramatically during the next 18 years, and the greatest increase in prevalence is expected to occur in Saudi Arabia (13.5-15.7%) (International Diabetes Federation, 2012). In Iraq, DM is considered common in Iraqi populations and its overall prevalence is 9.1% (Waad and Amjad, 2005) .The prevalence of hyperglycemia in Iraqis was reported to be 10.4% with an evident increase after the age 45 years (WHO 2005), while in 1979, a prevalence rate of 4.8% was reported in Iraqi rural populations (Foaud *et al*., 1979).

1.2.2.2 Aetiology of Type 2 Diabetes Mellitus

Type 2 diabetes is a heterogenous syndrome with many possible causes. It is due to the interaction of environmental factors with a genetic susceptibility to the disease, and it is clear that the relative contributions of genes and environment can differ considerably even

among individuals whose clinical phenotype is closely similar (Herder and Roden,2011).

Type 2 diabetes mellitus is characterized by three pathophysiological abnormalities; impaired insulin secretion, peripheral insulin resistance, and excessive hepatic glucose production (Powers, 2006).

The term "insulin resistance" indicates the presence of an impaired biological response to either exogenously administered or endogenously secreted insulin. This is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscles and by impaired suppression of hepatic glucose output. The insulin sensitivity is influenced by: age, weight, ethnicity, body fat (especially abdominal), physical activity and medication (Frier and Fisher, 2006). There is a moderate reduction in the total mass of pancreatic islet tissue in Type2DM, which is consistent with a measurable fall in plasma insulin concentration when related to the blood glucose level. While beta cell members are reduced by 20-30% in T2DM, alpha mass is unchanged and glucagon secretion is increased, which may contribute to the hyperglycemia (Herder and Roden , 2011).

The relative contributions of insulin resistance and β-cell dysfunction in the pathogenesis of Type 2DM vary among patients as well as during the course of the disease . Hyperglycemia can impair both insulin secretion and action; the so called "glucose toxicity" (DeFronzo, 1988). However, four main risk factors are considered as important in the etiology of T2DM; they are genetic factors , obesity, life style and early life malnutrition (American Diabetes Association, 2012).

Genetic Factors

Genetically, T2DM can be monogenic or polygenic. The monogenic forms, although relatively uncommon, are nevertheless important, and a number of the genes involved have been identified and characterized but these subtypes; such as maturity onset diabetes of the young (MODY) constitute less than 5% of all cases of diabetes (Frier and Fisher , 2006) . The genes involved in the common polygenic form or forms of the disorder have been far more difficult to be identified or characterized (Zimmer *et al*., 2001) . In this regard various genetic loci contribute to susceptibility, and environmental factors (such as nutrition and physical activity) further modulate phenotypic expression of the disease (Almind *et al*., 2001; Bell and Polonsky, 2001). The strongest evidence for T2DM susceptibility gene is for a locus designated "NIDDM1" on the short arm of chromosome 2. This locus accounts for about 30% of the genetic susceptibility among Mexican-American sibling pairs (Horikawa *et al*., 2000). Also the higher distribution of T45G polymorphism of adiponectin gene was detected among Iraqi type2 diabetic patients (Norrya and Ali ,2013).

The risk to develop the disease increases strikingly if there is a family history, especially among first-degree relatives. Furthermore, the concordance rate of T2DM among monozygotic twins is between 70 to 90%, which further confirms the genetic predisposition of the disease (Risérus and Willet, 2009) .

Obesity

Total body adiposity, a central fat distribution and the duration and time-course of developing obesity are all established risk factors for T2DM in both sexes , especially in those people who are genetically predisposed (Gregor and Hotamisligil, 2011) . Indeed, having a body mass index (BMI), which is calculated by dividing weight (in kilograms) by squared height (in meters) , of > 35 kg/m^2 increases the risk of developing diabetes over a 10-years period by a staggering 80-fold, as compared with thin individuals (BMI < 22 kg/m^2) (Lev-Ran, 1999). Obesity may have to be combined with genetic predisposition to insulin resistance or β-cell failure, or alternatively, there may be a common genetic predisposition that leads to both obesity and insulin resistance and so increase the risk of T2DM (Kahn *et al*., 2006).

The WHO has defined obesity in terms of body mass index (BMI) (WHO, 2000), and there is a strong curvilinear relation between BMI and relative body fat mass (Gallagher *et al.*, 2000). Table 1-1 summarizes the guidelines for classifying weight status by BMI (Klein and Romijn, 2008). These criteria for overweight and obesity represent imposed cutoff values between mortality rate and BMI and the prevalence of obesity-related disease such as diabetes, which begins to increase at BMI above 25.0 kg/m^2 (Colditz *et al*., 1995).

Table 1-1: Weight classification by body mass index.*

Weight Classification	Obesity Class	BMI(Kg/m^2)	
Underweight		<18.5 Low	Low
Normal		18.5-24.9	Normal
Overweight		25.0-29.9	Increased
Obesity	Obese	30.0-34.9	High
	Severity obese	35.0-39.9	Very High
	Morbidity obese	≥ 40.0	Extremely High

*(Klein and Romijn, 2008).

Several factors influence a BMI-related health risk; like persons with excess abdominal fat are at a higher risk for diabetes, hypertension, dyslipidemia, and ischemic heart disease than obese persons whose fat is located predominantly in the lower body (WHO,2008). Waist circumference is highly correlated with abdominal fat mass and is therefore, used as a marker of abdominal (upper body) obesity. Waist circumference values for men greater than 102 centimeters and for women greater than 88 centimeters have been proposed as cutoff values for an increased risk of a metabolic disease (National Institute of Health, 1998) .

Life Style

Sedentary life style is associated with high prevalence of diabetes in susceptible populations represented by habitual physical inactivity, high energy foods and smoking (American Diabetes Association, 2012). Mechanization and the replacement of physical active occupations may be important factors in raising the prevalence of T2DM in rural areas (Cock raw, 2000).

Malnutrition Early in Life

Malnutrition in utero and during the first year of life has been associated with a subsequent development of T2DM. It is proposed that malnutrition in utero may programme beta cell development and metabolic functions at a critical period, so predisposing to T2DM later in life. Smoking during pregnancy has also been implicated (Frier and Fisher, 2006).

1.2.3 The Proinsulin Hormone

Proinsulin is synthesized by the beta cell of the pancreas as a precursor molecule for insulin. Physiologically,virtually all proinsulin molecules are intracellularly cleaved by carboxypeptides into insulin and C-peptide.In healthy subjects, only a minor percentage of uncleaved intact proinsulin is released into the circulation. Progressive Insulin Resistance (IR) leads to an increased demand for insulin. Thus the cleavage capacity of the processing enzymes may be exhausted, and the intact precursor or partially processed proinsulin is secreted in addition to insulin and C-peptide. Intact proinsulin binds to the insulin receptor. However, it has only 10–20% of the glucose-

lowering effect of insulin but comparable adipogenetic activity (Breuer *et al*., 2010).

1.2.4 The Immune Response

The Diabetes Mellitus Type 2 is an inflammatory disease not only due to an innate immune response dominated by macrophage-mediated effects, but also by the adaptive immune response mediated by leukocytes (Clement *et al*.,2004) . A lymphocyte is a kind of white blood cell, and a landmark of the adaptive immune system. Small lymphocytes consist of T cells and B cells. T cells participate in the development of type 2 diabetes processes of insulin resistance, metabolic syndrome, T cells also produce proinflammatory cytokines, and growth factors, which modulate the local response and increase inflammation in type 2 diabetic patients (Wu *et al*.,2011). T cells are of two types ; helper T cell and cytotoxic T lymphocyte (CTLS). T helper cell (Th cells) assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages (Said *et al*., 2010). These cells are also known as Clusters of Differentiation 4+T (CD4+T) cells because they express the CD4 protein on their surface. The helper T cells become activated when they are presented with peptide antigens by major Histocompatibility Complex (MHC) class II molecules that are expressed on the surface of Antigen Presenting Cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response (Winter and Schatz, 2011). It is widely recognized that adaptive immunity CD4+ T cells can be differentiated into T-helper1(Th1),Th2, Th17, and T regulatory cells according to their cytokine profiles (Berrington *et al*., 2005) . Th1 cells

promote predominantly Cell Mediated Immunity (CMI) , Interleukin-12 (IL-12), Interferon –gamma (IFN–γ) and Tumor Necrosis Factor (TNF-a), also it induce delayed hypersensitivity reactions and activate macrophages. Nitric oxide (NO) synthesis by macrophage is induced by the interferon gamma (IFN –γ) and Tumor necrosis factor-alpha (TNF-α) and greatly increased when they act together (Lopamudra *et al*., 2001).Whereas Th2 derived cytokines (IL-3, IL-4, IL-5and IL-10) induce the production of particular classes of immunoglobulin antibodies and suppress Th1 cell activation (Ifere,2009). Th17, T helper cells preferentially producing interleukin-17, but not IFN-γ or IL-4. These three types of effector helper T cells: Th1, Th2, and Th17 which are regulated reciprocally to maintain a balance in immune-mediated disease (Musa *et al*., 2010).

Cytotoxic T cells (TC cells, or CTLs) destroy virally infected cells and tumor cells , and are also implicated in transplant rejection. These cells are also known as Cluster of Differentiation 8+T (CD8+ T) cells since they express the CD8 glycoprotein at their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of nearly every cell of the body . Cytotoxic T cells use various mechanisms to kill their targets, including direct cell-cell signaling via surface molecules and indirect signaling via various cytokines [interleukin (IL)-1,IL-2 and IL-12] (Faustman and Davis, 2009).

1.2.5 Cluster of Differentiation-Markers (CD-Markers)

CD- markers are used for the determination the identity of cell types based on the protein express on their cell surface .Using these markers, cells can be separated and stored based on their cell surface protein by application of flow cytometry .Depending on cell type, one marker alone or a combination of marker can be used to isolate a particular cell (Chia *et al*., 2010).

The CD- markers are glycoprotein characterized in two population of lymphocyte (T and B -lymphocyte) and Natural killer (NK) cells. Most T-helper cells express CD4,whereas most T- cytotoxic cells express CD8, NK cells express CD16 and CD56 ,and B cells express CD19,CD21,CD32 and CD35 (Ferreira *et al*.,2010). The CD4 cells involved in the regulation of the immune response and the T-CD8 cells have suppressive and cytotoxic activity .T- cell function involves the respective recognition of CD4 and CD8 by class II and class I MHC molecules, respectively which represent their natural ligands. The flow cytometry as well as alternative evaluation methods such as immunoperoxidase staining and immunofluorescent of lymphocytes enumeration , are based on the immunological detection of the markers of the cell surface with labeled specific monoclonal antibodies (Saleh, 2012).

The flow cytometry gives access to the percentage of CD4+ and CD8+ cells .The number of TCD4 and TCD8 decrease from birth and stabilize in the healthy adult . A mean value of a normal CD4 and CD8 count ranges from 500–1,000 cells/mm^3.When the CD4 or CD8 count is 350 cells/mm^3 or less, it’s time to consider treatment and when the count is fewer than 200

cells/mm^3 is one of the qualifications for a diagnosis of AIDS. The count can vary from day to day. It can also vary depending on the time of day and on the infections or illnesses. However CD4 and CD8 percentage is a more accurate measurement of the immune function, so the percentage that is greater than 29% usually means that the immune system is functioning normally, which mean that the CD4 count is roughly >500 cells/mm^3). The percentage of 14%-28% typically means the count is in the range of 200-500 cells/mm^3.When the count is below 200 cells/mm^3, the percentage is likely to be below 14%.(Riemann *et al* ., 2000).

1.2.6 Cytokines

Cytokines are soluble messenger molecules ,eg. Lymphokines (produced by lymphocyte) and interleukins (made by other white blood cells) that facilitate communication between different compartment of the immune system . Examples include Interferons ,Tumor Necrosis Factor- alpha (TNF-a), granulocyte -colony stimulating factor (G-CSF),granulocyte macrophage-colony stimulating factor (GM- CSF). These cause biological effects in destination cell population (e.g. activation, division or migration of destination cells) and often trigger inflammation (Gemmy, 2012).

The cytokines produced by variety of cells of the innate and adaptive immune system . Their major functional activities are concerned with the regulation of the development and behavior of the immune effector cells (Swardfager, 2010).The cells regulated by cytokines must express a receptor for the factor. Thus, cells are regulated by the quantity and type of cytokines to which they are exposed and by the expression of up regulation and down regulation of cytokine receptor .Cytokines act in concert with one

another to create synergistic effects that reinforce the other actions on a given cell .The interaction of multiple cytokines generated during atypical immune response are referred to cytokine cascade (Dowlati *et al* ., 2010).

1.2.6.1 Tumor Necrosis Factor-alpha (TNF- α)

Tumor necrosis factor-α , is a member of a group of cytokines that involved in systemic inflammation and it was discovered later independently as cachectin, a circulating mediator of wasting syndrome (cachexia) associated with chronic disease (Olszewski *et al*., 2007).

The primary role of TNF-a is the regulation of immune cells because of its ability to induce fever, apoptotic cell death, inhibit tumorigenesis ,viral replication , maintenance of secondary lymphoid organ structure, and host defense against various pathogens. So the TNF plays a critical role in bridging innate and adaptive immunity. However, its role in regulating the function of T regulatory cells or their impact on effector cells is presently unknown. Deregulation of TNF production has been implicated in a variety of human diseases including Alzheimer's disease, cancer, major depression, and Inflammatory disease (ID) (Swardfager *et al*., 2010).

It is a 26 kilodalton transmembrane protein that is cleaved into a 17 kilodalton biologically active protein that exerts its effects via type I and type II TNF-α receptors. Within adipose tissue, TNF-α is expressed by adipocytes and stromovascular cells (Olszewski *et al*., 2007).

Although initially suspected of playing a role in cachexia, TNF-α has now been implicated in the pathogenesis of obesity and insulin resistance (Hotamisligil , 2003). Adipose tissue expression of TNF-α is increased in

obese rodents and humans and is positively correlated with adiposity and insulin resistance. Although circulating concentrations of TNF-α are low relative to local tissue concentrations, plasma TNF-α levels have been positively correlated with obesity and insulin resistance in some studies but not others (Fernandez-Real and Ricart, 2003). Chronic exposure to TNF-α induces insulin resistance both in vitro and in vivo (Ruan and Lodish, 2003).

Several potential mechanisms for TNF-α's metabolic effects have been described. First, TNF-α influences gene expression in metabolically important tissues such as adipose tissue and liver (Ruan *et al*., 2002). In liver, TNF-α suppresses expression of genes involved in glucose uptake and metabolism and fatty acid oxidation and increases expression of genes involved in synthesis of cholesterol and fatty acids (Ruan *et al*., 2002). Second, TNF-α impairs insulin signaling, and this effect is mediated by activation of serine kinases that increase serine phosphorylation of insulin receptor substrate-1 and -2, making them poor substrates for insulin receptor kinases and increasing their degradation (Hotamisligil, 2003).

1.2.6.2 Interleukin-12(IL-12)

Interleukin-12 , is a cytokine produced by antigen presenting cells like Dendritic Cells (DC), macrophages also by NK cells. It plays a critical role in cell-mediated immunity. It affects a variety of stages in the immune response; it prompts NK cells and T cells to produce pro-inflammatory cytokines, such as IFN-γ, IL-2, IL-3 and TNF-α; it contributes to NK cell maturation (Blazhev *et al*., 2006); and, along with other pro-inflammatory factors, it stimulates CD4+CD25- T cell activation in the presence of regulatory T cells (Chueng *et al*.,2012). Interleukin-12 also regulates naive

T cell differentiation into T helper type 1 lymphocytes (Th1), and inhibits differentiation into T helper type 2 lymphocytes (Th2) (Kang *et al*., 2005). It has been documented that increased systemic inflammatory activity in patients with coronary artery disease is associated with a prominent Th1 response (Tan *et al*., 2013) Current data suggest that IL-12 plays a critical role in the pathogenesis of T1DM (Kang *et al*., 2005; Skarsvik *et al*., 2005), but the significance of IL-12 changes in the blood of patients with T2DM remains unclear. It has been observed that IL-12 plasma concentrations are elevated in T2DM (Winkler *et al*., 1998), and that IL-12 contributes to the process of atherosclerotic plaque formation and probably accelerates the development of macrovascular complications in T2DM (Hauer *et al*., 2005). Additionally, it has been noted that elevated glucose levels in diabetic animals stimulates inflammatory reactions related to IL-12 cytokine gene expression (Wen *et al*., 2006). However, it is not known whether factors related to the course of T2DM, such as metabolic compensation, beta cell secretory dysfunction, and insulin resistance affect IL-12 concentrations (Wegner *et al*., 2008).

1.2.7 Oxidative Stress

A diverse number of stimuli have been shown to induce apoptosis; many of them are also known to compromise the fine balance between intracellular oxidants and their defence systems (Kassab-Chekir *et al*., 2003).

Under aerobic situations, the participation of oxygen in redox reactions is unavoidable and a variety of highly reactive chemical entities are produced (Genestra, 2007). These are commonly referred to as Reactive Oxygen Species (ROS) and the list comprises the hydroxyl radical, hydrogen

peroxide, lipid peroxides, nitric oxide and superoxide. Many of these agents have a beneficial role in the cell but, when present in excess, the cell becomes oxidatively stressed (Halliwell, 2007).

A number of gross biochemical changes occur as a consequence of oxidative stress, the extent depending on the severity of the insult (Bahorun *et al* ., 2006). Oxidative overload causes gross cellular damage resulting in alteration of redox state (e.g. depletion of nucleotide coenzymes, disturbance of sulphur containing enzymes), saturation and destruction of the defense and repair systems. If the cellular balance is not restored, a number of pathological processes are elicited (Ceriello, 2008). Predominant processes resulting from oxidative stress include oxidative lipid degradation (lipid peroxidation), the loss of intracellular calcium homeostasis and alteration of metabolic pathways (Suneerat *et al*., 2010).

1.2.7.1 Free Radicals, Reactive Oxygen and Nitrogen Species

A free radical may be defined as a molecule or molecular fragments containing one or more unpaired electrons in it is outermost atomic or molecular orbital and are capable of independent existence (Valko *et al*., 2007).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radicals and other non-radical reactive derivatives (Pacher *et al*., 2007).The reactivity of radicals is generally stronger than non-radical formed from molecules by the homolytic cleavage of a chemical bond and via redox reactions. Once formed these highly reactive radicals can start a chain reaction (Fialkow *et al*., 2007). ROS and RNS include radicals such as superoxide (O2•−), hydroxyl (OH•), hydroperoxyl (HO2•), alkoxyl (RO•),

peroxyl (ROO•), nitric oxide (NO•), nitrogen dioxide (NO2•) and lipid peroxyl (LOO•); and non radicals like hydrogen peroxide (H2O2), hypochlorous acid (HOCl), ozone (O3), singlet oxygen (1O2), peroxynitrate (ONOO−), nitrous acid (HNO2), dinitrogen trioxide (N2O3), lipid peroxide (LOOH) (Pham-Huy et al.,2008). Non radicals are also termed as oxidants and capable to lead free radical reactions in living organisms easily. Radicals derived from oxygen are characterized as the most important class of radical species generated in living systems (Valko *et al*., 2006).

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Dalle-Donne *et al*., 2005). Superoxide (O2•−) radical is responsible for lipid peroxidation and also have the capability to decrease the activity of other antioxidant defense system enzyme such as glutathione peroxide (GPx); it causes damage to the ribonucleotide which is required for DNA synthesis (DeCoursey and Ligeti, 2005). The protonated form of O2• − is hydroperoxyl (HO2•), which is more reactive and able to cross the membrane and causes damage to tissue. Hydroxyl (OH•) radical is most reactive chemical species. It is a potent cytotoxic agent and able to attack and damage almost every molecule found in living tissue (Leonard *et al*., 2004). Hydrogen peroxide (H2O2) is not a radical but it produces toxicity to cell by causing DNA damage, membrane disruption and release calcium ions within cell, resulting in calcium dependent proteolytic enzyme to be activated. Hypochlorous acid (HOCl) is produced by the enzyme myeloperoxidase in activated neutrophils and initiates the deactivation of antiproteases and activation of latent proteases leading to tissue damage (Lobo *et al*., 2010). It has ability to damage biomolecules, directly and also decomposed to liberate toxic chlorine. Metal

induced generation of ROS attack DNA and other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Waris and Ahsan, 2006).

1.2.7.2 oxidative stress and Glycation reaction

Glycation is a reaction that takes place when simple sugar molecules, such as fructose or glucose, become attached to proteins or lipids without the moderation of an enzyme. This results in the formation of molecules known as advanced glycation endproducts (AGEs) (Fuentealba et al., 2009).

AGEs are a heterogeneous group of molecules formed from the non enzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. A series of subsequent reactions, including successions of dehydrations, oxidation-reduction reactions, and other arrangements lead to the formation of AGEs. A key characteristic of certain reactive or precursor AGEs is their ability for covalent crosslink formation between proteins, which alters their structure and function. Other major features of AGEs relate to their interaction with a variety of cell-surface AGE-binding receptors, leading either to their endocytosis and degradation or to cellular activation and pro-oxidant, pro-inflammatory events. A large body of evidence suggests that AGEs are important pathogenetic mediators of almost all diabetes complications. Since hyperglycemia is still considered the principal cause of diabetes complications, its deleterious effects are attributable among other things, to the formation of sugar-derived substances products (AGEs). A lowered glucose concentration will unhook the sugars from the amino groups to which they are attached; conversely, high glucose concentrations will have the opposite effect, if persistent (Glenn and Stirr,

2009). LDL-linked AGEs are significantly elevated in diabetic, as a result, are reported to exhibit greater AGE deposition in their arteries (Zhang et al., 2009).

The AGEs form at a constant but slow rate in the normal body, starting in early embryonic development, and accumulate with time. However, their formation is markedly accelerated in diabetes because of the increased availability of glucose (Kellow and Savige , 2012). AGEs may be less, or more, reactive than the initial sugars they were formed from. They are absorbed by the body during digestion with about 30% efficiency. Many cells in the body (for example, endothelial cells, smooth muscle, and cells of the immune system) from tissue such as lung, liver, kidney, and peripheral blood bear the Receptor for Advanced Glycation End-products (RAGE) that when binding AGEs, contributes to age- and diabetes-related chronic inflammatory diseases such as atherosclerosis, asthma, arthritis, myocardial infarction, nephropathy, retinopathy and neuropathy (Yan, 2007). The total state of oxidative and peroxidative stress on the healthy body, and the accumulation of AGE-related damage is proportional to the dietary intake of exogenous (preformed) AGEs, the consumption of sugars with a propensity towards glycation such as fructose and galactose (Fuentealba *et al*., 2009).

The Reactive Oxygen Species (ROS) generation, age, inflammatory stimuli, physical injury, hyperglycemia lead to AGE formation which consider a key first step in a broad array of injury settings (Kellow and Savige, 2012). AGE enhanced the attraction of the inflammatory cells, such as polymorphonuclear leukocytes, polymorphonuclear phagocyte , and lymphocytes by the release of S100/calgranulins and/or amphoterin as a transducing signal for the Receptor of Advanced Glycation End-products

(RAGE), thereby allowing them to engage the receptor in an autocrine and paracrine manner triggers a new wave of inflammatory and cell stress reactions , causes the further generation of ROS; such ROS may be cause further AGE generation, inflammation, and ROS production, also to sustain the cycle of stress in a wide range of cell types, such as Endothelial Cell (EC), Smooth Muscle Cell (SMC), Mononuclear Phagocyte (MP),Peripheral Blood Mononuclear Cell (PBMC) , podocytes, neurons, and glia, and, thus, eventually cause tissue dysfunction and damage (Ramasary *et al*., 2005).

Figure (1.1) Specific consequences of Advanced Glycation End-product accumulation. AGE: Advanced Glycation End-product (AGE). AR: Aldose Reductase.EC: Endothelial Cell. NADPH: Nicotinamide Adnine Dinucleotide phosphate- oxidase. PBMC: peripheral blood mononuclear cell. PMNs: Polymorphonuclear leukocyte .ROS: Reactive Oxygen Species. SMC:Smooth Muscle Cell. MMPs: Matrix metalloproteinase. PMNs:Mononuclear Phagocyte. MPO: Myeloperoxidase (Ramasamy *et al* ., 2005).

1.2.8 Apoptosis Detection Method

1.2.8.1 DNA Fragmentation Analysis

Apoptosis is a form of cell death characterized by cell shrinkage, chromatin condensation, fragmentation of the nucleus and bubbling of the plasma membrane (known as “blebbing”), long-lasting maintenance of plasma membrane integrity, lack of inflammatory responses to the dying cell, with the formation of apoptotic bodies that are consumed by macrophages or neighboring cells (Taylor *et al* ., 2008).

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis. This assay involves extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis (Elmore, 2007). These result in a characteristic “DNA ladder” with each band in the ladder separated in size by approximately 180 base pairs (Galluzzi and Kroemer, 2008).

Since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis (Kroemer *et al*., 2009). Additionally, DNA fragmentation can occur during preparation making it difficult to produce a nucleosome ladder and necrotic cells can also generate DNA fragments (Shemarova, 2010; Kaufmann *et al*., 2012).

1.2.9: Flow cytometry

Flow cytometry is a laser based, biophysical technology employed in cell counting, sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic

detection apparatus. It allows simultaneous smultiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second (Khong and Restifo, 2002).

Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in basic research, clinical practice and clinical trials. A common variation is to physically sort particles based on their properties, so as to purify populations of interest (Hallett *et al*., 2008).

1.2.9.1 Principle of flow cytometry

A beam of light (usually laser light) of a single wavelength is directed onto a hydrodynamically-focused stream of liquid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescence detectors (Sherr, 2000). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source.

This combination of scattered and fluorescent light is picked up by the detectors, and, by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle (Paietta, 2002). FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane

roughness). This is because the light is scattered off of the internal components of the cell. Some flow cytometers in the market have eliminated the need for fluorescence and use only light scatter for measurement. Other flow cytometers form images of each cell's fluorescence, scattered light, and transmitted light (Jacobberger, 2001).

Figure 1-3 Scattered and emitted light signals are converted to electronic pulses that can be processed by the computer.

2. Materials and Methods

2.1 Materials

2.1.1 Equipment

The used equipment and their sources are given in Table (2-1)

Table (2-1): The used equipments and their sources .

Name of Equipment	Company	Origin
Autoclave	P-Selecta/Medic lane	Spain
Automatic micropipettes	Gillson P10,P100,P1000	Germany
Balance	MettlerAE240	Swiss
Blood collection plain tubes	AFMH	England
Blood collection conical tubes	AFMH	England
Cooling centrifuge	Tomy Seiko company	Japan
Cover slips	MAI	Germany
Digital camera	Sony	Japan
Distillator	American	USA
EDTA containing tubes	AFMH	England
Electrophoresis power supply	ATTO	Japan
Eppendorff bench centrifuge	Nether land and Hinz Gmb 2000	Germany
Eppendorff tube	Eppendorf	Germany
Flow cytometry	Apogee	England
Gel electrophoresis	Bio-Rad	Italy

Hemocytometer	Boeco	Germany
ecocs0 r chmgthgiL	Olympus	Japan
Magnetic stirrer with hot plate	Lassco	India
Micro ELISA system (reader).	Thermo	Germany
Microscope Slides	MAI	Germany
multichannel micropipettes	Gilson	France
Pipette tips	Gilson	France
pH meter	Orient	USA
Plastic disposable syringes; 5ml	Meheco	China
Printer	Epson	UK
Refrigerator and freezer (-20°C)	Arcelik	Turkey
Sensitive balance	Sartorius	Germany
Spectrophotometer	Cintra 5-GBC	France
UV light transiluminator	Ultra violet products institute	USA
Volumetric cylinders	Volac	England
Volumetric Flasks	Volac	England
Vortex	Clay Adams	Germany
Water bath	Memmert	Germany

2.1.2 Chemicals

The used chemicals and their sources are given in Table (2-2):

Table (2-2): The used chemicals and their sources .

Name of chemical	Origin
Absolute Ethanol	Fluka
Agarose	Sigma
Ammonium Chloride (NH_4CL)	Fluka
Anti_CD4 Marker(cat.T9-359)	ExBio
Anti_CD8 Marker(cat.T9-209)	ExBio
Bromo Phenol Blue(loading buffer)	BDH
Boric Acid	BDH
Cholesterol Kit (cat.AT 80106)	Biolab
Cholesterol_HDL Kit (cat.AT 80106)	Biolab
Deionized Water	Promega
DNA Ladder Marker(100pb)	promega
Ethidium bromide (EtBr)	Sigma
Ethylene Diamine Tetra Acetic Acid (EDTA)	HiMedia
QIAGEN DNA blood mini Kit (cat.51104)	QIAGEN
Glucose Kit (cat.EC 12249)	Biolab
Glycerol	Fluka
Interleukin-12(IL-12) ELISA Kits (cat. D1200)	R&D System
Lymphopreb	Sigma

Normal Saline	ADWIC
Oxalic Acid	Fluka
Phosphate buffer saline	Oxoid/England
Proinsulin hormone Kit ELISA(cat.DE1560)	De Medi tec
Potassium Bicarbonate ($KHCO_3$)	Fluka
Trichloroacetic Acid (TCA)	Fluka
Triglyceride Kit (AT80019)	Biolab
Thiobarbituric Acid (TBA)	Fluka
Trypan blue	BDH
Trise Base	Sigma
Tumor Necrosis Factor_Alpha(TNF_a) ELISA Kits(cat. DTA00C(	R&D System

2.2 Subjects

2.2.1 Type 2 Diabetes Mellitus Patients

The study was conducted on 50 type 2 diabetes mellitus (T2DM) patients, 26 females and 24 males, (30-60 years) randomly selected from those attending the National Diabetes Center for Treatment and Research at Al-Mustansiriya University between December 2012 and January 2013.

A well-structured questionnaire was filled for every subject and patient (Appendix I) after full clinical exanimation by their consultant physicians. Patients were subjected to detailed history for collection of demographic data and recording of relevant medical history and medications. Thorough clinical examination including neurological examination, height and weight measurement for calculation of body mass index were also done for all

patients .Any patient with a history of smoking was excluded. They had no history of alcohol drinking. The diagnosis of T2DM was made on the basis of the recommended criteria by WHO (1999).

2.2.2 Control subjects

For the purpose of comparisons, 30 healthy control subjects comparable to diabetes mellitus patients in respect to age (30-60 year) and gender (18 females and 12 males), were included in the study. The controls were selected among subjects who were apparently healthy in terms of non-diabetic, no other endocrine disorders or metabolic kidney diseases and were free of acute illness or infection at time of sampling. Also, they had no history of smoking or alcohol drinking.

2.2.3 Characteristics of patients and subjects

Type 2 diabetes mellitus patients and controls were characterized in terms of age, gender, family history of diabetes, body mass index (BMI), waist-to-hip (W-H) ratio. The patients were also checked for duration the of disease. The BMI was calculated by dividing weight (kilogram) by the squared height (meter)[National Institute of Health(1998)].

2.3 Collection of blood samples

From patients and control subjects, in fasting state by venipuncture, using a 5 ml disposable syringe between 8 to 9 A.M, 3ml of blood were obtained and dispensed in a plain tubes and left for an hour to clot at room temperature (22°C). Then, it was centrifuged at 3000 rpm for 10 minutes to collect serum. The serum was divided into aliquots in eppendorff tubes for measuring the lipid profile (fasting blood glucose , cholesterol , HDL cholesterol ,LDL cholesterol and triglyceride) the serum were stored in deep freezer (-20◦c)

and used later for measuring the cytokines (TNF-a and IL-12(.From the same patients and controls , a second 3 ml were obtained in (EDTA) tubes for the measurement of Hb A_{1C}, proinsulin hormone , lymphocytes separation and DNA extraction .

2.4 Laboratory Methods

2.4.1 Preparation of Solutions and Buffers

2.4.1.1 Solutions used in lymphocytes Separation

2.4.1.1.1 Preparation of Red blood cell lysis buffer

Red blood cell lysis was prepared by dissolving the following chemical in appropriate amount of water, the pH was adjusted to 9 and the volume was completed to250 ml of distilled water:

2.0725 g	Ammonium Chloride (NH4CL)
0.25 g	Potassium Bicarbonate(KHCO3)
0.00925 g	EDTA

and then autoclaved and stored at 4◦c (Aspaller *et al*.,1972)

2.4.1.1.2 Preparation of Phosphate Buffer Saline (PBS)

The solution was prepared by dissolving one tablet of PBS in appropriate amount of distilled water, the pH was adjusted to 7.2 and the volume was completed to 100 ml distilled water. Then the solution was autoclaved (121◦C, 20 min) and stored at 4◦C until use (Marlise, 1997).

2.4.1.1.3 Preparation of Trypan Blue Stain (1%)

The stain was prepared by dissolving 1g of the stain powder in 100 ml phosphate buffer saline (PBS), then filtered using filter paper whatman No.1 The clear solution was diluted immediately before use in a ratio of 1:10 with PBS(Ad 'hiah, 1990).

4.1.2 Solutions used in Agarose gel electrophorsis

1-Tris-borite-EDTA (TBE) Buffer: To prepare 10x TBE solution, the components used were : 108 g of Tris-Base, 55g of Borric acid, 40ml of 0.5M EDTA (pH=8.0) in an appropriate amount of D.W, pH was adjusted to 7.8 and volume completed to 1 liter with distilled water. The solution was sterilized by autoclave and stored at room temperature (Sambrook *et al*.,2001).

2- Ethidium bromide solution (10mg/ml): It was prepared by dissolving 1g of Ethidium bromide powder in 100 ml of a sterile D.W., and the bottle was kept in a dark (Maniatis *et al*., 1982).

3-Loading buffer: It was prepared by dissolving 0.25g of bromo phenol blue –xylene cyanole dye in 50 ml D.W, 30ml of glycerol was added. The volume was completed with 100 ml distilled water and stored at 4˚C (Manaitis *et al*.,1982).

2.5 Determination of Fasting Blood Glucose (FBG)(Glucose kit, Biolab)

a) An aliquot (10µl) of the serum was added to tubes containing 1ml of reagent R, then it were mixed well and incubated at 37ºC for 10 min.
b) An aliquot (10µl) of the serum was added to tubes containing 1ml of Standard, then it were mixed well and incubated at 37ºC for 10 min (Standard).
c) An aliquot 1ml of reagent R was added to the tubes and incubated at 37ºC for 10 min (Blank).
d) The samples and standard , were read at (505 nm) wave length against the Blank. The color is stable for at least 30 minutes.
e) The Glucose concentration (mg/dl) was calculated in the sample as follow: Glucose mg/dl = $\frac{(\quad)\qquad\qquad(\qquad\qquad)}{(\quad)}$

2.6 Determination the Cholesterol level (Cholesterol kit, Biolab)

a) An aliquot (10µl) of the serum was added to tubes containing 1ml of reagent R, then it were mixed well and incubated at 37ºC for 5 min.

b) An aliquot (10µl) of the serum was added to tubes containing 1ml of Standard, then it were mixed well and incubated at 37ºC for 5 min (Standard).

c) An aliquot (10µl) of distilled water was added to tube containing 1ml of reagent R and incubated at 37ºC for 5 min (Blank).

d) The samples and standard were read at (500 nm) wave length against the Blank. The color is stable for 1 hour.

e) The Cholesterol concentration (mg/dl) was calculated in the sample as follow:

$$\text{Cholesterol mg/dl} = \frac{(\quad)\qquad\qquad(\qquad\qquad)}{(\quad)}$$

2.7 Determination of High Density Lipoprotein Cholesterol level (HDL Cholesterol) (HDL Cholesterol kit, Biolab).

a_An aliquot (10 µl) of the serum was added to tubes containing 1ml of reagent R, then it was mixed well and incubated at 37ºC for 5 min.

b_ An aliquot (10 µl) of the serum was added to tube containing 1ml of Standard , then it was mixed well and incubated at 37ºC for 5 min Standard.

c_ An aliquot (10µl) of distilled water was added to tube containing 1ml of reagent R and incubated at 37ºC for 5 min (Blank).

d_ The samples and standard, were read at (500 nm) wave length against the Blank. The color is stable for 1 hour.

e_ The HDL Cholesterol mg/dl was calculated in the sample as follow:

HDL Cholesterol mg/dl $\frac{(\quad)\qquad\qquad(\qquad\qquad)}{(\quad)}$

2.8 Determination the Low Density Lipoprotein Cholesterol level (LDL) (Tietz, 1995)

LDL cholesterol= Total cholesterol _Triglyceide/5_HDL Choleterol

2.9 Determination of Triglycerides level (Triglycerides kit, Biolab)

a. An aliquot (10µl) of the serum was added to tubes containing 1ml of reagent R, then it were mixed well and incubated at 37ºC for 5 min.

b. An aliquot (10µl) of the serum was added to tubes containing 1ml of Standard, then it were mixed well and incubated at 37ºC for 5 min (Standard).

c. An aliquot (10µl) of distilled water was added to tubes containing 1ml of reagent R and incubated at 37ºC for 5 min (Blank).

d. The samples and standard were read at (500 nm) wave length against the Blank. The color is stable for 1 hour.

e. The Triglyceride mg/dl was calculated in the sample as follow:

Triglycerides mg/dl = $\frac{(\quad)\qquad\qquad(\qquad\qquad)}{}$

2.10 Determination of Glycosylated hemoglobin A_{1C} (Hb A_{1C}) (Winterhalter *et al*.,1981)

2.10.1 Lysate solution prepration

An aliquot (1ml) EDTA-treated anticoagulant blood were washed three times with 8.5% normal saline and were lysed by addition equal volume of distilled water .

2.10.2 Estimation of Hb A_{1C}

Approximately (0.5 ml)of saline_ washed red blood cells were lysed with 1.8 ml of water and 0.4 ml of carbon tetrachloride with vigorous shaking at room temperature .The hemolysate was free of cellular debris by centrifugation, and the Hb concentration was subsequently adjusted to 50 mg/ml with distilled water. To 20 ml of this hemolysate was added 1.0 ml of 1.0 N oxalic acid. After mixing, the solution was incubated at 100◦C for 4.5 hours and subsequently cooled at room temperature. One ml of 40% Trichloroacetic Acid (TCA) was added, and, after mixing, the precipitation was removed by centrifugation. From the resulting clear supernatant, 2ml are mixed with 0.5 ml of saturated Thiobutri Acid (TBA) solution prepared by dissolving 0.05 ml of TBA in 1000 ml distilled water at 60◦C for 1 hour. After mixing the solution was kept at 40◦C for 30 minute, then cooled at room temperature. The reaction was measured at 443 nm, and the mean glycosylation of hemoglobin A1C was determined using an extinction coefficient of $6x10^{-2}$ at 443 nm.

2.11 Determination of Proinsulin Hormone

•Kits Content :

1**.** Microtiter Plate: 1 x 96 wells pre-coated with streptavidin anti-insulin monoclonal antibody (ready-to-use).

2. proinsulin standards.

3. Controls (low and high).

4. Sample Diluent.

5. Enzyme Conjugate 11x concentration1.

6. Conjugate Diluent.
7. Assay Buffer.
8. Substrate Solution.
9. Washing buffer stock (40x).
10. Stop Solution (1N acidic solution).

- **Preparation of solution**

1_**Washing solution**: An aliquot (30 ml) of washing concentrate was diluted with 1170 ml distilled water to final volume of1200 ml.

2_**Conjugate solution**: The Enzyme conjugate was diluted 1:1ml with conjugate diluent per strip.

3_**standard solution**: The standard vials were diluted with 1.0 ml of distilled water.

4_**control solution**:The control vials were reconstituted with 2.0 ml of distilled water.

5_**Specimen solution**: An aliquot (10µl) of serum was diluted with 90µl of sample diluent .

- **Assay procedure**

1_The desired number of microliter well was secured in the holder .

2_ An aliquot (100µl) of each standard , control , and sample were dispensed into appropriate wells.

3_ An aliquot (100µl) assay Buffer was dispensed to each well and mixed for 10 seconds.

4_The plate was covered with a plate sealer and was incubated overnight (16-24hours) at 4◦C in a humidity chamber.

5-The wells were rinsed 3 times with diluted wash solution (350µl per well then the wells were stroked sharply on absorbent paper to remove residual droplets .

6-An aliquot (100µl) of diluted enzyme conjugate was dispensed to each well.

7-The plate was incubate at 60 minute at room temperature (without covering the plate).

8-The wells were rinsed three times with diluted wash solution (350µl per well). The wells were stroked sharply on absorbent paper to remove residual droplets.

9- An aliquot (100µl) of substrate solution was added to each well .

10-The plate was incubated at 30 minute at room temperature .

11-The enzymatic reaction was stopped by adding 50µl of stop solution to each well.

12-The absorbance was read at a wave length of 450 nm using ELISA reader within 30 minutes.

- **Calculation**

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample Figures

(2.1), using a curve fitting equation

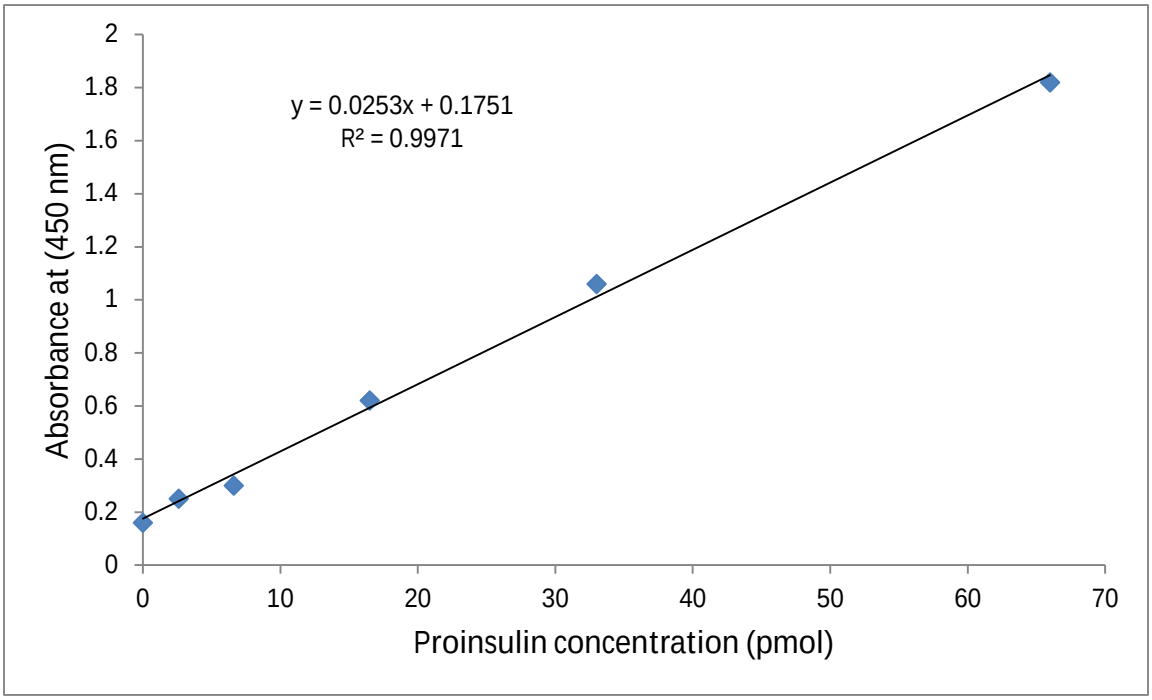


Figure (2.1) Standard curve of Proinsulin hormone.

2.12 Analysis of T-Lymphocyte Subset by Flow cytometry

2.12.1 Lymphocytes Separation

The Lymphocytes were separated from blood sample by density gradient centrifugation according to the protocol described by Erices *et al*. (2003) with some modification as shown in the figure (2.2).

1- Fresh Blood was diluted in a proportion of 1:1 with phosphate buffer saline.

2-The diluted blood was carefully layered into conical tubes containing the lymphoprep solution.

3- The mixture were centrifuged at 4°C at 2700 rpm for 30 min to isolate the lymphocytes .

4-The upper layer was drew off using a clean Pasteur pipette, the lymphocytes rich zone was isolated, transferred into a new 15 ml conical tubes.

5- An aliquot (1ml) of the cell lysis buffer was added and the conical tubes were centrifuged at 2700 rpm at 4◦c for 15 min.

6-The mixture were washed twice with phosphate buffer saline through centrifugation at 2700 rpm at 4◦c for 15 min, the supernatant was discarded, phosphate buffer saline was added again and the mixture were centrifuged again at 2700 rpm for 15 min and the final pellet was re-suspended in a clean ependorff tubes.

Figure (2.2): Isolation of lymphocytes from blood sample.

2.12.2 Counting and Viability Assessment of Lymphocytes

Counting the cells were performed according to porakishvili *et al.* 2004. Cells were examined under phase contrast microscope (10x magnification) to monitor cells, then cells were collected by centrifugation at 2000 rpm for 5 min and resuspended with 5 ml complete phosphate buffer saline (PBS) . An aliquot (10µl) of cell suspension was mixed wih 90µl of trypan blue for 3 min and a drop allowed to spread under the cover slip of a haemocytometer taking care not to over fill it and making sure that the cover slip was firmly in place, using the light microscope at low power. Total numbers of cells (viable and dead) were calculated in the four Neumbaur hemocytometer chamber as following (Doyle and Griffiths, 1998):

The cell viability of the samples was determined. The cells with 90% viability were ready to test, and those with less than 90% cell viability were discarded.

At the same time , the number of lymphocyte were counted by light microscope and the cells concentration was adjusted to 2×10^6 cell/ml in 1 ml (PBS).

2.12.3 Detection of (CD4+ and CD8+)T cells (Littman,1987)

Immunofluorescent staining was used to identify lymphocyte. Two sets of single staining monoclonal antibodies labeled with fluorescein isothiocyanate (FITC) in the order of anti_CD4 and anti_CD8 were used as follow:

1-An aliquot (100 µl) of the resuspend lymphocytes cells were transferred into a clean eppendorff tubes.

2-An aliquot (20 µl) of anti- CD4 and anti-CD8 markers labeled with fluorescein isothiocyanate (FITC), were added into separated eppendorff tubes then it was incubated for 20_30 minute at room temperature in a dark place**.**

3-After incubation, unreacted CD4+ T cells and CD8+ T were removed by washing the cells with 1 ml of Phosphate Buffer Saline (PBS), the eppendorff tubes were centrifuged at 2700 rpm for 10 minute.

4-The supernatants were removed and the cells were suspended with 400 µl of Phosphate Buffer Saline (PBS) for analysis by flow cytometry.

2.13 Serum Levels of Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-12(IL-12).(TNF-α and IL-12 ELISA Kits, R&D system).

• **Kit Contents**

1. Microtiter Plate: 1 x 96 wells pre-coated with anti-human TNF-α monoclonal antibody or IL-12 (ready-to-use).
2. Anti-human TNF-α and IL-12 antibody conjugated to biotin.
3. Avidin conjugated to horseradish peroxidase (HRP).
4. TNF-α and IL-12 standards.
5. Calibrator diluents (animal serum with buffer).
6. Washing buffer stock (buffered surfactant) (20X).
7. Buffered solution with H2O2 (A).
8. Buffered solution with TMB (B).
9. Stop solution (2N sulfuric acid solution; H2SO4) .

• **Assay procedure for TNF-α**

Before carrying out the assay procedure, the kit was left at room temperature (18-25ºC) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

i. Serial concentrations (0, 31.25, 62.5, 125, 250, 500, 1000 and 2000) pg/ml of the standard were made using the diluent.

ii. An aliquot (50µl) of (Biotin) was added to the pre-coated wells.

iii. An aliquot (200 µl) of the standard and serum was added into the appropriate well and then mixed well, covered and incubated for 120 minutes at room temperature.

iv. The wells were washed with five cycles of washing (350µl/well/wash) using the washing solution, with the aid of a microtiter plate washer. The washing buffer was prepared by diluting 60 ml of the washing buffer stock up to 1200 ml with distilled water.

v. An aliquot (100 µl) of avidin (HRP) was added to each well. After mixing the contents of wells, the plate was covered and incubated for 120 minutes at room temperature.

vi. The washing step was repeated (step iv).

vii. The substrate solution was prepared no more than 15 minutes before the end of incubation period (step v) by mixing equal volumes of A and B. Then, 200 µl of substrate solution was added to each well. After mixing the contents of wells, the plate was covered and incubated for 20 minutes at room temperature.

viii. An aliquot (100 µl) of stop solution was added to each well and the absorbance was read at a wave length of 450 nm using ELISA reader within 30 minutes.

• **Assay Procedure for IL-12**

Before carrying out the assay procedure, the kit was left at room temperature (18-25ºC) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kit's leaflet, which are summarized in the following steps:

i. Serial concentrations (0, 31.25, 62.5, 125, 250, 500 and 1000) pg/ml for IL-12 of the standard were made using the diluent.

ii. An aliquot (50µl) of (Biotin) was added to the pre-coated wells.

iii. An aliquot (200 µl) of the standard and serum was added into the appropriate well and then mixed well, covered and incubated for 120 minutes at room temperature.

iv. The wells were washed with five cycles of washing (350µl/well/wash) using the washing solution, with the aid of a microtiter plate washer. The washing buffer was prepared by diluting 60 ml of the washing buffer stock up to 1200 ml with distilled water.

v. An aliquot (100 µl) of avidin (HRP) was added to each well. After mixing the contents of wells, the plate was covered and incubated for 120 minutes at room temperature.

vi. The washing step was repeated (step iv).

vii. The substrate solution was prepared no more than 15 minutes before the end of incubation period (step v) by mixing equal volumes of A and B. Then, 200 µl of substrate solution was added to each well. After mixing the contents of wells, the plate was covered and incubated for 20 minutes at room temperature.

viii. An aliquot (100 µl) of stop solution was added to each well and the absorbance was read at a wave length of 450 nm using ELISA reader within 30 minutes.

• Calculation: The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for the sample (Figure 2-3) (Figure 2-4) using a curve fitting equation.

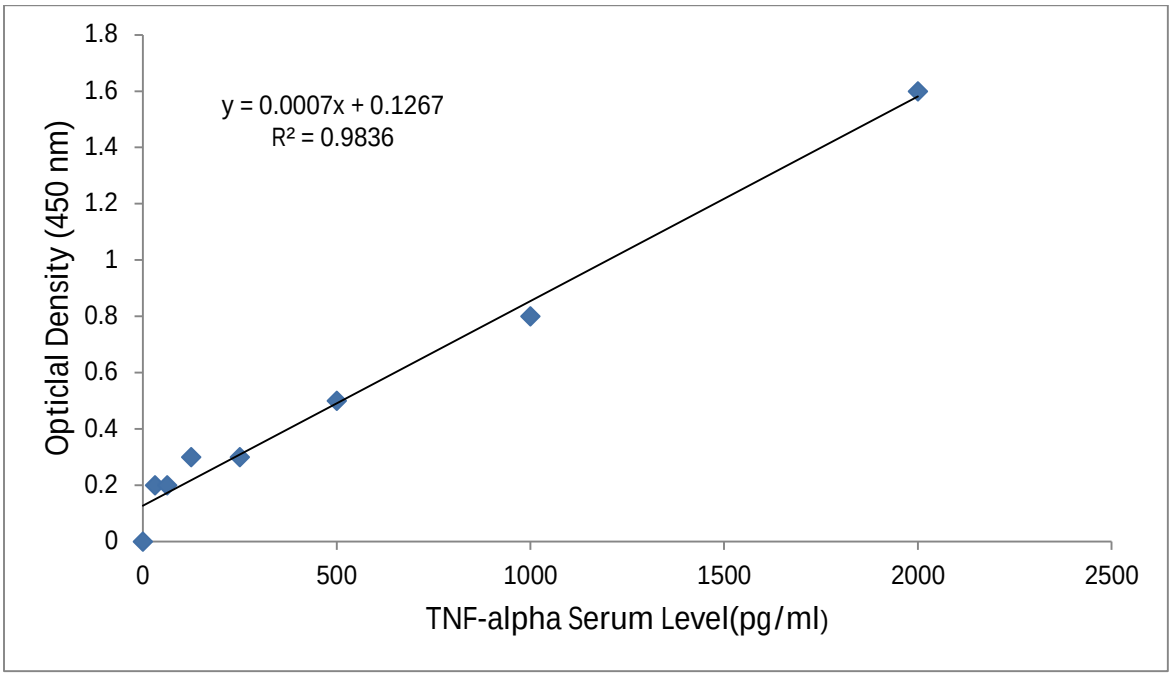


Figure (2.3) Standard curve of TNF-a

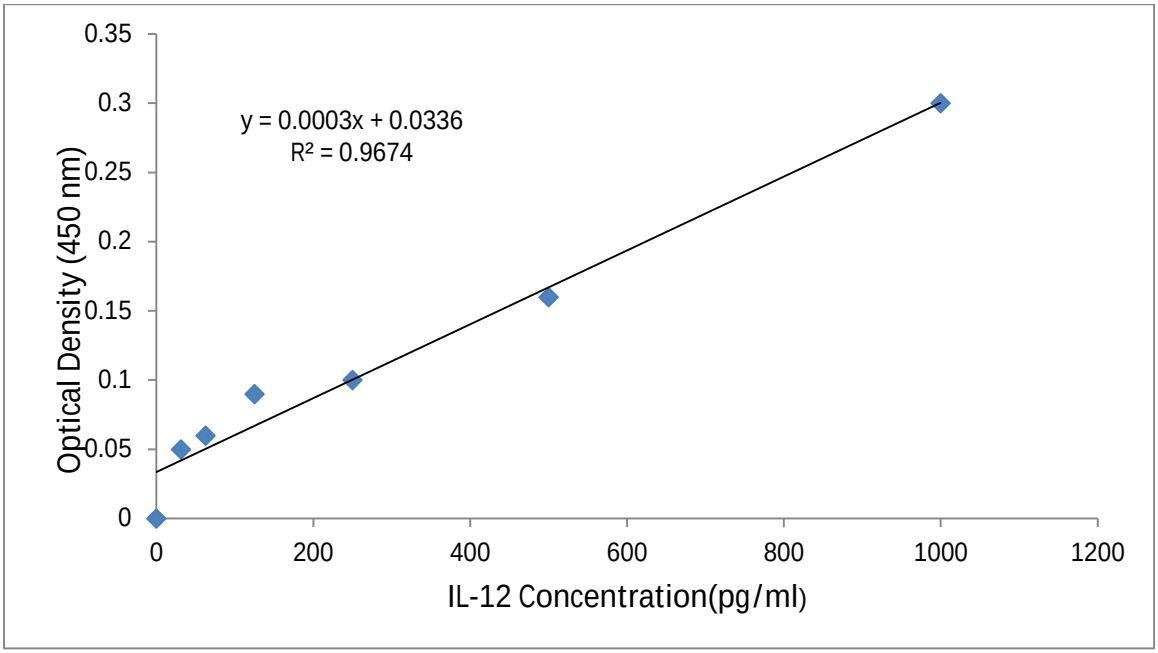


Figure (2.4) Standard curve of IL-12

2.14 Isolation of DNA

- Kits contents
- QIAGEN protease or (proteinase K).
- Lysis Buffer (AL).
- Elution Buffer (AE).
- Washing Buffer 1 (AW1).
- Washing Buffer2 (AW2).
- QIAGEN mini spin columns.
- QIAGEN collection tubes.

•Protocol of DNA Isolation (DNA extraction kit, QIAGEN)

1. An aliquot (20µl) of QIAGEN protease (or proteinase K) was pipetted into the bottom of 1.5 ml micro centrifuge tubes .

2. An aliquot (200µl) of bloods were added to the micro centrifuge tubes.

3. An aliquot(200µl) of the Lysis Buffer (AL) was added to the samples. Then they were mixed by pluse-vortexing for 15 seconds and incubated at 56◦c for 10 minute.

4.The microcentrifuge tubes were centrifuged briefly to remove drops from the inside of the lid.

5. An aliquot(200µl) ethanol was added to the samples, and they were mixed again by pluse-vortexing for 15 seconds .

7. The mixture was carefully applied from step 6 to the QIAGEN mini spin columns (in a 2 ml collection tube) and centrifuged at 8000 rpm for 1minute .The QIAGEN mini spin columns were placed into a clean 2ml collection tube and the tube containing the filtrate was discarded.

8. An aliquot (500µl) of the washing buffer 1(AW1) was added to the mini spin columns and centrifuged at 8000 rpm for 1minute . The mini spin columns were placed in a clean 2ml collection tubes ,and discard the tubes containing the filtrate.

9. An aliquot (500µl) of washing buffer2(AW2) was added to the mini spin columns and centrifuged at full speed (20,000xg;14,000 rpm) for 3 minute.

10. The mini spin columns were placed in a new 2ml collection tubes and the old collection tubes were discarded with the filtrate, then Centrifuged at full speed for 1 minute.

11.The mini spin columns were placed in a clean 1.5 ml microcentrifuge tubes , and the collection tubes were discarded . An aliquot (200µl) of the Elution Buffer (AE) was added to the mini spin columns , incubated at room temperature (15_25◦c) for 1min ,and then centrifuged at 6000xg(8000 rpm) for 1 minute. The DNA were collected in microcentrifuge tubes and stored at-20◦c.

2.15 Agarose Gel Electrophoresis (Sambrook *et al*., 1989).

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of extracted DNA.

2.15.1 Reagent of Gel Electrophoresis

- Agarose
- 1X TBE Buffer
- Loading Buffer
- Ethidium Bromide

2.15.2 Protocol of Gel Electophoresis

2.15.2.1 Preparation of Agarose Gel

A quantity of (0.8) g agarose was completely dissolved in 50 ml of 1x TBE buffer solution , then 0.5 ml of ethidium bromide solution (0.5µg/ml) was added.

2- The agarose was stirred in order to be mix and avoid making bubbles.

3- The solution was left to cool down at 50 – 60 ^{o}C.

2.15.2.2 Casting of the Horizontal Agarose Gel

After sealing both edges of the gel tray with a cellophane tapes and fixing the comb in 1 cm away from one edge, the agarose solution was poured into the gel tray. The agarose was allowed to solidify at room temperature for 30 minutes. The fixed comb was carefully removed and the gel tray was placed in the gel tank. The tank was filled with 1 X TBE buffer until it reached 1-2 mm over the surface of the gel.

2.15.2.3 DNA Loading and Electrophoresis

DNA (7 µl) was mixed with 3 µl of Loading buffer . Samples are loaded carefully into the individual wells of the gel, and then electrical power was turned on at 70 volt for 1 hour, afterwards the DNA moved from cathode (-) to anode (+) poles. The ethidium bromide stained bands in the gel are visualized using UV transiluminator at 340 nm and photographed.

2.15.2.4 Estimation of DNA Concentration (Sambrook *et al*.,1989)

DNA samples that showed acceptable integrity are diluted to 1:10 or 1:20; afterwards the optical density was read with spectrophotometer at wave length 260 nm. The concentration of the DNA in µg /ml of samples was calculated according to the following equation:

(OD.260 nm x dilution factor x 50 µg /ml = ( ) µg /ml))

For measuring the purity of DNA, reading was taken at wave length 280 nm as: The purity of DNA = A 260 / A 280 = ~ 2

2.16 DNA Fragmentation Analysis

The DNA fragmentation was used to determine apoptosis induction by observation of biochemical change. DNA fragmented technique was done by a method described by Rodrigues *et al*. (2009).

The DNA in the cell pellet was extracted using QIAGEN kit , 2 µg of DNA and 6 µl of DNA ladder loaded alongside the DNA samples as a standard are analyzed by gel electrophoresis described previously (3.15) but on 1.8% w/v agarose gel containing 0.75% w/v ethidium bromide.

The gel electrophoresis was run at 250 voltages for 1 minute and 20 voltages for 4 h. After electrophoresis, DNA fragment was analyzed by using UV-laminated camera

2.17 Statistical Analysis

The values of the investigation parameters were given in term of mean ± standard division , and differences between means assessed by analysis of variance (ANOVA) , least significant differences (LSD) and Duncan test . The difference was considered significant when the probability value was equal or less than 0.05.

The value of the body weight and blood glucose level before and after treatment were expressed as mean ± SD . The differences was considered significant when the probability value was equal or less than 0.05. All statistical analysis of investigated parameter was assessed using the statistical package (SPSS v. 7.5). (Sorlie, 1995)

3. Results and Discussion

3.1 General Parameters in Type II Diabetes Mellitus

3.1.1 Age, Gender, Body Mass Index, Waist to Hip ratio, The duration of disease.

The Type 2 Diabetes mellitus(T2DM) patients showed a significant increase in BMI in comparison with controls (31.80 *vs*. 29.70 kg/m^2) .The W-H ratio showed a significant ($P \leq 0.01$) increase in Type 2 diabetic patients (1.02 *vs*. 0.95) Table (3-1).

Table (3-1) General parameters in healthy controls and Type2 diabetes.

Variables	Healthy controls (n=30)	Type2diabetic Patients (n=50)	P-Value
Age	48.40±1.31	49.48±1.03	N.S
Gender(F/M)	18/12	26/24	
BMI(kg/m^2)	29.70±0.71	31.80±0.80	≤0.001
Waist-to-Hip Ratio	0.95±0.01	1.02±0.02	≤0.01
Duration of disease(years)	–	9.7±2.1	–

Data are represented as mean ± SD.,

BMI(body mass index)

The age, gender distribution duration of the disease were divided into three groups as shown in Table (3-2).

Table (3-2) Age and gender distribution of Type 2 diabetic patients.

parameter	Gender			Duration of disease (years)
Age	male	female	total	
(30-39)	6	7	13	7
(40-49)	10	10	20	8
(50-60)	8	9	17	10
Total	24	26	50	

3.1.2 The biochemical parameters in diabetic patients

The fasting blood glucose (FPG) (204.13*vs*.133.64), cholesterol (188.5 *vs*. 134.3) , low density lipoprotein (LDL) (86.06 *vs*. 65.06) , the triglyceride (165.8 *vs*. 96.8) and the glycosylated hemoglobin A1C (9.50 *vs*. 5.80) showed a significant ($P \leq 0.001$) increases in diabetic patients as compared to controls . Only the high density lipoprotein (HDL) (48.2 *vs*. 61.04) shows a significant decrease ($P \leq 0.001$) in diabetic patients when compared with controls. The fasting concentration of proinsulin was higher in diabetic patients as compared to healthy controls (10.8 *vs*. 8.0) Table (3-3).

Table (3-3): Biochemical profile of healthy controls and diabetic patients.

Variables	Healthy controls (n=30)	Type 2 diabetic patients (n=50)	p-value
fasting blood glucose (mg/dl)	133.64±39.4	204.13±119.2	≤0.001
Cholesterol (mg/dl)	134.3±23.9	188.5±85.9	≤0.001
HDL Cholesterol (mg/dl)	61.04±52.4	48.2±13.3	≤0.001
LDL Cholesterol (mg/dl)	65.06±37.9	86.06±27.7	≤0.001
Triglyceride (mg/dl)	96.8±33.9	165.8±25.12	≤0.001
Hb A1C%	5.80±0.12	9.50±0.25	≤0.001
Proinsulin hormone (pmol/l)	8.0±2.4	10.8±2.8	≤0.001

Data was represented as mean ± SD.,Hb A_{1C}: glycosylated hemoglobin A_{1C}.HDL: high density lipoprotein. LDL: low density lipoprotein

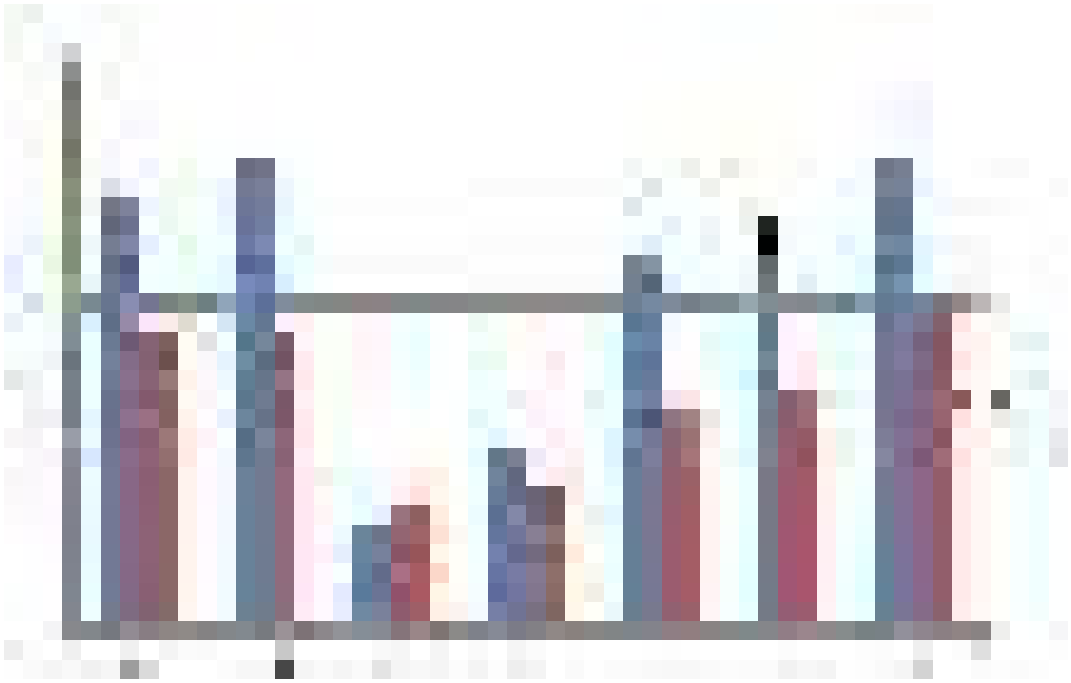


Figure (3.1) Biochemical profile of diabetic patients and healthy controls. TC: Total cholesterol. FBG: fasting blood glucose .HDL: high-density lipoprotein cholesterol .LDL:low-density lipoprotein cholesterol.TG: triglycerides . HbA_{1C} : glycosylated hemoglobinA_{1c} . pro: proinsulin hormone in healthy controls and diabetic patients.

A high level of HbA_{1c} (7 - 9.9%) was observed in more than half of diabetic cases (52.0%), while 40.0% of them had a very high level of HbA_{1c} (≥10%) (Figure 3.2).

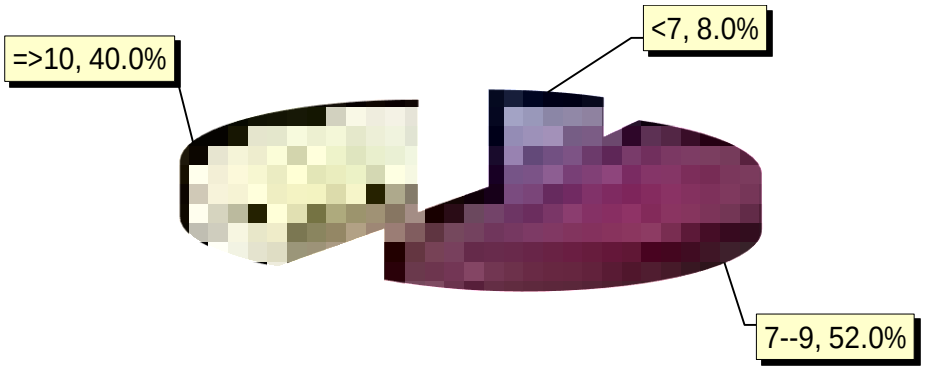


Figure (3.2): Diabetic patients distribution by HbA_{1c} categories.

Patients with diabetes tend to be dyslipidemic with high Fasting blood glucose (FBG), high plasma triglyceride (TG), and high Low Density Lipoprotein(LDL),with a decreased High Density Lipoprotein (HDL).

It is well documented that reduced HDL cholesterol levels are associated with an increased risk of coronary heart disease (CHD) (Jessica *et al*., 2011). A number of functions of HDL particles may contribute to direct cardio protective effects, including promotion of cellular cholesterol efflux and direct antioxidative and anti-inflammatory properties. In addition, hyperinsulinemia and hypertriglyceridemia are independently associated with low levels of HDL .

The long standing hyperglycaemia is broadly related to the diabetic complications seen in the diabetics (Brownlee, 2005). Acute hyperglycemia has numerous effects on the cardiovascular system. It demonstrated reduced coronary collateral blood flow in the setting of moderately severe hyperglycemia, and the markers of inflammation, a well-recognized manifestation of oxidative stress which induce the cell death, have also been observed to increase in response to intermittent elevated glucose levels (Kawahito *et al*., 2009).

Increased the level of the LDL appears to be related to a number of physicochemical and metabolic properties of these particles, including reduced LDL receptor affinity, greater propensity for transport into the sub-endothelial space and susceptibility to oxidative modifications(Davidson *et al*.,2008). Although these are *in vitro* findings, they support the concept that small LDL level contributes to arterial damage in patients with the characteristic dyslipidemia associated with diabetes (Nissen *et al*., 2008) .

The evidence for a relationship between plasma triglyceride levels and the risk of cardiovascular disease (CAD) is largely based on epidemiologic studies. For each 1-mmol/l increase in plasma triglyceride there is a 32% increase in coronary disease risk for men and a 76% increase in risk for women (Krauss, 2004).

Although many factors play a role in the accelerated atherosclerosis observed in diabetes, LDL is the main cholesterol bearing lipoprotein, is major determinant of atherosclerosis in patients with diabetes (Abbasi *et al*., 2007). Lipid profile depends on ethnic groups, genetic and environmental factors as well as severity and duration of the diabetic patients (Karter *et al*., 2002) .

The patients with Type 2 diabetes had high proinsulin level. This result is in agreement with that of (Pfützner and Forst , 2011) as they had reported elevated absolute proinsulin levels as well as disproportionate hyperproinsulinaemia in patients with type 2 diabetes.

Two major hypotheses have been expounded to explain the hyperproinsulinaemia in type 2 diabetes: some investigators have speculated that the increased release of proinsulin in such patients might result from an intrinsic defect in proinsulin processing, leading to an increased release of immature insulin precursors and thus contributing to the impairment in B-cell function in type 2 diabetes .Alternatively hyperproinsulinaemia may be caused by an increased secretory demand on the B-cells, leading to depletion of the ‘readily releasable’ insulin granule pool and mobilisation of insulin granules from the ‘reserve pool’, which is thought to contain greater amounts of immature insulin precursors (Breur *et al*., 2010).

The measurement of proinsulin levels could lead to the diagnosis of insulinoma for blood glucose levels ranging from 2.5 to 3.3 mmol/l during the fast test or when evaluated after an overnight fast (Hirshberg *et al*., 2000). Thus the fasting intact proinsulin is a reliable and robust biomarker for beta cell dysfunction ,insulin resistant (IR), and cardiovascular risk in T2DM patients (Vezzosi *et al*., 2007; Breuer *et al* ., 2010) .

3.2 Lymphocyte subsets counting by Flow Cytometry

3.2.1 T-helper Cells (CD4+)

The results revealed that the percentage mean of CD4+T cells by Apogee flow cytomtry was lower in the diabetic patients Type2 (17.19±4.4)% in comparison with the percentage mean of the same cells also by Apogee flow cytometry in controls (66.25±2.71)% with high significant differences ($p \leq 0.001$). Table(3-4) Figure (3.3).

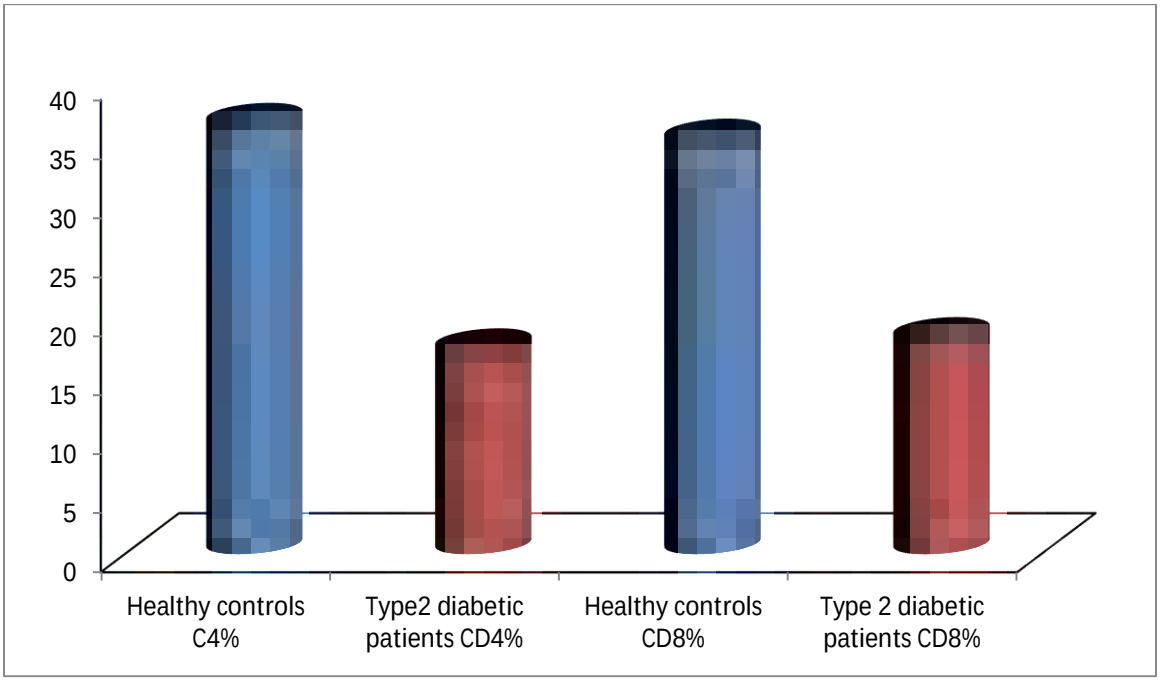
3.2.2 T-Cytotoxic Cells (CD8+)

The percentage mean of CD8+T cells by Apogee flow cytomtry was lower in the diabetic patients Type2 (18.14±4.25)% in comparison with the percentage mean of the same cells also by Apogee flow cytometry in controls (34.92±2.81)% with significant differences ($p \leq 0.001$) Table (3- 4) Figure (3.3) .

Table (3-4) Lymphocyte subsets in Type 2 Diabetes mellitus Patients and healthy controls .

Cell subset	Healthy Controls (n=30)	Type2 diabetic patients (n=50)	p-Value
CD4+%	66.25±2.71	17.19±4.4	≤0.001
CD8+%	34.92±2.81	18.14±4.25	≤0.001

Number are mean ±SD.



Figure(3.3) The percentage mean of CD4 and CD8 in healthy controls compared Type 2 diabetic patients.

CD4+ T-cell frequency was analysed by flow-cytometry in fifty diabetic patients . Flow cytometry is proving to be a powerful tool for examining changes in immune parameters in diabetic patients. The analysis of peripheral blood CD4+ T cells in healthy controls expresses higher level (66.25±2.71), than the diabetic patients which express low level (17.19±4.4) figure (3.4) (Appendix II) .

Healthy Controls **Type 2 diabetic patients**

Figure (3.4) Histogram of CD4+T lymphocyte in healthy controls and Type 2 diabetic patients by Apogee flow cytometry .

CD8+ T-cell frequency was analyses by flow-cytometry in the same fifty Diabetes Mellitus patients. The sample provided here shows decreased in CD8+ T subsets. The analysis of peripheral blood CD8+ T cells in diabetic patients expresses low level (18.14±4.25) , while in the controls it expresses higher level (34.92±2.81) figure (3.5) (Appendix III) .

Healthy controls **Type2 diabetic patients**

Figure (3. 5) Histogram of CD8+ T lymphocyte in healthy controls and Type2 diabetic patients by Apogee flow cytometry .

The present study using the flow cytometry demonstrated a significant decrease in CD4+ and CD8+ T lymphocytes subset in patients with Diabetic Mellitus type 2 when compared with healthy controls . These results seem to agree with Bagdade and Bulger (2004) , Thornton (2005) and Younger (2006) who reported a significant decrease in the percentage of T-helper(CD4) and T-cytotoxic (CD8) in type2 diabetic patients.

The helper T-lymphocytes (CD4+) and cytotoxic T-lymphocytes (CD8+) exert a prominent role in disease pathogenesis. The chronic inflammation is associated with the development and progression of type 2 DM, implying that immunologic and inflammatory mechanisms may play a pivotal role in the disease process (Hedman *et al.*, 2008).

The reduced number of CD8 T cells and CD4 T cells in peripheral blood of type2 diabetic patients may indicate that these patients are suffering from hypoimmune responsiveness and may have an increased incidence of infections with a wide variety of pathogens (Saleh , 2012) . An enhanced susceptibility to infections is well known to occur in diabetic individuals against a variety of infectious agents have long been recognized (Leegaard *et al*., 2011). So the imbalance in cell mediated immunity in patients with type 2 diabetes mellitus may suggest a possible relationship between long duration of the disease and abnormal cellular immunity. This may be reflected in a lowered immunocompetence.

The CD4+T and the CD8+ T cells also might be influenced by persistent poor glycaemic control , which leads to chronic hyperglycemia. The oxidation of elevated levels of glucose within the cell stimulates production of ROS and increases oxidative stress (Brownlee,2005). An increased

generation of ROS such as superoxide, hydrogen peroxide and hydroxyl radical is the cause of oxidation and modification of structure of cellular proteins, nucleic acids, and membrane lipids, so the hyperglycemia may induce oxidative stress condition inside the cells which may stimulates cell death of various cells including lymphocytes (Arya and Tripathi,2011). Also the high glucose levels inhibit proliferation of peripheral mononuclear cells (Otton *et al* , 2002) because hyperglycemia lead to AGE formation, AGE enhanced the attraction of the inflammatory cells, such as lymphocytes by the release of S100/calgranulins and/or amphoterin as a transducing signal for the Receptor of Advanced Glycation End-products (RAGE), thereby allowing them to engage the receptor in an autocrine and paracrine manner triggers a new wave of inflammatory and cell stress reactions , causes the cycle of stress in a wide range of cell types. As well as the hypoinsulinaemia, or underlying disease can exert enough stress to affect the immune cell population and influence adversely the outcome of T2DM , as an anti-infectious response is a potential mechanism of lymphocyte apoptosis (Otton *et al*., 2004 ; Chowdhry *et al*., 2007 and Musa *et al*.,2010) that may be responsible for the reduced numbers of CD4+ and CD8+ T cells in patients with diabetes mellitus type2.

The integration phase of apoptosis is triggered in response to an induction phase ; this phase is controlled by oncogenes and anti-oncogenes of the bcl-2 family (Gurzov and Eizirik , 2011). As the expression of bcl-2, an anti-apoptotic member of the bcl-2 family, was significantly decreased in lymphocytes from the diabetic patient as compared with cells from the healthy subject greatly corroborate with the lymphocyte death (Otton *et al*., 2004).

3.3 Serum Level of Tumor Necrosis Factor_alph (TNF_ α) and Interleukin12(IL-12)

The serum levels of (TNF_ α) demonstrated a significant (p≤ 0.001) increased in T2DM patients as compared to controls (117.6 *vs*. 97.06) (Table 3-5) (figure 3.6) and the serum levels of IL-12 also demonstrated a significant (P ≤ 0.001) increase in T2DM patients as compared to controls (58.02 *vs*. 28.05) (Table 3- 6) (figure 3.7).

Table(3-5) Serum levels of Tumor Necrosis Factor-alpha (TNF_ α) and Interleukin12 (IL12) in Type 2diabetic patients and healthy controls.

Parameters	Healthy controls (n=30)	Type 2 diabetic patients (n=50)	P_Value
TNF_a(pg/ml)	97.06±22.61	117.6±33.9	≤ 0.001
IL12(pg/ml)	28.05±21.4	58.02±14.43	≤ 0.001

Data are expressed as mean ± SD.,(IL12) Interleukin 12 ,TNF(Tumor necrosis factor).

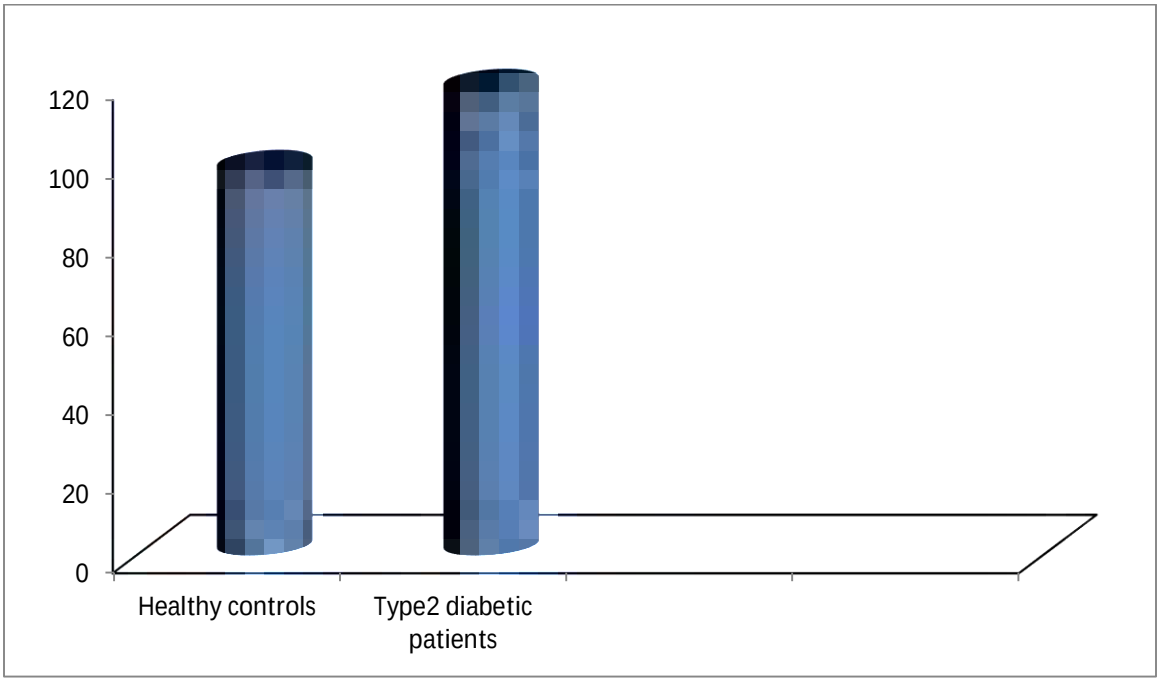


Figure (3.6) Serum levels of TNF_a (Tumor necrosis factor_alpha) in the healthy controls and Type 2 diabetic patients .

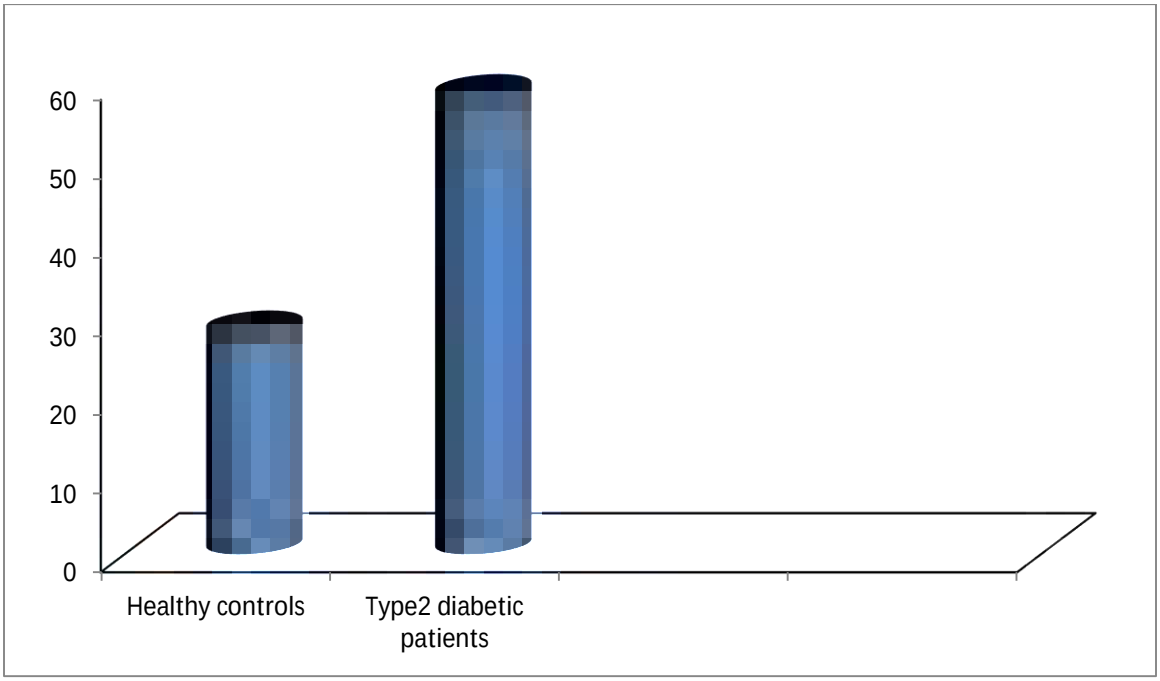


Figure (3.7) Serum levels of Interleukin 12(IL-12) in healthy controls and Type 2 diabetic patients.

The results demonstrated an increased level of TNF-α in T2DM patients. This finding agrees with other investigators who reported similar findings (Rajala and Scherer, 2003; Hotamisligil *et al*., 2003; Fernandez-Real and Ricart, 2003). Furthermore, Xu *et al*. (2002) and Kern *et al*. (1995) demonstrated a high expression of TNF-α in obese animals and obese human with T2DM, while Catalan *et al*. (2007) suggested that elevated pro-inflammatory cytokine level found in obese T2DM subjects is related mainly to obesity rather than to T2DM.

The present study demonstrated that the increased serum level of TNF-α in T2DM patients was associated with increased poinsulin hormone levels and BMI. Bruun *et al* . (2003) suggested that the high level of circulating TNF-a is an indicating of hyperinsulinemia .Furthermore Yaturu *et al*. (2006) demonstrated a positive correlation between TNF-α and high BMI.

The production of TNF- α may limit the activity of the T regulatory cells and foster induction of immune reactivity and the effector phase of lymphocyte responses . So a decrease in TNF production may result in enhanced T regulatory function that limits immune reactivity. In this way, TNF may play an important instructive role in controlling adaptive immunity (Valencia *et al*., 2006).

Interleukin-12 showed a significant increase in level of T2DM patients as compared to controls. Wegner *et al*.(2008) demonstrated that elevated serum IL-12 levels in T2DM was related to the excessive proinsulin secretion. Therefore, this cytokine may play a critical role in the pathogenesis of T2DM, since IL-12 is important in immune response to infections. It has been shown that in the absence of infection, IL-12 induced autoreactive T

cell responses might predispose to self-destructive immunity but the significance of IL-12 changes in the blood of patients with T2DM remains unclear. The IL-12 accelerates the development of macrovascular complications in the disease (Hauer *et al*., 2005). Additionally, it has been noted that elevated glucose levels in diabetic animals stimulates inflammatory reactions related to IL-12 cytokine gene expression (Wen *et al*., 2006). However, it is not known whether factors related to the course of T2DM, such as metabolic compensation, beta cell secretory dysfunction and insulin resistance affect IL-12 concentrations, but in a recent study, a multiple regression analysis revealed that the IL-12 serum level in T2DM primarily was dependent upon fasting pro-insulin concentration (Wegner *et al*., 2008).

It has been suggested that cytokines released by monocytes/macrophages, including IL-1beta, IL-12 and tumor necrosis factor alpha (TNF-α) could have an initial role in islet beta-cell damage (Blazher *et al*., 2006).

Proinflammatory cytokines may be increased by hyperglycemia in subjects with impaired glucose tolerance, this result was confirmed, because all diabetics had elevated serum level of TNF_a and IL12 .However, the TNF_a and IL12 cytokine is stimulated by stress hyperglycemia (Cheung et al., 2012) which lead to AGEs formation that may be the mean reason of interleukins increases in addition to T regulatory dysfunction (Valencia *et al*., 2006) despite of decrease in the T lymphocyte cells that stimulated the realease of interleukines.

3.4 DNA Fragmentation in Type 2 diabetic patients

Blood samples were subjected to DNA isolation procedure . The band integrity and DNA concentration were found to be different according to the yielded amount of genomic DNA and its purity Figure (3-8) which depend on the amount of white blood cells in the blood samples . In addition ,the use of fresh blood samples were found to be better ,therefore ,the DNA isolation should be applied as early as possible.

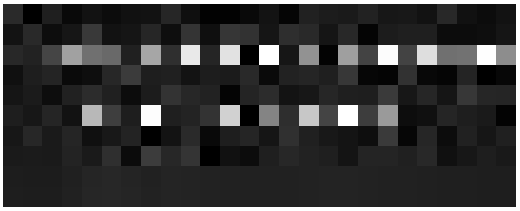


Figure (3.8):Chromosomal DNA bands which extracted from human blood samples on 0.8% agarose at 70 voltage for one hour.

DNA Fragmentation is one of the hallmarks of the late stage of apoptosis process .The smear shape pattern on gel electrophoresis indicating double strand breakage of the DNA detected in 20% of the patients in severe damage according to the molecular weight (MW) of the ladder . Compared to the control and MW of ladder, the patient of DM had higher prevalence of DNA double-strand breaks in their leukocyte .The DNA ladder of DM patient was clearly observed in Figure (3.9) .

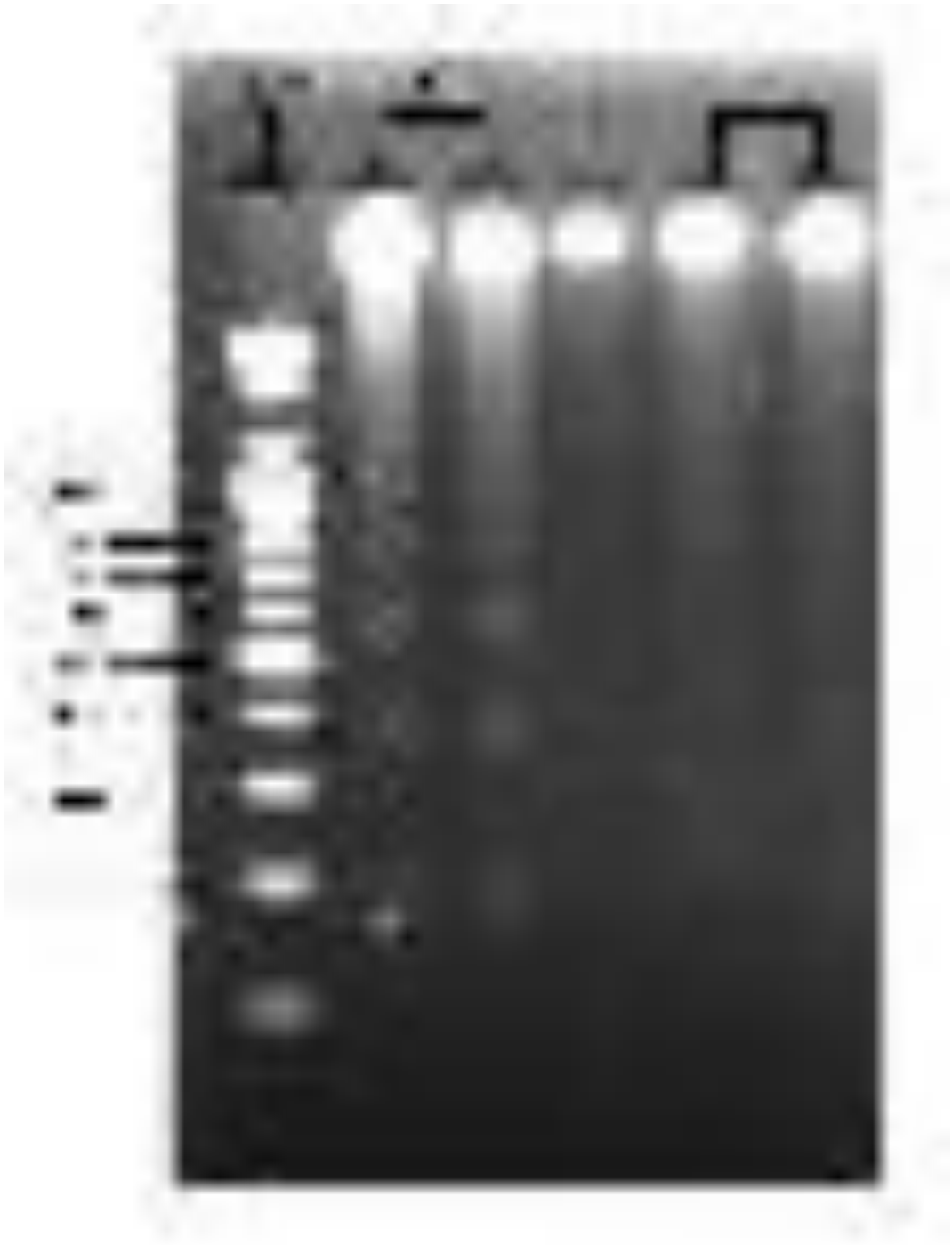


Figure (3.9) Electrophorsis pattern of separated DNA from Type2 diabetes mellitus and controls . Lane1:shows shaped DNA damage of 20% diabetic patients (200bp).Lane 2 shows intact DNA from healthy people (control). Lane 3 shows negative result for diabetic patient samples. On 1.8% agarose gel at 24 voltage for 4 hour.

It has been suggested that oxidative stress induces premature senescence, caused by rapid telomerase shortening resulting from DNA damage and double strand break (Bertram and Hass, 2008) .Many studies have concluded that diabetes may have an oxidative effect on DNA level of the cell (Marua *et al* ., 2001;Honma *et al*., 2003; Kumar *et al.*, 2007;Xu *et al.,* 2008 and Bagatini *et al*.,2008).

The result of DNA fragmentation using agarose gel electrophoresis and image analysis revealed that about 20% of diabetic patients had DNA damage , which may be depend either on the severity and chronicity of the diabetes mellitus disease or on the reactive oxygen species generation (ROS) (Ghoneim and Al-Maliki , 2010).

The Hyperglycemia results in oxidative stress due to increased production of reactive oxygen species (ROS), and decrease antioxidative mechanisms (Shetty *et al*., 2013). The hyperglycemia enhance the non-enzymatic glycosylation reaction (glycation) of proteins, this results in the formation of a metabolites, known as Advanced Glycosylation End products (AGE) which enhanced the generation of ROS (Kawahito *et al*., 2009). Therefore the excessive levels of ROS lead to the damage of proteins, peroxidation of lipids, also cause strand breaks in DNA and base modifications including the oxidation of guanine residues and severe metabolic dysfunction (Sohair *et al*., 2012).

In diabetic patients, the generated of free radical are electrically charged molecules (having unpaired electron) which causes them to seek out and capture electrons from other substances in order to neutralize themselves. These radical are formed under normal conditions in minute quantities and

are rapidly scavenged by natural cellular defense mechanism, which increases in diabetes, therefore are considered as important results of oxidative stress (Ghoneim and AL-Malki , 2010).

Conclusions

1_Immune modulation accompanying metabolic dysfunction can adversely affect outcome of a type 2 diabetes mellitus. The decrease in CD4+ T cells and in CD8+ Tcells in patients with Type 2 diabetes mellitus may reflect an abnormal immunoregulatory mechanism in parallel with an impaired metabolic processes and may lead to enhanced beta cell damage and increased susceptibility to infections, accelerated atherosclerosis.

2_ The cytokines (TNF-α and IL-12) have a positive correlation with BMI and obesity in patients with type2 diabetic patients.

3_ Hyperglycemia contributes to increased generation of ROS, which in one way, necessary for inflammatory response to defend organisms against infection agents. In contrast, high levels of cellular ROS could damage many critical cellular components, such as protein, lipids and DNA, and eventually cause cellular injuries that might lead to a variety of clinical abnormalities, including cardiovascular disease, diabetes, aging and cancer.

Recommendations

1_ The macrophage dysfunction is a likely mechanism underlying common diabetic complications such as increased susceptibility to infections, accelerated atherosclerosis and disturbed wound healing, therefore studying the functional profiles of these cells are required in diabetic patients and healthy controls.

2_ Future investigations including other cytokines such as IL-6, IL-18, interferon (IFN)-γ and transforming growth factor-Beta1 (TGF-β1) will help clarify the role of interleukins in the etiopathogenesis of Type 2 diabetes mellitus.

3_Further studies about the CD4 and CD8 genes during T cell development could provide insights into both the mechanism of regulation of CD4 and CD8 genes, and the process of lineage commitment.

4- Detection the double strand breaks that could cause the cell death by apoptosis in patients with diabetes type2 .

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Questionnaire

No. : No. of file:

Name: Age: Gender:

Date of onset of diabetes: Family history:

Present history (any medical problem or medication):

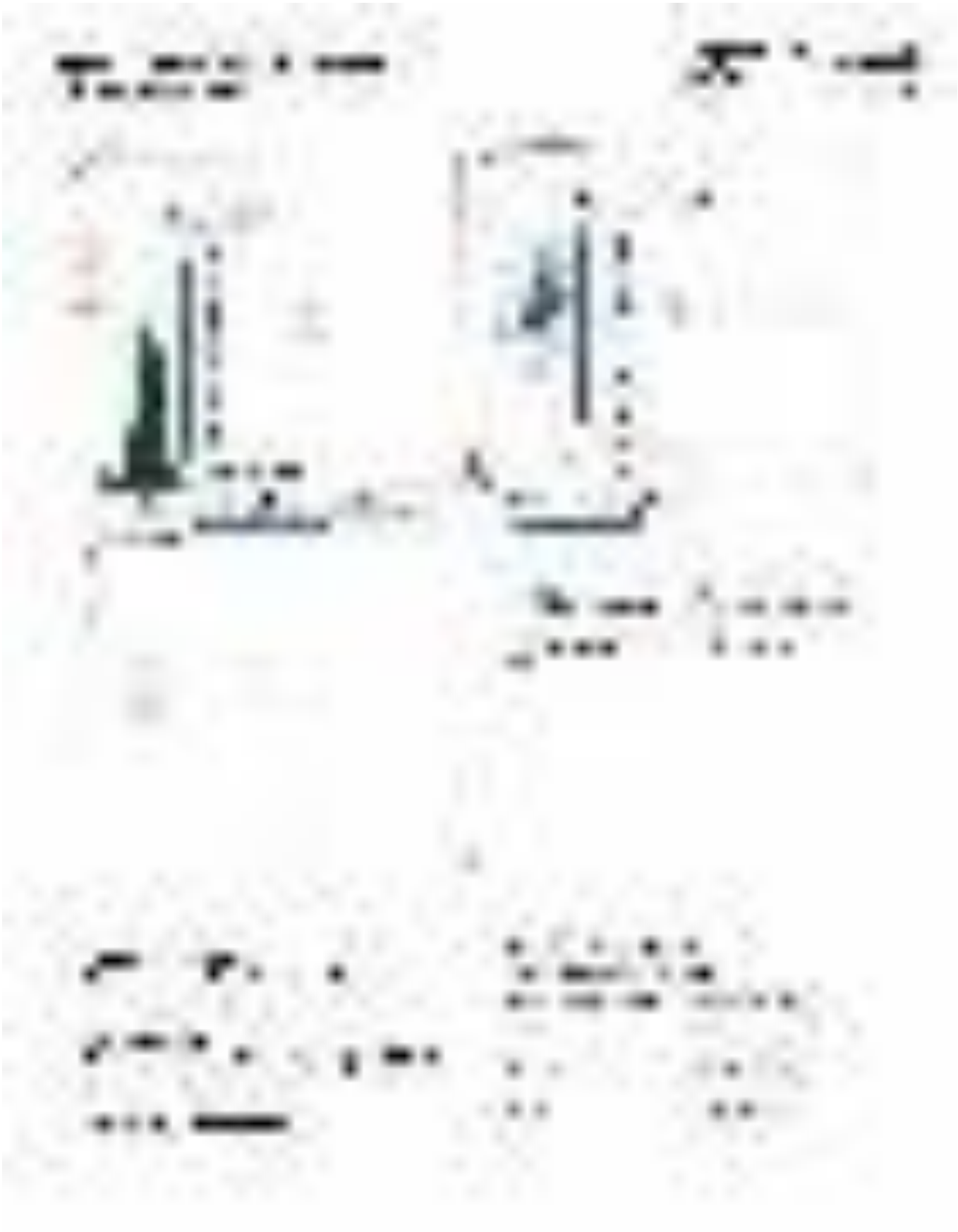
Type of treatment: Oral Insulin

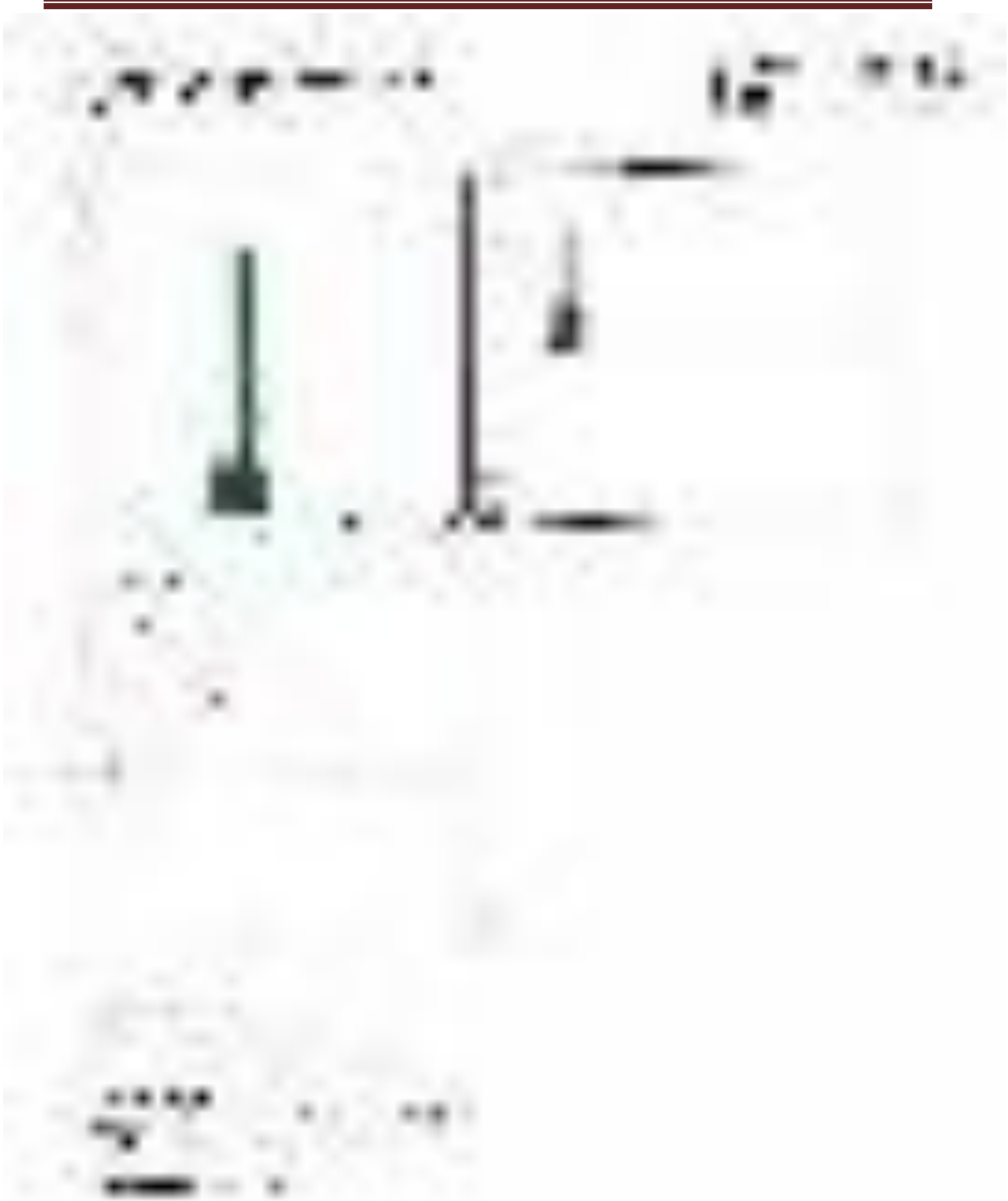
Past history (medical, surgical and hospital admission):

Blood pressure: Smoking:

Weight: Height: BMI:

Waist: Hip: W-H Ratio:





الخلاصة

المرض السكري من النوع الثاني هو اضطراب لمتلازمة الأيض يتميز بفرط الدم المزمن والاضطرابات من الكربوهيدرات والدهون والبروتين الناتجة عن عيوب في افراز الانسولين وعمل الانسولين او كليهما, متوسط جزء كبير في المناعة البدائية والحركيات الخلوية وكان مقرر في هذه الدراسة هي تحديد النمط الظاهري للخلايا المناعوية المساعدة والخلايا المناعوية السامة باستخدام الجسام المضادة وحيدة النسلة .ودور الحركيات الخلوية من خلال تحديد المستوى المصلي للحركيات (TNF- α and IL-12) هورمون الانسولين وكذلك دراسة تجزئة الحمض النووي .

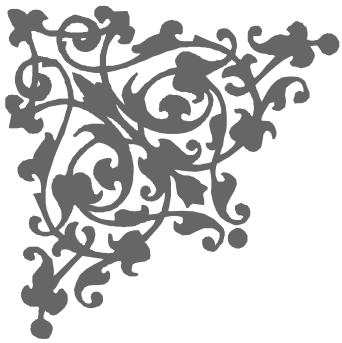
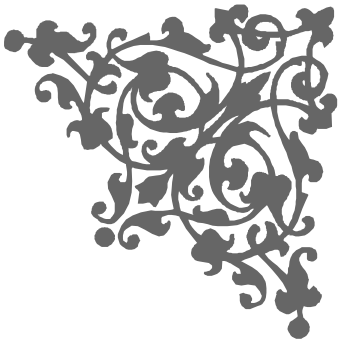
جمعت خمسين عينة دم لمرضى السكري النوع الثاني من المركز الوطني السكري للعلاج والبحاث في الجامعة المستنصرية للفترة من كانون الول 2102 الى كانون الثاني 2102. اخذت ايضا ثلثين عينة دم من اشخاص اصحاء لغرض المقارنة متطابقين بالجنس والعمر والخلفية العرقية من قبل المرض لمدة المرض وايضا من قياس السمنة بواسطة مؤشر كتلة الجسم ونسبة الخصر الى الورك و الكولسترول الدهون الثلثية والكلوكوز ونسبة الهيموكلوبين الكلي.

من قياس المعدل النسبي للخلايا المساعدة والخلايا السامة بواسطة جهاز التدفق الخلوي اظهرمعدل نسبة الخلايا المساعدة) 17.19% vs. 55.26%) ومعدل نسبة الخلايا السامة (18.14% vs. 34.92%) انخفاضا معنويا عالي p≤0.001 في مرضى السكري مقارنة بالاصحاء. وقد تم تحديد مستوى الحركات الخلوية بواسطة عدة الليزا وقد اظهرت الحركيات الخلوية (TNF- α and IL-12) زيادة معنوية عالية p≤0.001 في مرضى السكري مقارنة بالاصحاء (117.6 vs. 97.06 pg/ml) وايضا مستوى IL-12 اظهر زيادة (p≤0.001) في مرضى السكري مقارنة بالاصحاء (58.02 vs. 28.05 pg/ml). وايضا كشف ان 21% من مرضى السكري لديهم ملف الحمض النووي بواسطة تحليل تجزئة الحمض النووي عن طريق

الخلاصة

التحليل الكهربائي على 1.8% للهلام في 01 فولطية لمدة اربع ساعات هذا الضرر في الدنا الذي من المفكن يعود الى ردة فعل الجذور الحرة وشدة وطول المرض.

كاستنتاج تبين ان ضعف الخلايا المناعية هي السائدة في مرضى السكري النوع الثاني وان من المفكن ان الخلل في التركيز السكري وزيادة في السكولين والهابات الكامنة ممارسة الضغط على عدد الخلايا اللمفاوية والنأثير سلبا على نتائج مرضى السكري النوع الثاني وقد يعكس هذا على الهلية المناعية وضعف التمثيل الغذائي كما ينظر في مرضى السكري النوع الثاني.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَإِذْ قَالَ رَبُّكَ لِلْمَلَائِكَةِ إِنِّي خَالِقٌ

بَشَرًا مِنْ

صَلْصَالٍ مِنْ حَمَإٍ مَسْنُونٍ فَإِذَا

سَوَّيْتُهُ وَنَفَخْتُ

فِيهِ مِنْ رُوحِي فَقَعُوا لَهُ سَاجِدِينَ

صدق الله العظيم

سورة الحجر 22-28

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تقييم بعض الجوانب المناعية لمرضى السكري النوع الثاني

رسالة

مقدمة الى كلية العلوم قسم التقانة الاحيائية جزء من متطلبات

نيل درجة الماجستير في التقانة الاحيائية

من قبل

علاء عبد الجبار عبد الرزاق

بكالوريوس تقانة احيائية/جامعة النهرين 9002

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ذو القعدة 0121