العلاقة بين بروتين ج الفعال ومضادات الأكسدة في داء السكري

أطروحة مقدمة من قبل

لميس طالب هادي تاج الدين

بكلوريوس طب و جراحة عامة(جامعة بابل، ٢٠٠١)

إلى مجلس كلية الطب ـ جامعة بابل

وهي جزء من متطلبات نيل درجة الماجستير في الكيمياء الحياتية السريرية

٢٠٠٧م ١٤٢٨ هـ

THE RELATIONSHIP BETWEEN C-REACTIVE PROTEIN AND ANTIOXIDANTS IN DIABETES MELLITUS

A THESIS SUBMITTED By

Lamis Talib Hadi Taj- Aldeen

M.B. Ch.B. (University of Babylon, ٢٠٠١)

TO THE COUNCIL OF THE COLLEGE OF MEDICINE, UNIVERSITY OF BABYLON,

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CLINICAL BIOCHEMISTRY

١٤٢٨ A.H. **٢٠٠٧ A.D.**

بسم الله الرحمن الرحيم

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (١) خَلَقَ الْإِنسَانَ مِنْ عَلَقٍ (٢) اقْرَأْ وَرَبُّكَ الْأَكْرَمُ (٣) الَّذِي عَلَّمَ بِالْقَلَمِ (٤) عَلَّمَ الْإِنسَانَ مَا لَمْ يَعْلَمْ (٥)

صدق الله العلي العظيم

سُورة العَلَق

الآية (١ – ٥)

Dedication

To:

My parents: examples of integrity , hard work , and love ;

My sisters and brothers: who have greatly supported me ;and

My husband: who fills my life with kindness, support, happiness, and love.

Acknowledgements

I am greatly indebted to my supervisors Prof. Dr. Mufeed J. Ewadh and Prof. Dr. Hussein S. Al-Janabi for their continuous help, guidance and encouragement.

Sincere thanks also go to Miss Khawla Abdul Hamza (High Diploma) as well as Mr. Adul Samie Alta'ee(Assistant Lecturer) and the Teaching Staff of the Department of Biochemistry for their encouragement, support, and help. I would like to thank Dr. Ammal Merza (Ph.D.)and Dr. Hatim Abdul Lateef (Assistant Professor) for their help and advice.

I highly acknowledge the help of my colleagues Rafid S. Ewadh and Ossama F. Mzahim.

Special thanks also go to the staffs of the Diabetic Clinic, the wards, and the laboratory unit of biochemistry in Mirjan Teaching Hospital and Babylon Hospital of Pediatric and Maternity for their help.

الخلاصة

تتناول هذه الرسالة دراسة دور ارتفاع مستوى السكر على ارتفاع مستوى بروتين ج الفعال من خلال دراسة العلاقة بين تركيز بروتين ج الفعال ومضادات الأكسدة (الكلوتاثايون المختزل ، فيتامين ج المختزل).

شملت الدراسة ٨٧ مريضاً مصاباً بداء السكري: بينهم ٢٧ مريضاً مصاباً بداء السكري النوع الأول (١٠ ذكور و ١٧ إناثاً) و٦٠ مريضاً مصاباً بداء السكري النوع الثاني (٢٧ ذكوراً ،٣٣ إناثاً). أما مجموعة السيطرة فتكونت من ٣٩ شخصاً من الأصحاء .

تمت دراسة الخصائص الإحصائية (الجنس والعمر) والسريرية والكيميائية الحياتية لهذه المجموعات ووجد أن توزيع الفئة العمرية للمرضى المصابين بداء السكري النوع الثاني ظهر على أعلى مستوى له في الفئة العمرية من (٥٠ – ٥٩) سنة، بينما كان التوزيع متساوياً للفئات العمرية من – ٣٩) (١٠ سنة بالنسبة لمرضى السكري النوع الأول .

وتبين أن مرضى السكري من النوع الأول الذين ليس لديهم وزن زائد يكونّون (٧٤٪) من العدد الكلي لمرضى السكري النوع الأول. من جانب آخر فإنّ مرضى السكري من النوع الثاني من ذوي الوزن الزائد يكونّون (٧٢٪) من العدد الكلي لمرضى السكري النوع الثاني.

ودلت النتائج على أن كلا النوعين الأول والثاني لمرضى داء السكري لديهم انخفاض معنوي بمستويات مضادات الأكسدة (الكلوتاثايون المختزل، فيتامين ج المختزل) بالمقارنة مع ما هو عليه في مجموعة السيطرة.

كما تبين أن مستويات بروتين ج الفعال فوق المستوى الطبيعي تكّون (١٣.٧٩٪) من العدد الكلي لمرضى السكري وهؤلاء المرضى أما يعانون من مضاعفات داء السكري أو مصابين بالتهاب أو كانوا حديثي التشخيص بداء السكري، وتبين من ذلك أنه ليس كل مرضى داء السكري الذين يعانون من ارتفاع مستوى السكر لديهم ارتفاع في مستوى بروتين ج الفعال, ولوحظ أن ارتفاع مستوى بروتين ج الفعال أكثر حدوثاً بين الإناث منه بين الذكور.

ودلت النتائج أيضاً على وجود علاقة معنوية عكسية بين تركيز بروتين ج الفعال ومضادات الأكسدة (الكلوتاثايون المختزل، فيتامين ج المختزل) في كلا النوعين من مرضى السكري، لكن العلاقة كانت أقوى وذات معنوية أكثر بين مرضى السكري النوع الأول منها بين مرضى السكري النوع الثاني، ويعزى ذلك إلى الارتفاع المعنوي لمستوى السكر بين مرضى السكري من النوع الأول من ذوي المستوى المرتفع لبروتين ج الفعال بالمقارنة مع مستوى السكر بين مرضى السكري من النوع الثاني من ذوي المستوى المرتفع لبروتين ج الفعال.

نستنتج من ذلك أن الارتفاع المزمن لمستوى السكر ليس كافياً لتحفيز حدوث الالتهاب (المستدل عليه بواسطة ارتفاع مستوى بروتين ج الفعال)، على الرغم من أنه يسهم بصورة غير مباشرة في حدوثه من خلال الإجهاد التأكسدي (المستدل عليه بواسطة انخفاض مستويات مضادات الأكسدة) بين الأشخاص المصابين بداء السكري لكلا النوعين الأول والثاني.

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List of Abbreviations

Abbreviation	*Meaning*
A.D.	(Anno Domini) after Christ
ADP	Adenosine diphosphate
ADP-ribose	Adenosine diphosphate – ribose
AFR	Ascorbate free radical
AGE	Advanced glycation endproduct
AIDS	Acquired immuno deficiency syndrome
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
AR	Aldose reductase
ATP	Adenosine triphosphate
BMI	Body mass index
^{o}C	Celsius degree
CAT	Catalase
CBF	Complete blood film
CRP	C-reactive protein
DCIP	Dichloro indo phenol
DFU	Diabetic foot ulcer
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
DNPH	Dinitrophenyl hydrazin
DTCS reagent	٢,٤-dinitrophenye hydrazine + thiourea + copper sulfate reagent
DTNB	٥,٥' dithiobis (٢-nitrobenzoic acid)
ECG	Electro cardiograph
EDRF	Endothelium derived relaxation factor
$EDTANa_{٢}$	Ethylene diamine tetra acetic acid disodium
e.g.	(L. example gratia) for example
EIA	Enzyme immuno assay
ESR	Electron spin resonance
F	Female
FPG	Fasting plasma glucose
FSG	Fasting serum glucose
GLUT٤	Glucose transporter ٤
GOD	Glucose oxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase

Abbreviation	*Meaning*
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GUE	General urine examination
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
hs CRP method	High sensitivity C-reactive protein method
IDDM	Insulin dependant diabetes mellitus
i.e.	(L.id est) that is
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IHD	Ischaemic heart disease
IL-١	Interleukin-١
IL-٦	Interleukin-٦
IRS-١	Insulin receptor substrate-١
JNK	C- Jun NH_2 – terminal kinase
LDL	Low density lipoprotein
M	Male
M-HPO_3	Meta- phosphoric acid
MODY	Maturity onset diabetes of the young
MPO	Myelo peroxidase
NaCl	Sodium chloride
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF_kB	Nuclear factor kappa B
NH_2CH_2COOH	Amino acetic acid (glycine)
$NO^\bullet$	Nitric oxide radical
NS	Not significant
NIDDM	Non insulin dependant diabetes mellitus
$O_2^\bullet$	Superoxide radical
$^1O_2$	Singlet oxygen
O_3	Ozone
OGTT	Oral glucose tolerance test
$OH^\bullet$	Hydroxyl radical

Abbreviation	*Meaning*
$ONOO^-$	Peroxy nitrate
P	Probability
Pi	Inorganic phosphate
PKC	Protein kinase C
POD	Peroxidase
PUFA	Poly unsaturated fatty acid
PVD	Peripheral vascular disease
r	Correlation coefficient
R١	Reagent ١
R٢	Reagent ٢
R٤	Reagent ٤
RDA	Recommended dietary allowance
RNS	Reactive nitrogen species
$RO^\bullet$	Alkoxyl radical
$ROO^\bullet$	Peroxyl radical
ROS	Reactive oxygen species
RSG	Random serum glucose
RTI	Respiratory tract infection
RvitC	Reduced vitamin C
SD	Standard deviation
SDH	Sorbitol dehydrogenase
Sig.	Significancy
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
TCA	Trichloro acetic acid
TNF-α	Tumor necrosis factor - α
Tris	Tris (hydroxy methyl) amino methane
TvitC	Total vitamin C
UTI	Urinary tract infection
UVA	Ultraviolet alpha
UVB	Ultraviolet beta
VLDL	Very low density lipoproteins
WHO	World health organization
xg	The gravitational force exerted on the homogenate being centrifuged

Abstract

The study done to explain the role of hyperglycaemia in raising C-reactive protein level through studying the relationship between the concentration of C-reactive protein and antioxidants (reduced glutathione, reduced vitamin C).

The study has been conducted on ٨٧ patients with diabetes mellitus: ٢٧ of them with type ١ DM (١٠ males and ١٧ females) and ٦٠ patients with type ٢ DM (٢٧ males and ٣٣ females). Thirty-nine healthy subjects are considered a Control Group.

Demographic (sex, age), clinical, and biochemical characteristics have been studied in these groups. The peak level of age group distribution in type ٢ DM is between (٥٠ – ٥٩) years old, whereas in type ١ DM the age group is equally distributed between (١٠ – ٣٩) years old. Also it has been revealed that type ١ DM patients that being non obese constitute (٧٤٪) of the total number of type ١ DM patients. On the other hand, type ٢ DM patients that being obese constitute (٧٢٪) of the total number of type ٢ DM patients.

The results show that both types ١ and ٢ diabetic patients have significant decrease in their antioxidants levels (reduced glutathione, reduced vitamin C) in comparison with that of Control Group.

Also, it has been revealed that C-reactive protein levels above the normal value constitute (١٣.٧٩٪) of the total number of diabetic patients, and they have been found to be either have diabetic complications, infection, or be newly diagnosed diabetics, and not all diabetic patients with hyperglycaemia have raised C-reactive protein level. It has been found also that raised C-reactive protein levels are higher in female sex than male.

Also, the results have been revealed an inverse significant relationship between C-reactive protein concentration and antioxidants (reduced glutathione, reduced vitamin C) in both types ١ and ٢ diabetic patients, but the relation is stronger and more significant in type ١ DM patients than that in type ٢ DM patients, this has been attributed to the significant higher hyperglycaemic level of type ١ DM patients with raised C-reactive protein in

comparison with that of type ٢ DM patients with raised C-reactive protein level.

We concluded that chronic hyperglycaemia is not sufficient to induce inflammation (reflected by raised C-reactive protein), although it contributes to it indirectly through oxidative stress (reflected by decreased antioxidants levels) in persons with established diabetes mellitus of both types ١ and ٢.

Certification

We certify that this thesis entitled " **The Relationship between C-Reactive Protein and Antioxidants in Diabetes Mellitus** " was prepared under our supervision at the College of Medicine, University of Babylon as a partial requirement for the degree of Master of Science in Clinical Biochemistry.

Signature:	Signature:
Name: Dr. Mufeed J. Ewadh	Name: Dr. Hussein S. Al-Janabi
Title: Professor	Title: Professor
Address: President of University of Karbalaa	Address: College of Medicine, University of Kufa
Date: / /	Date: / /

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature:
Name: Dr. Kadhim J. K. Al-Hamdani
Title: Lecturer
Address: Head of Department of Biochemistry College of Medicine, University of Babylon
Date: / /

Examining Committee Certification

We, the Examining Committee, after reading this thesis entitled " **The Relationship between C-Reactive Protein and Antioxidants in Diabetes Mellitus** " and, examined the student "**Lamis Talib Hadi Taj-Aldeen**" in its content, find it adequate as a thesis for the degree of Master of Science in Clinical Biochemistry.

Chairman
Signature:
Name: Dr. Oda M.Y. Al-Zamely
Title: Professor
Address: College of Science, University of Babylon
Date: / / ٢٠٠٧

Signature:	Signature:
Name: Ala H. Haider	Name:Dr. Sahib A. Al-Atrakchi
Title: Assistant Professor	Title: Assistant Professor
Address: College of Medicine, University of Babylon	Address: College of Medicine, University of Karbalaa
Date: / / ٢٠٠٧	Date: / / ٢٠٠٧
(Member)	**(Member)**

Signature:	Signature:
Name: Dr. Mufeed J. Ewadh	Name: Dr. Hussein S. Al-Janabi
Title: Professor	Title: Professor
Address: President of University of Karbalaa	Address: College of Medicine, University of Kufa
Date: / / ٢٠٠٧	Date: / / ٢٠٠٧
(Supervisor)	**(Supervisor)**

Approved for the college committee on postgraduate studies.

Signature:

Name: Dr. Ali K. Al-Shaely

Title: Assistant Professor

Address: University of Babylon

(Dean of the College of Medicine)

Date: / / ٢٠٠٧

Conclusions

١- Both types١ and ٢ diabetic patients have significant decrease in their antioxidants levels (GSH,RvitC) in comparison with those of the healthy Control Group.

٢- Raised C-reactive protein is higher in the female sex .

٣- An inverse significant relationship is found between C-reactive protein concentration and antioxidants (GSH, RvitC) in both types ١ and ٢ diabetic patients.

٤- Chronic hyperglycaemia is not sufficient to induce inflammation (reflected by raised C-reactive protein), although it contributes to it indirectly through oxidative stress(reflected by decreased antioxidants levels of GSH and RvitC) in people with established diabetes of both types ١ and ٢ .

Recommendations

١- The results in the present study imply the indirect contribution of hyperglycaemia to inflammation (reflected by raised C-reactive protein) through oxidative stress (reflected by decreased antioxidants levels of GSH and RvitC) in people with established diabetes of both types١and ٢. Improving glycaemic control and supplementation with antioxidants can therefore reduce inflammatory response. Such a study has important implications for the treatment of diabetic patients.

٢- Application of advanced methods in future studies for the determination of C-reactive protein like high-sensitivity analytic method (hsCRP)or immunological methods including nephlometry and enzyme immuno assay (EIA) and the application of high performance liquid chromatography (HPLC) for the determination of reduced glutathione and reduced vitamin C .

٣- Further studies on the relation of other inflammatory markers like (interleukin-٦ IL-٦ and tumor necrosis factor α TNF-α) to hyperglycaemia and oxidative stress reflected by measuring free radicals by electron spin resonance (ESR) spectroscopy in diabetic patients.

١.١ Related Origin of Diabetes Mellitus:-

Diabetes mellitus is the most common disorder associated with pancreatic islet dysfunction of the endocrine portion (Bullock, ١٩٩٦). The pancreas consists of very different organs contained within one structure. The acinar portion of the pancreas has an exocrine function. The endocrine portion consists of islets of Langerhans (Murray *et al.,* ١٩٩٦).

١.١.١ Anatomy and Physiology of the Pancreas :-

The pancreas is an elongated retroperitoneal gland that lies in the upper portion of the posterior abdominal wall. It resembles a fish, with its head and neck lying in the C-shaped curve of the duodenum, its body extending horizontally behind the stomach, and its tail touching the spleen. Figure (١.١) shows the relation of the pancreas to other abdominal organs (Bullock, ١٩٩٦).

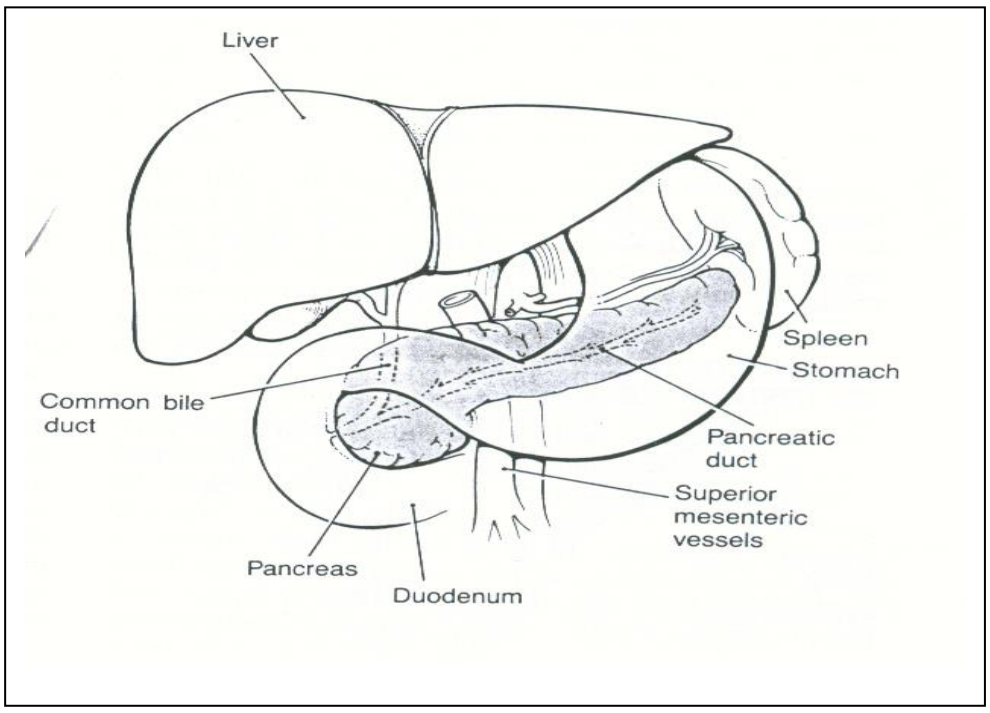

Figure (١.١):Relation of the pancreas to other abdominal organs.

The head is at the level of the second lumber vertebra, makes up about ٣٠٪ of the gland, and lies within the concavity of the duodenum. The neck, the narrowed portion between the head and the body, joins to the body, which accounts for the largest portion of the gland. The tail tapers off from the body. The pancreas is firm and has a characteristic lobular appearance with a light yellow and slightly pink coloration. In adult, the total length of the pancreas is

١٢ to ٢٠ cm, with a width of ٣ to ٥ cm and a maximal thickness of ٢ to ٣ cm. Its weight is between ٦٠ and ١٤٠ g.

The pancreas is composed of the acini (grape like formations), which represent the exocrine portion of the pancreas and form the largest mass (about ٨٠٪) of the gland, which secrets digestive juices into the duodenum. The major enzymes secreted include trypsin, chymotrypsin, peptidases, amylases, lipases, phospholipases, and elastase. The endocrine portion of the pancreas, the islets of Langerhans, is embedded between exocrine units like small islands (Bullock, ١٩٩٦).

In humans, there are ١-٢ million islets. The cells in the islets can be divided into types according to their staining properties and morphology. There are at least ٤ distinct cell types in humans :A, B, D and F cells. A, B, and D cells are also called α, ß and δ cells. The B cells which are the most common and account for ٦٠ % – ٧٥ % of the cells in the islets are generally located in the center of each islet (Ganong, ١٩٨٩).

Insulin, from Latin *insula* " island " (Nelson and Cox, ٢٠٠٥), was linked to diabetes in ١٩٢١ by Banting and Best (Murray *et al.,* ١٩٩٦). Insulin is a polypeptide hormone produced by the B-cells of the islets of Langerhans. It is one of the most important hormones coordinating the utilization of fuels by tissues. Its metabolic effects are anabolic (Champe and Harvey, ١٩٩٤). Insulin acts on three main target tissues: liver, muscle, and adipose tissues (Baynes and Dominiczak, ٢٠٠٥). Table (١.١) shows the effect of insulin on cellular metabolism (Smith *et al.,* ١٩٩٨).

Table (١.١) : The effects of insulin on cellular metabolism.

Tissue	Processes Activated by Insulin	Processes Inhibited by Insulin

Liver	**Uptake of amino acids and glycerol production of NADPH**	**Glycogenolysis** **Gluconeogenesis**
	Synthesis of glycogen, proteins, triglycerides, and VLDL	
Muscle	**Uptake of glucose and amino acids**	**Triglyceride utilization**
	Synthesis of glycogen	
Adipose	**Uptake of chylomicrons and VLDL and of glucose**	**Lipolysis**
	Utilization of glucose	

١.٢ Diabetes Mellitus (DM) :-

Diabetes mellitus is a group of diseases characterized by an elevated blood glucose level (hyperglycaemia) resulting from defects in insulin secretion, in insulin action, or both (WHO,٢٠٠٢). Diabetes mellitus is not a pathogenic entity but a group of aetiologically different metabolic defects. Common symptoms of diabetes are lethargy from marked hyperglycaemia, polyuria, polydipsia, weight loss, blurred vision and susceptibility to certain infections. Severe hyperglycaemia may lead to hyperosmolar syndrome and insulin deficiency to life-threatening ketoacidosis. Chronic hyperglycaemia causes long - term damage, dysfunction and failures of various cells, tissues, and organs.

Long - term complications of diabetes are :

١) Macroangiopathy: ischaemic heart disease (IHD), stroke, peripheral vascular disease (PVD).

٢) Microangiopathy : retinopathy, nephropathy.

٣) Neuropathy : peripheral neuropathy, autonomic neuropathy.

٤) Cataract.

٥) Diabetic foot.

٦) Diabetic heart (WHO,٢٠٠٢).

١.٢.١ Etymology (Terminology) :-

" Diabetes " is a Greek word meaning " a passer through; a siphon "

" Mellitus " comes from the Greek word "sweet". Apparently, the Greeks named it thus because the excessive amount of urine that a diabetic (when in a hyperglycaemic state) would excrete attracted flies and bees because of the glucose content (Nursing Study. com,٢٠٠٣).

١.٢.٢ History of Diabetes Mellitus :-

Writing about diabetes goes back more than ٣٠٠٠ years, to the Ebers Papyrus, in which afflicted people were described as passing frequent and large amounts of urine. Ayur Veda described the sweetness of the urine and noted that ants were attracted to it. He wrote of weakness, emaciation, polyuria and carbuncles in affected people. Aretaeus, a first – century Greek physician, was credited with naming the disorder. He described the disease as a " melting down of the flesh and limbs into urine ". Lipemia of diabetic blood was noted by Helmunt some time between ١٥٧٣ and ١٦٦٤ A.D.. The first diagnostic sign of the disease was established by Thomas Willis in the seventeenth century, when he tasted the urine of his patients and noted its

sweetness. A French physician, Michel Chevreul, discovered that the sweetness was caused by sugar (Bullock *et al.,* ١٩٩٦).

In ١٨٦٩, Langerhans, while a medical student, identified the islets but did not understand their function (Murray *et al.,* ١٩٩٦). The discovery of insulin, which began with an accidental observation, when in ١٨٨٩ Oskar Minkowski and Josef Von Mering, had a friendly disagreement about whether the pancreas, known to contain lipases, was important in fat digestion in dogs. To resolve the issue, they began an experiment on fat digestion. They surgically removed the pancreas from a dog, but before their experiment got any further, Minkowski noticed that the dog was now producing far more urine than normal. Also, the dog's urine had glucose levels far above normal. These findings suggested that lack of some pancreatic product caused diabetes (Nelson and Cox, ٢٠٠٥).

The link between islets and diabetes was suggested by De Mayer in ١٩٠٩ and by Sharpey – Schaffer in ١٩١٧, but it was Banting and Best who proved this association in ١٩٢١.These investigators used acid – ethanol to extract from the tissue an islet cell factor that had potent hypoglycemic activity. The factor was named " insulin ", and it was quickly learned that bovine and porcine islets contained insulin that was active in humans. Within a year, insulin was in wide spread use for the treatment of diabetes and proved to be life saving (Murray *et al.*, ١٩٩٦).

١.٢.٣ Diagnosis of Diabetes Mellitus :-

The National Diabetes Data Group and World Health Organization have issued diagnostic criteria for the diagnosis of diabetes mellitus :

١) Symptoms of diabetes plus random blood glucose concentration ≥١١.١ mmol/L (٢٠٠ mg / dl)[a] or,

٢) Fasting plasma glucose ≥ ٧. ٠ mmol/L (١٢٦ mg/dl)[b] or,

٣) Two – hour plasma glucose ≥ ١١.١ mmol/L (٢٠٠ mg/dl) during an oral glucose tolerance test[c].

[a] : Random is defined as without regard to time since the last meal.

[b] : Fasting is defined as no caloric intake for at least ٨ hours.

[c] : The test should be performed using a glucose load containing the equivalent of ٧٥ g anhydrous glucose dissolved in water; not recommended for routine clinical use.

Note: In the absence of unequivocal hyperglycaemia and acute metabolic decompensation, these criteria should be confirmed by repeated testings on different days (Kasper *et al.,* ٢٠٠٥).

The diagnostic strategy for diabetes mellitus is illustrated in Figure (١.٢) (WHO, ٢٠٠٢).

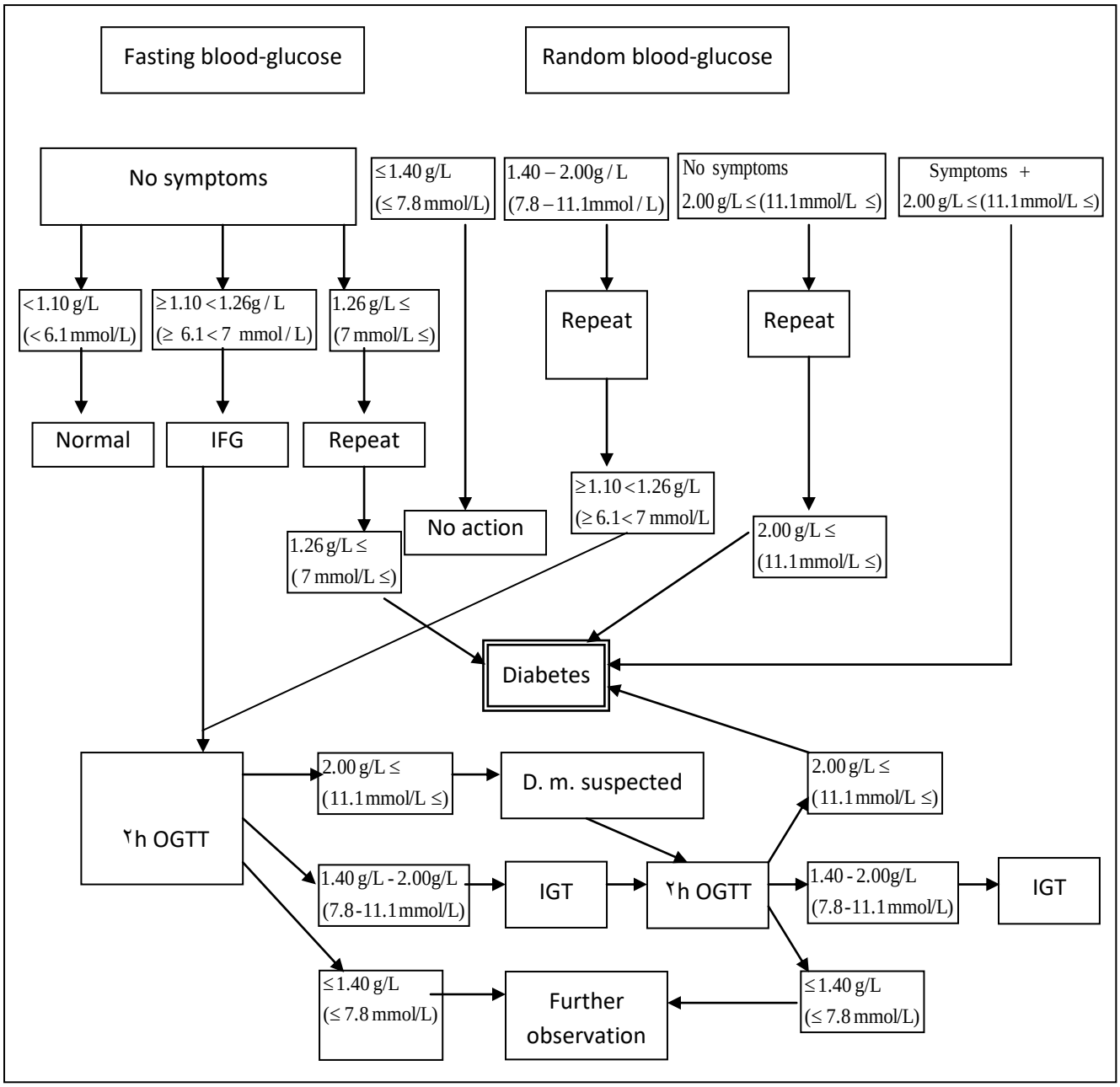

Figure (١.٢) : Diagnostic strategy for diabetes mellitus.

١.٢.٤ Classification of Diabetes Mellitus(DM):-

The following new classification system identifies four types of diabetes mellitus: type ١, type ٢, "other specific types", and gestational diabetes which is mainly based on the aetiology of DM :

I. Type ١ diabetes mellitus (B-cell destruction, usually leading to absolute insulin deficiency).

A- Immune mediated.

B- Idiopathic.

II. Type ٢ diabetes mellitus (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly insulin secretory defect with insulin resistance).

III. Other specific types of diabetes :

A-Genetic defects of islet B-cell function (e.g. maturity onset diabetes of the young ''MODY'').

B- Genetic defect in insulin action.

C- Diseases of the exocrine pancreas.

D- Endocrinopathies.

E- Drug-or chemical- induced diabetes.

F- Infections.

G- Uncommon forms of immune-mediated diabetes.

H- Other genetic syndromes.

IV. Gestational diabetes mellitus.

(WHO, ٢٠٠٢; Kasper *et al.,* ٢٠٠٥).

١.٢.٥ Changes in the Classification System :-

Two features of the current classification of DM diverge from previous classifications. First, the terms insulin-dependent diabetes mellitus (IDDM) and non insulin- dependent diabetes mellitus (NIDDM) are obsolete. Since many individuals with type ٢ DM eventually require insulin treatment for the control of glycaemia, the use of the term NIDDM generates a considerable confusion.

The second difference is that age is not a criterion in the classification system. Although type ١ DM most commonly develops before the age of ٣٠, an autoimmune beta cell destructive process can develop at any age.

It is estimated that between ٥% and ١٠٪ of individuals who develop DM after age ٣٠ have type ١A DM. Likewise, type ٢ DM more typically develops with increasing age, but it also occurs in children, particularly in obese adolescent (Kasper *et al.,* ٢٠٠٥).

Arabic numerals are specifically used in the new system to minimize the occasional confusion of type " II " as the number " ١١" (Mayfield, ١٩٩٨).

١.٢.٦ Type ١ Diabetes Mellitus :-

Type ١ Diabetes mellitus (formerly called type I, IDDM or juvenile diabetes) is characterized by an autoimmune process, usually leading to absolute insulin deficiency. The onset is usually acute, developing over a period of a few days to weeks. Over ٩٥ percent of persons with type ١ DM develop the disease before the age of ٢٥. A family history of type ١ DM, gluten enteropathy (celiac disease) or other endocrine disease is often found (Mayfield, ١٩٩٨). However, symptoms appear abruptly when ٨٠% to ٩٠٪ of the B-cells have been destroyed. At this point the pancreas fails to respond adequately to ingestion of glucose, and insulin therapy is required to restore metabolic control, many patients show a transient disappearance of diabetes and little or no insulin therapy is required. This remission results from a temporary return of insulin secretion, which may last for weeks or months. Ultimately, most patients lose all B-cell function and require insulin therapy (Champe and Harvey, ١٩٩٤).

١.٢.٧ Type ٢ Diabetes Mellitus :-

Type ٢ Diabetes mellitus (formerly called NIDDM, type II or adult onset) is characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the beta cell. This is the most common form of DM and is highly associated with a family history of diabetes, older age, obesity, and lack of exercise. It is more common in women especially women with a history of gestational diabetes. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Defective beta cells become exhausted, further fueling the cycle of glucose intolerance and hyperglycaemia (Mayfield, ١٩٩٨).

Weight reduction and dietary modifications often correct the hyperglycemia of type ٢ diabetes. Oral hypoglycemic agents or insulin therapy may be required to achieve satisfactory plasma glucose levels (Champe *et al.,* ٢٠٠٥). Table (١.٢) shows the characteristic features of type ١ and type ٢ diabetes (WHO,٢٠٠٢; Champe *et al.,* ٢٠٠٥; Haslett *et al.,* ١٩٩٩).

Table (١.٢):

Characteristic features of type ١ and type ٢ diabetes mellitus.

Characteristics	Type ١ Diabetes	Type ٢ Diabetes
Typical age of onset (years)	**< ٣٥**	**> ٣٥**
Duration of symptoms	**Weeks**	**Months to years**
Genetic predisposition	**Low**	**High**
Antibodies to B-cells	**Yes (٩٠-٩٥ %)**	**No**
Body habitus	**Normal / Wasted**	**Obese**
Plasma insulin / C-peptide	**Low / Absent**	**High**
Main metabolic feature	**Insulin deficiency**	**Metabolic syndrome with insulin insensitivity**
Insulin therapy	**Responsive**	**High doses required**
Insulin secretagogue drugs	**Unresponsive**	**Responsive**
Family history of Diabetes	**Uncommon**	**Common**
Prevalence	**١٠٪ - ٢٠٪ of Diagnosed Diabetes**	**٨٠٪ - ٩٠٪ of Diagnosed Diabetes**
Acute complication	**Ketoacidosis**	**Hyperosmolar coma**

١.٢.٨ Metabolic Disturbances in Diabetes :-

The hyperglycaemia of diabetes develops because of an absolute (type ١ diabetes) or a relative (type ٢ diabetes) deficiency of insulin, resulting in decreased anabolic and increased catabolic effects. In both type ١ and type ٢ diabetes, insulin's actions are also impaired by the insensitivity of target tissues. While this is a fundamental defect in type ٢ diabetes, hyperglycaemia can also induce insulin resistance through glucose toxicity.

Decreased anabolism results in hyperglycaemia, which leads to glycosuria, osmotic diuresis and consequent salt and water depletion. Increased catabolism is represented by increased glycogenolysis, gluconeogenesis and lipolysis, which will result in hyperketonaemia and consequently acidosis occurs and the patient gets diabetic ketoacidosis (Haslett *et al.,* ١٩٩٩).

The counter – regulatory hormones secretion will also increase, which includes glucagons, cortisol, growth hormone, and catecholamines. These hormones are catabolic and increase hepatic glucose production initially by enhancing the breakdown of glycogen to glucose (glycogenolysis) and later by stimulating the synthesis of glucose (gluconeogenesis) (Burtis and Ashwood., ١٩٩٦).

١.٣ Oxidative Stress :-

Oxidative stress has been defined as an imbalance between pro-oxidants and antioxidants. Either an increase in the production of oxidants or a deficiency in the antioxidant defense system could disturb this balance, causing oxidative stress (Langseth, ١٩٩٥). However, it is closely associated with aging and a number of diseases including cancer, cardiovascular disease, diabetes and diabetic complications (Atalay and Laaksonen, ٢٠٠٢).

Oxidative stress may be localized, for instance in the joints in arthritis or in the vascular wall in atherosclerosis, or can be systemic e.g. in systemic lupus erythematosus (SLE), and possibly diabetes (Baynes and Dominiczak, ٢٠٠٥). Figure (١.٣) shows oxidative stress which results from an imbalance between pro-oxidant and antioxidant forces (Baynes and Dominiczak, ٢٠٠٥).

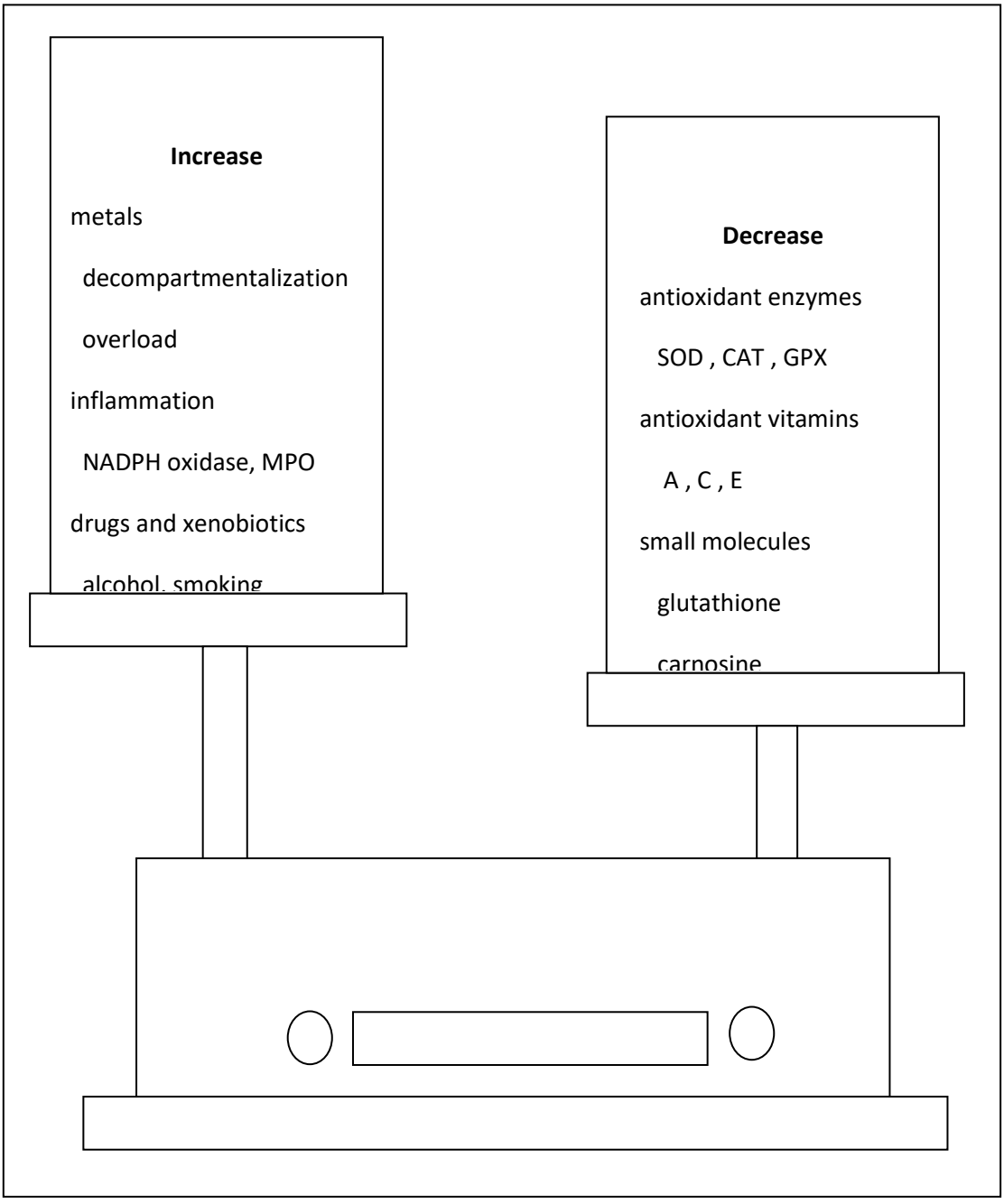

Figure (١.٣) :Oxidative stress which results from an imbalance between

pro-oxidant and antioxidant forces.

١.٣.١ Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) :-

In the body certain molecules called reactive oxygen species (ROS) and reactive nitrogen species (RNS) are normally produced as part of the defense system and as the by-products of cellular metabolic processes utilizing oxygen. These reactive species include free radicals or certain molecules which may be oxidizing agents or convertible to free radicals. Many factors can cause the body to produce more reactive species than are needed. These include smoking, drinking alcohol, too much fat in the diet, too much sun exposure, too many pollutants in the air, and even too much exercise (Wahlqvist and Wattanapenpaiboon, ١٩٩٩).

A free radical is any chemical species that has one or more unpaired electrons. Many free radicals are unstable and highly reactive. Free radicals are not always harmful. It is important to note that they also serve a useful purpose in the human body, for example, free radicals play a role in the destruction of disease causing microbes by specialized blood cells called Phagocytes (Langseth, ١٩٩٥). To combat microbial infections, phagocytes produce and release toxic oxygen radicals to kill invading bacteria in a process known as “respiratory burst”, the phagocytes then engulf killed bacteria. In an acute infection, the production of oxygen radicals which cause the killing of bacteria are efficient processes; however, in prolonged infections, phagocytes tend to die, releasing toxic oxygen radicals that affect surrounding cells (Devlin, ٢٠٠٢).

Reactive oxygen species (ROS) are partially reduced reactive forms of oxygen. They include oxygen-containing radicals such as super oxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and alkoxyl

radical ($RO^{\bullet}$), and non-radical species that can produce oxygen –containing radicals during their reaction, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), ozone (O_3), and hypochlorous acid (HOCl) which result from the reaction of H_2O_2 with Cl^- (Baynes and Dominiczak, ٢٠٠٥; Murray *et al.,* ٢٠٠٣; Alta'ee, ٢٠٠٣). Among the ROS hydroxyl radicals ($OH^{\bullet}$) are the most reactive and damaging species (Baynes and Dominiczak, ٢٠٠٥).

Reactive nitrogen species (RNS) include nitric oxide ($NO^{\bullet}$), also known as the endothelium derived relaxation factor (EDRF), which has a role in the regulation of vascular tone. ($NO^{\bullet}$) reacts with ($O_2^{\bullet}$) to form the strong oxidant, peroxy nitrate ($ONOO^-$), which is a RNS that has many of the strong oxidizing properties of ($OH^{\bullet}$) (Baynes and Dominiczak, ٢٠٠٥).

١.٣.٢ The Antioxidant Defense System in Human Body :-

In general, an antioxidant has been defined as any substance that, when present in low concentrations compared with that of an oxidizable substrate, can delay or inhibit the oxidation of that substrate (Al-Ameri, ٢٠٠٢), whereas Wahlqvist and Wattanapenpaiboon have defined antioxidants as substances that reduce oxidation and so counteract the reactive species (Wahlqvist and Wattanapenpaiboon, ١٩٩٩).

The human body has several mechanisms for defense against the radicals and other ROS. The various defenses are complementary to one another because they act on different oxidants or in different cellular compartments. One important line of defense is a system of enzymes, including glutathione peroxidases, superoxide dismutases, and catalase, which decrease the concentration of the most harmful oxidants. Nutrition plays a key role in maintaining the body's enzymatic defenses against free radicals. Several

essential minerals including selenium, copper, manganese, and zinc are involved in the structure or catalytic activity of these enzymes. A second line of defense is small-molecular-weight compounds which act as antioxidants; that is , they react with oxidizing chemicals, reducing their capacity for damaging effects. Some, such as glutathione, ubiquinol and uric acid, are produced by normal metabolism. Other small-molecular-weight antioxidants are found in the diet, the best known being vitamin E, vitamin C and carotenoids. Some foods also contain other antioxidant substances although these substances have no known nutritional function; they may be important to human health because of their antioxidant potency. Most of the antioxidants found in these foods are phenolic or polyphenolic compounds (Langseth, ١٩٩٥).

Another line of defense is to keep free levels of potentially redox-active metal ions at minimal. This can be achieved by the sequestration or the chelation of transition metals e.g. by binding to strong and transport proteins which minimize the free ions within the cells, then prevent the formation of highly reactive radicals (Mathkor, ٢٠٠٢).

١.٣.٣ The Toxic Effects of ROS :-

The free radicals are extremely reactive and unstable, and enter into reactions with proteins, lipids, carbohydrate, particularly with key molecules in membranes and nucleic acids. Moreover, free radicals initiate autocatalytic reaction whereby molecules with which they react are themselves converted into free radicals to propagate chain of damages. The effect of these reactive species are wide-ranging, but three reactions are particularly relevant to cell injury :

I. Lipid peroxidation of membranes.

II. Oxidative modification of proteins.

III. Lesions in deoxyribonucleic acid (DNA) (Mathkor, ٢٠٠٢).

Diabetes causes an increased oxidative stress in various tissues as evidenced by increased levels of oxidized DNA, proteins, and lipids. Besides damaging the functions of these molecules, oxidative stress also triggers a series of cellular responses, including the activation of protein kinase C (PKC), transcription factor NF-κB, and c-Jun $NH^{٢}$-terminal kinase (JNK) stress-associated kinases, and so forth. Inappropriate activation of these important regulatory molecules would have deleterious effects on cellular functions, and it is though to contribute to the pathogenesis of various diabetic complications (Chung *et al.,*٢٠٠٣). Figure (١.٤) shows the free radical damage (Bagchi and Puri, ١٩٩٨).

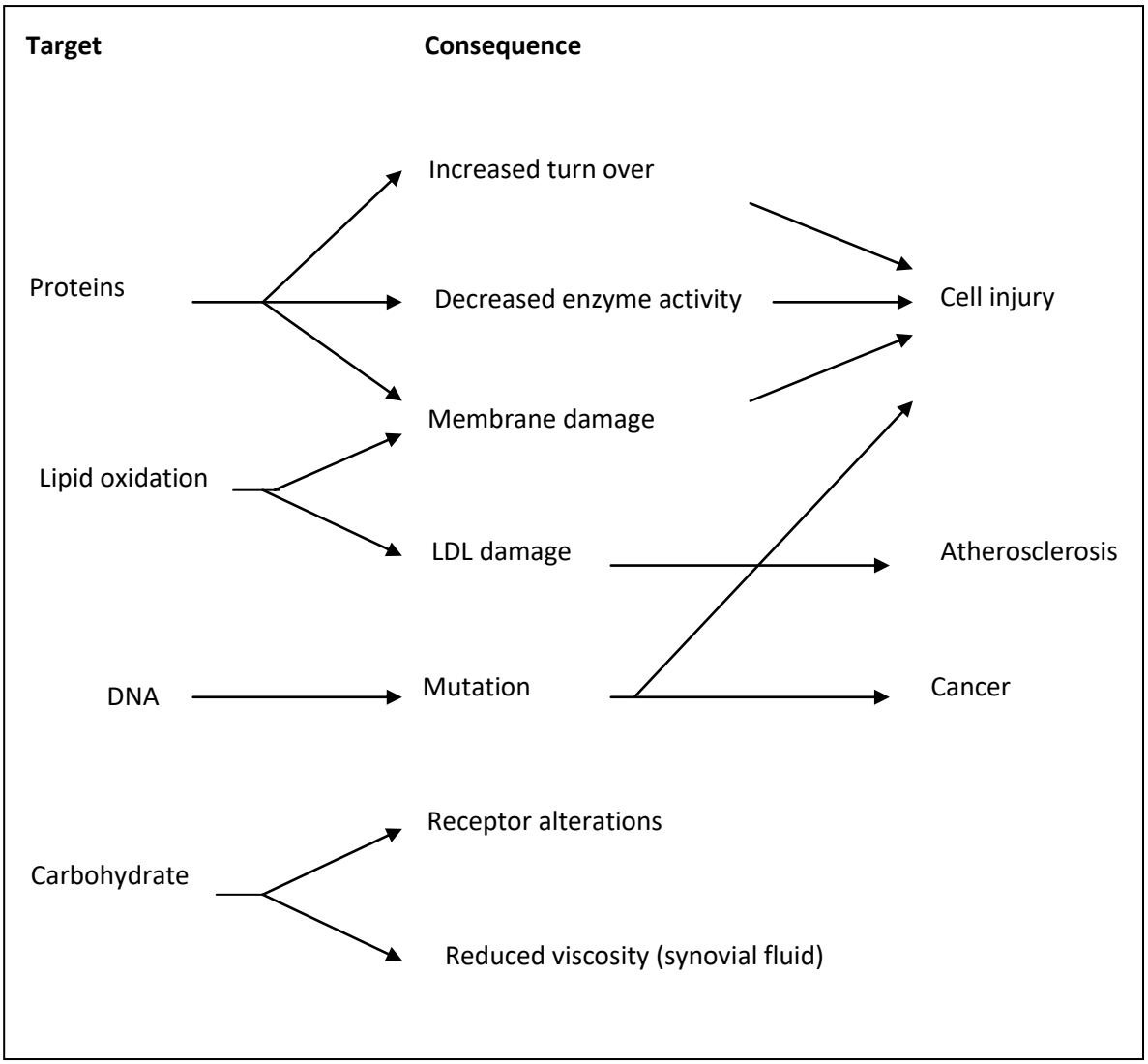

Figure (١.٤) : Free radical damage.

١.٣.٤ Mechanisms for Increased Oxidative Stress in Diabetes Mellitus :-

The mechanisms behind the apparent increased oxidative stress in diabetes are not entirely clear. Accumulating evidence points to a number of interrelated mechanisms, increasing production of free radicals such as superoxide or decreasing antioxidant status (Atalay and Laaksonen, ٢٠٠٢). It is most likely the combined effects of increased levels of reactive oxygen species (ROS) and decreased capacity of the cellular antioxidant defense system (Chung et al,. ٢٠٠٣). Oxidative stress may occur in several ways :

I. Increase generation of ROS.

II. Decreased antioxidant defense.

III. Alterations in enzymatic pathways.

IV. Ischaemic – reperfusion injury, hypoxia and pseudohypoxia.

Further possible sources of oxidative stress in diabetes mellitus are :

I. Inadequate diet -derived antioxidant.

II. Lack of dietary proteins may lead to inadequate synthesis of metal ion binding protein.

III. Exposure to drugs or toxins that are metabolized to produce free Radicals (Al-Ameri , ٢٠٠٢ ; Szaleczky *et al*., ١٩٩٩ ; Atalay and Laaksonen, ٢٠٠٢).

Figure (١.٥) shows the mechanisms for increased oxidative stress in diabetes mellitus (Atalay and Laaksonen, ٢٠٠٢).

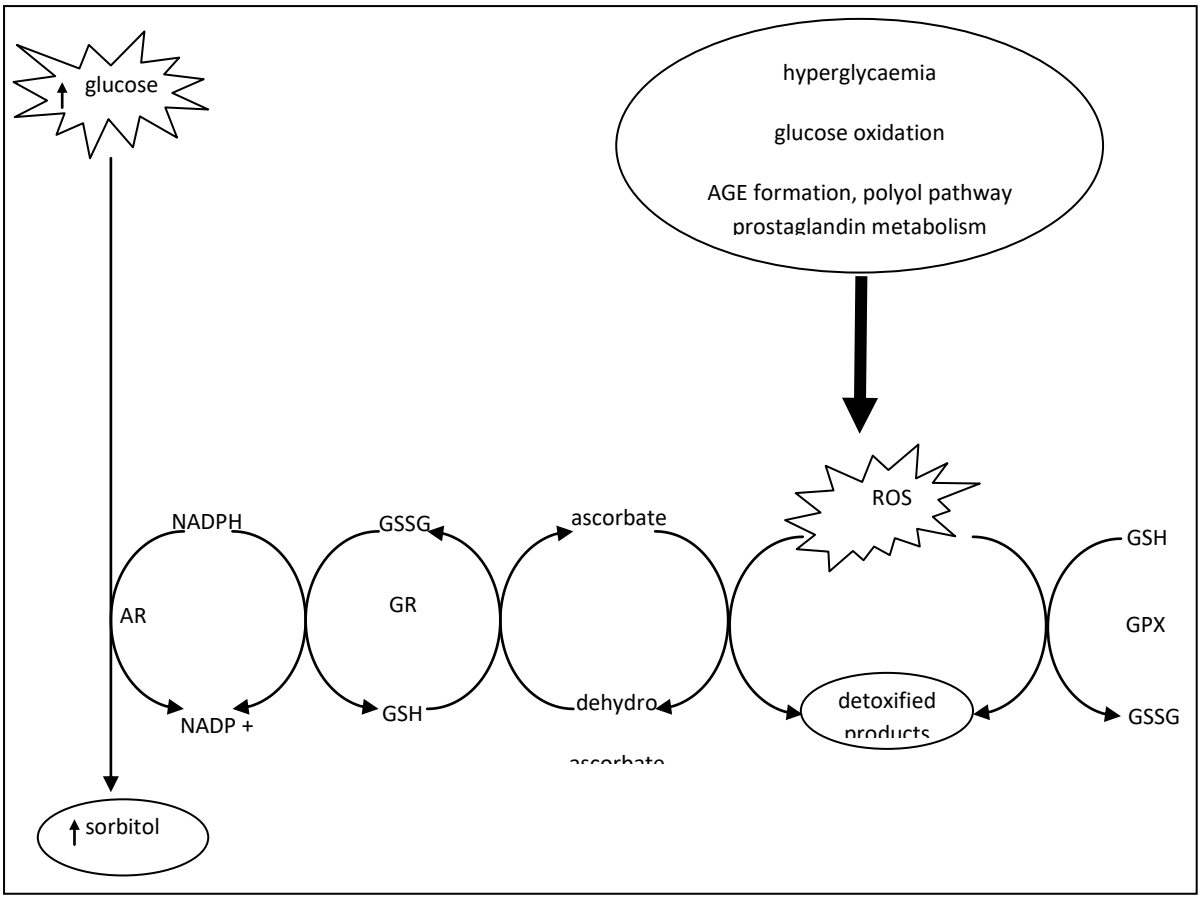

Figure (١.٥) :The mechanisms for increased oxidative stress in diabetes mellitus.

١.٣.٥ The Mechanism of Free Radicals in Beta Cells Destruction :-

Beta cells are prone to be destroyed by free radicals because of the low antioxidant enzyme nature. Immune cells such as macrophages, T and B cells are believed to produce free radicals that cause damage to beta cells of the pancreas. There are two mechanisms :

I. The infiltration of macrophages produces superoxide as a primary source of free radicals. The superoxide can be further converted to more active radicals, hydroxyl radicals, which attack cellular membrane and cause DNA breakage. The consequence of DNA breakage leads to cell death if the cells fail to repair the damage; also the activation of poly (ADP-ribose) synthetase, which is a nuclear enzyme, depletes the DNA levels in cells,

inhibiting proinsulin synthesis and, in addition, increasing the sensitivity of the cells to free radicals.

II. Cytokines are released by T-cells, and macrophages, in the insulin secreting cells and induce the formation of intra cellular free radicals causing selective damage to beta cells (Al-Ameri, ٢٠٠٢).

١.٣.٦ The Mechanism of Reactive Oxygen Species in Insulin Resistance :-

Insulin resistance is a condition where a given amount of insulin produces less than the expected response; therefore, more insulin is required to produce the ' normal ' effect (Baynes and Dominiczak, ٢٠٠٥). Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance. Defective beta cells become exhausted, further fueling the cycle of glucose intolerance and hyperglycaemia (Mayfield, ١٩٩٨). By affecting the amount of proton donors within the cell, protons are pumped across the inner mitochondrial membrane through certain complexes of electron transport system, hyperglycaemia would increase electrochemical potential difference across the inner mitochondrial membrane and would thus lead to an increased production of reactive oxygen species (ROS). ROS would change cellular signaling patterns, the most important cause of insulin resistance. Signaling transduction defects include: defective tyrosine phosphorylation, reduced insulin receptor substrate-١ (IRS-١) level, decreased phosphatidyl inositol-٣ kinase leads to defective cellular translocation of the glucose transporter ٤ (GLUT٤) to cell membrane, decreased activity of key enzymes such as pyruvate dehydrogenase or glycogen synthase , leading to the development of insulin resistance and impaired insulin secretion (Baynes and Dominiczak, ٢٠٠٥).

١.٤ Vitamin C (Ascorbic Acid) :-

Vitamin C is L-ascorbic acid (chemically : ٢- oxogulonolactone). The two hydroxyl groups have acidic properties. By releasing a proton, ascorbic acid therefore turns into its anion, ascorbate (Kodman and Roehm, ٢٠٠٥). The word "ascorbic" means ' without scurvy ' (Grodner *et al.,* ٢٠٠٠). The discovery of vitamin C is associated with the search for the cause of scurvy, a potentially fatal disease that weakens the body's connective tissues and also causes inflammation to them (Grodner *et al.,* ٢٠٠٠). It was first isolated in ١٩٢٨, by the Hungarian biochemist and Nobel Prize winner Szent-Gyor Gyi (Iqbal *et al.,*٢٠٠٤). Vitamin C is an essential nutrient in human beings, the higher primates, the guinea pigs and fruit eating-bats. In some other animals, a specific pathway exists for its synthesis (Baynes and Dominiczak, ٢٠٠٥). It has been found that the cause of human inability to synthesize ascorbic acid is the absence of the active enzyme, L-gulonolacton oxidase (Iqbal *et al*., ٢٠٠٤; Murray *et al.,* ١٩٩٦). Both L-ascorbic acid and L-dehydroascorbic acid are physiologically active forms of vitamin C (Iqbal *et al*., ٢٠٠٤).

The principal pathway of oxidation which leads to turn over of ascorbic acid is believed to involve in the removal of two electrons in succession and to yield first the ascorbate free radical (AFR) and then dehydroascorbate. Two molecules of AFR may react together to form one molecule of ascorbate and one of dehydroascorbate. Alternatively, AFR may be reduced by a microsomal NADH-dependant enzyme, mono-dehydro-L ascorbate oxidoreductase to ascorbate (Iqbal et al*.,* ٢٠٠٤). Figure (١.٦) shows ascorbic acid as an antioxidant (Spencer, ٢٠٠٥).

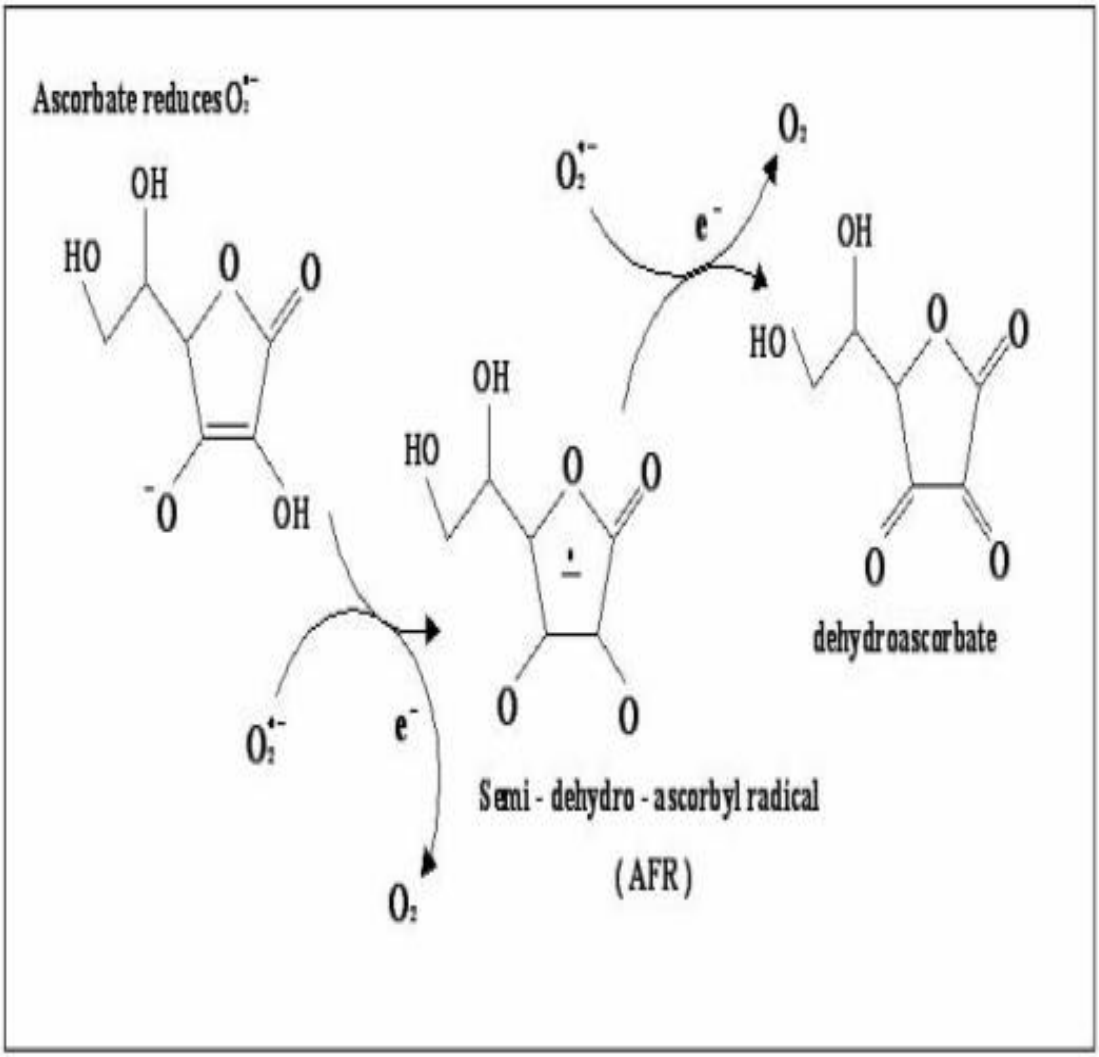

Figure (١.٦): Ascorbic acid (vitamin C) as an antioxidant.

Vitamin C is a water-soluble vitamin. This vitamin is a free radical scavenger and interacts with free-radicals in the water compartment of cells as well as in the fluids between cells. It is considered one of the most important antioxidants in extra cellular fluids. Vitamin C has a sparing effect on vitamin E as it regenerates vitamin E from the tocopheroxyl radical after it has neutralized free radicals. Its protective effects extend to cancer, coronary

artery disease, arthritis and aging (Bagchi and Puri,١٩٩٨). Figure (١.٧) shows the antioxidant network (Spencer, ٢٠٠٥).

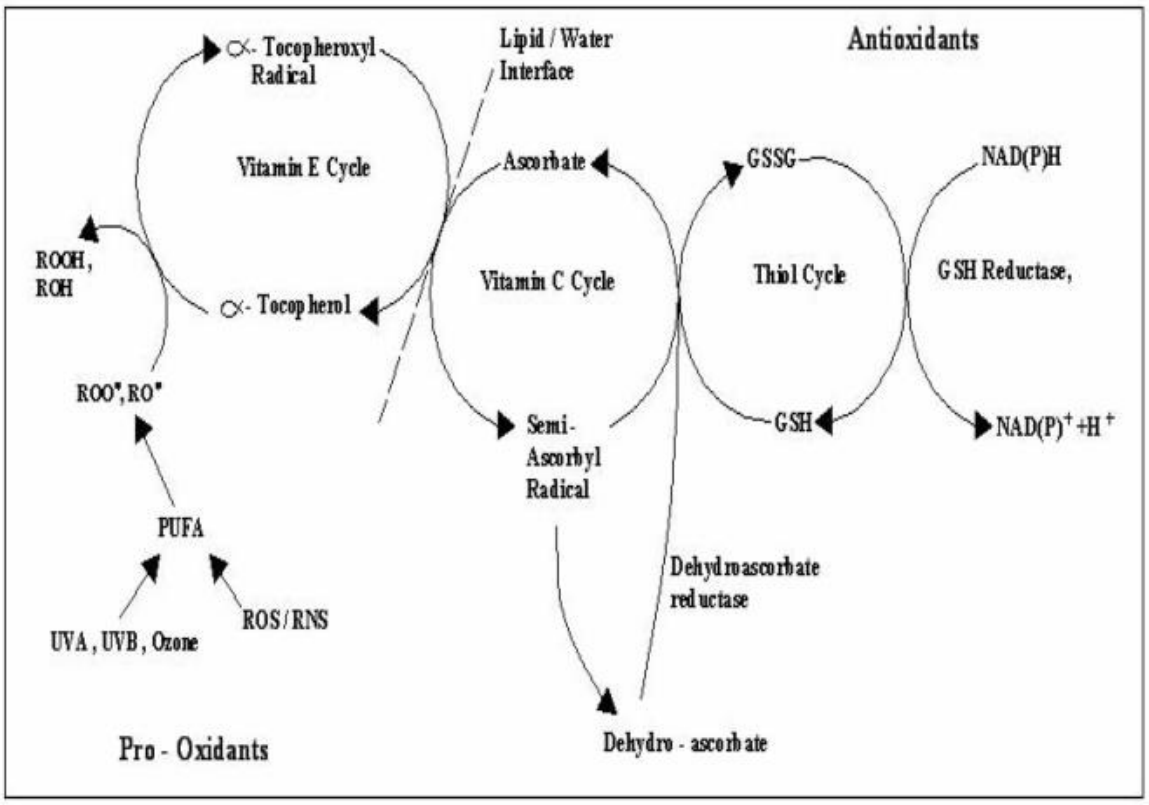

Figure (١.٧): The antioxidant network.

The most clearly established and critical functional role for ascorbic acid is as a cofactor for protocollagen hydroxylase enzyme for collagen synthesis ; vitamin C may also be involved in tyrosine metabolism , microsomal drug metabolism , synthesis of epinephrine and anti-inflammatory steroids by the adrenals , synthesis of bile acid , folic acid metabolism , and leukocyte functions. Absorption of $Fe^{+٢}$ is enhanced by the presence of vitamin C (Burtis and Ashwood, ١٩٩٦; Kodman and Roehm, ٢٠٠٥). The Recommended Dietary Allowance (RDA) for vitamin C has varied from ٤٥ mg to ٦٠ mg for adults. Presently, the RDA is ٦٠ mg but recommendations world-wide vary (Grodner *et al.,* ٢٠٠٠).

١.٤.١ Vitamin C (Ascorbic Acid) in Diabetes Mellitus :-

Although the biosynthetic relationship between glucose and ascorbic acid is absent in man, the glucose / insulin system does influence ascorbic acid. In particular, insulin promotes the active cellular uptake of ascorbic acid where as hyperglycaemia inhibits renal ascorbic acid reabsorption (Cunningham,١٩٩٨).

Vitamin C is considered the principal water-soluble antioxidant; it quenches the hydroxyl free radical, considered the most dangerous of all free radicals. It also reduces the formation of advanced glycation end products (AGEs), and it slows the conversion of glucose to sorbitol, another type of sugar that is a big contributor to diabetic complications (Smith, ٢٠٠٣).

A number of research groups report significantly decreased ascorbic acid concentrations in both types ١ and ٢ diabetic patients when compared to healthy control subjects (Banerjee , ١٩٨٢; Stankova *et al.,* ١٩٨٤; Will and Byers, ١٩٩٦). It has been reported that diabetic individuals have low levels of vitamin C in the plasma and in the white blood cells (Iqbal *et al.,* ٢٠٠٤).

Cunningham has found that leukocyte ascorbic acid is lower in type ١ DM compared with non diabetics (Cunningham, ١٩٩٨). Ahmad and associates have found significant low levels of ascorbic acid in type ٢ DM (Ahmad *et al.,* ٢٠٠٣). Iqbal and associates have reported in their review that ascorbic acid also has been tested as an antioxidant to inflammatory reaction in mice. High doses given after but not before the injury would successfully suppress edema. Vitamin C strengthens the immune system by stimulating the activity of antibodies and immune system cells such as phagocytes and neutrophils, and it protects the immune system against damage by the free

radicals released by the body in its fight against the infection. It has also been shown to decrease the bacteriological activity (Iqbal *et al.,* ٢٠٠٤).

١.٥ Glutathione :-

Glutathione is present in almost all living organisms: plants, animals, and some bacteria (Bannai and Tateish, ١٩٨٦; Nelson and Cox, ٢٠٠٥). Glutathione was discovered by F.G.Hopkins in ١٩٢١ (Orrenius and Moldēus, ١٩٨٤). Most of the intracellular glutathione exists in thiol form (GSH), although mixed disulfides (mainly G-SS-protein), thioethers and, to a lesser extent, glutathione disulfide (GSSG) contribute to the total cellular pool of glutathione. The bulk of intracellular GSH is found in the cytosol, but the existence of a minor mitochondrial pool of GSH has also been demonstrated. Moreover, both GSH and GSSG are present in various body fluids, including plasma, bile, and the glomerular filtrate, although at much lower concentrations (Orrenius and Moldēus, ١٩٨٤).

GSH is a tripeptide with a structure of γ-L-glutamyl-L-cysteinylglycin, which is characterized by the reactive thiol group and the γ-glutamyl bond which makes it resistant to normal peptidase activity (Bannai and Tateishi, ١٩٨٦ ; Orrenius and Moldēus, ١٩٨٤).

The intracellular level of this tripeptide varies with the growth, nutritional state, and hormonal balance of the organism (Moron *et al.,* ١٩٧٩).GSH is synthesized in two steps catalyzed by γ-glutamyl cysteine synthetase (reaction ١) and glutathione synthetase (reaction ٢) respectively :

γ-glutamyl cysteine synthetase

L-glutamate + L-cysteine +ATP ⇌ γ-L-glutamyl-L-cysteine + ADP + Pi (reaction ١)

glutathione synthetase

γ - L-glutamyl- L- cysteine + glycine + ATP ⇌ GSH + ADP + Pi (reaction ٢)

Reaction١ is considered rate limiting and inhibited by GSH, suggesting a physiologically significant feedback control of GSH synthesis (Bannai and Tateishi, ١٩٨٦). In normal conditions, the ratio of GSH/GSSG is high but in oxidative stress the ratio of GSH/GSSG is low (Spencer, ٢٠٠٥).

The GSH is recycled by an NADPH - dependent enzyme GSH reductase. The NADPH, provided by the pentose phosphate pathway, maintains a ≈ ١٠٠ : ١ ratio of GSH : GSSG in the cell (Baynes and Dominiczak, ٢٠٠٥). Low concentrations of GSH have been implicated in numerous pathological conditions, including diabetes, alcoholic liver disease, AIDS, acute hemorrhagic gastric erosions, cataracts, Parkinson diseases, xenobiotic-induced oxidative stress and toxicity, and aging (Richie *et al.,* ١٩٩٦). More recently, GSH has been demonstrated to regulate gene transcription involved in the pathogenesis of cancer, diabetes, and atherosclerosis (Al-Ameri, ٢٠٠٢). However, it has been called the "master antioxidant", which is considered the major endogenous antioxidant produced by the cell. It participates directly in the neutralization of free radicals, reactive oxygen compounds, and maintains exogenous antioxidants such as vitamins C and E in their reduced (active) forms. In addition, through direct conjugation, GSH plays a role in the detoxification of many xenobiotics (foreign compounds) both organic and inorganic. GSH is also

considered an essential component of the human immune response. The proposed mechanisms of immune enhancement include:

I. Optimizing macrophage functions,

II.Offsetting oxidative damage associated with lymphocyte monoclonal

expansion, and

III.Stabilizing the mitochondrial membrane thereby, reducing apoptosis

in lymphocytes. (Petrosino, ٢٠٠٥).

Other physiological functions of GSH include the maintenance of membrane integrity and cytoskeletal organization, involvement in protein and DNA synthesis, modulation of protein conformation and enzyme activity and promotion of neurotransmitter release (Orrenius and Moldēus, ١٩٨٤).

١.٥.١ Reduced Glutathione (GSH) in Diabetes Mellitus :-

Reduced glutathione (GSH) is a non - specific reduction agent and plays an important role in oxidation mechanisms. It participates in antioxidative defense system as a free radical inactivator (Atamer *et al.,* ١٩٩٨).

The depletion of GSH has been speculated to be an important contributing factor to some serious human diseases, such as chronic renal failure, diabetes, Parkinson's disease and cataract formation (Lomaestro and Malone, ١٩٩٥). Tessier and associates have observed that oxidized glutathione (GSSG) increases in the serum of diabetic patients and GSH / GSSG ratio decreases (Tessier *et al.,* ١٩٩٩). Al-Ameri has observed in his study that type ٢ DM patients have lower antioxidants levels , GSH in

between them , as compared with control subjects (Al-Ameri, ٢٠٠٢). Atamer and associates have found that erythrocyte GSH levels of type ٢ diabetes are significantly lower as compared with control subjects (Atamer *et al.,* ١٩٩٨).

١.٦ C-reactive protein (CRP) :-

C-reactive protein is the first reported acute-phase protein in humans. It belongs to a highly conserved phylogenetically ancient family called pentraxins, which have five identical non-covalently linked subunits (Das *et al.,* ٢٠٠٣). It was first described in ١٩٣٠ (Burtis and Ashwood, ١٩٩٦). It is so named because it precipitates with the C substance, a polysaccharide of pneumococci (Bishop *et al.,* ٢٠٠٥). CRP is mainly synthesized in the liver (Fischbach,٢٠٠٠). Its synthesis is triggered by certain cytokines including interleukin-٦, interleukin-١, growth factor B , and some other extracellular signaling molecules (Das *et al.,* ٢٠٠٣). Normally, it is present only in trace amounts in serum, but it can increase by as much as ١٠٠٠-flod in response to injury or infection (Pepys, ١٩٨١; Werner, ١٩٦٩; Fischbach, ٢٠٠٠). Large amounts of CRP can also appear in peritoneal, pleural, pericardial, and synovial body fluids (Fischbach, ٢٠٠٠). Its level is elevated in infections and other disorders associated with tissue damage and inflammation (Kallio, ١٩٩٧; Clyne and Olshaker, ١٩٩٩).

Measurements of CRP levels in serum are of value in many clinical situations including :

I. Assessment of the activity, extent , and course of inflammatory processes.

II. Differentiation of viral / bacterial infections, CRP rises up during

bacterial infection to higher levels than in viral infection.

III. Monitoring its level is used to assess results of various treatment regimens.

IV. Monitoring patients who are immuno - suppressed either from chemotherapy or from disease for potential bacterial infection.

V. Prognostic marker for coronary heart disease in patients with unstable angina and is also used as a marker of cardiovascular risk.

VI. In obstetric patients with premature rupture of membranes, a rise in CRP can give early warning of intrauterine infections.

VII. CRP rises up during the chronic course of the diseases (diabetes mellitus, chronic brucellosis, chronic viral hepatitis-B and C).

VIII. Early detection of complication in post-operative patients.

IX. For the differentiation between active disease and infection in Systemic lupus erythematosus (SLE), an elevated CRP indicates infection, not inflammation caused by SLE it self, where the level of response to active disease has previously been established.

X. For the differentiation between infection and graft -versus- host disease in bone marrow transplant patients.

(Craig *et al.,* ٢٠٠٢ ; Das *et al.,* ٢٠٠٣ ; Pagana and Pagana,٢٠٠٢).

The nature of non specific elevation of CRP is an important obstacle for its wide clinical application , as it can not be used for specific disease diagnosis, but Das and associates (٢٠٠٣) have found that human CRP is glycosylated in different clinical conditions, keeping their pentraxin structures

almost the same as the native CRP, but some minor changes in their amino-acid sequence and glycosylation make them differ chemically, which may be essential for their proper biological function. These observations may pave the way for overcoming many important obstacles for the clinical use of CRP measurements and for new diagnostic approaches for using CRP as a potential clinical marker (Das *et al.,* ٢٠٠٣).

١.٦.١ C-reactive protein in Diabetes Mellitus :-

Diabetes mellitus (DM) is a chronic disorder, and comprises a group of syndromes with abnormal carbohydrate metabolism, all characterized at some point by hyperglycaemia. Changes in serum protein levels in diabetes are related to inflammation, secondary complications, and to the metabolic effects of abnormal insulin and glucose level (Craig *et al.,* ٢٠٠٢; Pickup, ٢٠٠٤). CRP is moderately elevated in DM (Craig *et al.,* ٢٠٠٢). Its concentrations are lowest among those with newly or previously diagnosed diabetics (Ford, ١٩٩٩).

Previous studies have suggested that CRP is involved in the development of type ١ DM Chase and associates have found that type ١ DM is an immuno inflammatory disorder and elevated CRP levels is associated in the development of type ١ DM (Chase *et al.,* ٢٠٠٤). Amrani and associates have reported that cytokines including IL-١, IL-٦, and TNF-α are present in the inflammatory infiltrate of pancreatic islets, and have a potential role in beta cell destruction particularly when present in combination (Amrani *et al.,* ٢٠٠٠). Schram and associates have found that inflammatory activity, measured by inflammatory markers (CRP, IL-٦, and TNF-α), is increased in type ١ diabetes and may predispose to vascular disease (Schram *et al.,* ٢٠٠٣). Many studies have approved that CRP also plays a role in the development of type ٢ DM (Pickup, ٢٠٠٤; Thorand *et al.,* ٢٠٠٣; Pradhan *et al.,* ٢٠٠١). Schulze and

associates have found that high plasma levels of CRP are associated with an increased risk of incident cardiovascular events among men with type ٢ diabetes (Schulze *et al.,* ٢٠٠٤). Although the mechanism of the CRP elevation is unknown, they suggest that it might be related to the activation of macrophages, increased oxidative stress, or induction of cytokines (Chase *et al.,* ٢٠٠٤).

١.٧ The Relation between C-reactive protein and Oxidative Stress in Diabetes Mellitus :-

Diabetes mellitus is associated with an increased production of reactive oxygen species and a reduction in antioxidant defenses (Bonnefont-Rousselot, ٢٠٠٤). The reactive oxygen species create oxidative stress, which damages molecules and activates a number of signaling molecules such as protein kinase C or the transcription factor NFκB (NFκB is a key determinant of inflammatory response).

(Baynes and Dominiczak, ٢٠٠٥; Chung *et al.,* ٢٠٠٣; Nishikawa *et al.,* ٢٠٠٠).

It has also been found that oxidants such as hydrogen peroxide activate NFκB and IL-٦ in cultured monocytes and endothelial cells (Jain *et al.,* ٢٠٠٣). Shurtz-Swirski and associates have found that type ٢ diabetic patients are exposed to oxidative stress and chronic inflammation (partially because of the primed state of their polymorph nuclear leukocytes). The existence of chronic inflammation in diabetes is mainly based on the increased plasma concentrations of C-reactive protein, fibrinogen, interleukin-٦, interleukin-١, and tumor necrosis factor (Shurtz-Swirski *et al.,* ٢٠٠١). Bloomgarden has reported that free radicals are accompanied by an increase in inflammatory markers, ultimately leading to decreased metabolic insulin response in a variety of tissues and to endothelial dysfunction (Bloomgarden, ٢٠٠٤).

Yasunari and associates have found that there is a significant direct relationship between monocyte oxidative stress and serum CRP in diabetes (Yasunari *et al.,* ٢٠٠٢). Block and associates have found that CRP has a significant positive relation with both biomarkers of lipid peroxidation plasma (malondialdehyde and F٢-isoprostane), while plasma ascorbic acid is significantly and inversely related to both biomarkers in healthy adults (Block *et al.,* ٢٠٠٢). Esposito and associates have found that inflammatory cytokine concentrations (plasma IL-٦ and TNF-α) are acutely increased by hyperglycaemia in humans. The effect is abolished by the infusion of the antioxidant glutathione, suggesting that hyperglycaemia-induced cytokine production is mediated by reactive oxygen species (Esposito *et al.,*٢٠٠٢).

Aims of the Study

١- Study the antioxidants levels(reduced glutathione,reduced vitamin C) in both

types ١ and ٢ diabetic patients.

٢- Study the inflammatory marker (C-reactive protein) in both types ١ and ٢

diabetic patients.

٣- Study the relation of glucose level with C-reactive protein level in both types ١ and ٢ diabetic patients.

٤- Study the relationship between C-reactive protein concentration and antioxidants (reduced glutathione, reduced vitamin C) in both types ١ and ٢ diabetic patients.

٢.١ Materials :-

٢.١.١ Patients and Conditions of The Study :-

The period covering the practical side of the study was from the ٢٧th of November ٢٠٠٤ to the ١٧th of May ٢٠٠٥. It was performed at the Laboratory of Biochemistry Department, College of Medicine, University of Babylon. The study was conducted on ٨٧ patients from the diabetic clinic in Mirjan Teaching Hospital, as well as from Babylon Hospital of Pediatric and Maternity. ٢٧ patients were with type ١ DM (١٠ males and ١٧ females) whose ages ranged from ١٠ – ٧٤ years old, and ٦٠ patients were with type ٢ DM (٢٧ males and ٣٣ females) whose ages ranged from ٣٠ – ٨٥ years old.

The medical history of each patient was taken regarding age, gender, duration of DM, type of treatment, , history of ischaemic heart disease, history of any other illness, and smoking status. Measurements of their height and weight were done to calculate their body mass index (BMI) and blood samples were also drawn. Regarding the type of DM, patients were classified according to the characteristic features of type ١ and type ٢ DM, in which patient features goes with type ١ DM considered mostly type ١ DM patient, whereas

patient features goes with type ٢ DM considered mostly type ٢ DM patient because definite classification by measuring plasma insulin level or antibodies to B-cells was not available for us. All patients underwent full physical examinations, and investigations: fasting serum glucose (FSG), random serum glucose (RSG), complete blood film (CBF), general urine examination (GUE), blood urea, serum creatinine, and electrocardiograph (ECG). Other investigations were done according to the condition for which the diabetic patient was admitted to hospital.

٢.١.٢ Healthy Subjects :-

Thirty-nine healthy subjects were taken as a control group (١٦ males and ٢٣ females). They did not have any history of chronic disease and did not take any treatment for chronic disease, history of smoking was taking, and their ages ranged from ١١ – ٦٥ years old. Measurements of their height and weight were also done to calculate their body mass index (BMI) and blood samples were drawn.

٢.١.٣ Blood Sampling :-

Venous blood samples were drawn from healthy control subjects and diabetic patients using disposable syringes in the sitting position. Five ml of blood was obtained from each subject, and was pushed slowly into plain disposable tube without anticoagulant, blood was allowed to clot for ١٠-١٥ minutes, the clot shrinks and serum was obtained by centrifuging ٢٥٠٠ xg for approximately ١٠ – ١٥ minutes.

٢.١.٤ Chemicals :-

All chemicals were highly purified and used as supplied without further purification.

Table (٢.١): Chemical compounds used in this study.

Chemicals	Production
❖ ٢,٤-Dinitrophenyl hydrazine (٢,٤-DNPH)	BDH (England)
❖ ٥,٥' Dithiobis (٢-nitro benzoic acid) (DTNB)	Fluka (Switzerland)
❖ Ascorbic acid standard	Merck (Germany)
❖ Copper sulfate anhydrous	Fluka (Switzerland)
❖ C-reactive protein (CRP) latex test – kit	Standard (Germany)
❖ Distilled water (DW)	Laboratory distillator (England)
❖ Ethylene diamine tetra acetic acid disodium ($EDTANa_٢$)	Gainland Chemical (England)
❖ Glucose enzymatic colorimetric test – kit	Biocon GLU (Germany)
❖ Glutathione standard (GSH)	Sigma – Aldrich chemie (Germany)
❖ Glycine	Fluka (Switzerland)
❖ Meta-phosphoric acid ($m\text{-}HPO_٣$)	Merck (Germany)
❖ Methanol	Fluka (Switzerland)
❖ Sodium chloride (NaCl)	Fisher Scientific (U.K.)
❖ Sulfuric acid ($H_٢SO_٤$)	Fluka (Switzerland)
❖ Thiourea	Fluka (Switzerland)
❖ Trichloro acetic acid (TCA)	Hopkin and Williams (England)
❖ Tris (hydroxymethyl) amino methane	Merck (Germany)

٢.١.٥ Instruments and Materials :-

Table (٢.٢): Instruments and materials used in this study.

Instruments and Materials	Production
❖ Balance (for chemical compounds)	Sartorius AG GOTTINGEN (Germany)
❖ Body weight balance	Japan,UK
❖ Centrifuge	-Griffin and George BS (Britain) -Kokusan (Japan)
❖ Distillator	Bibby Science Products Limited (England)
❖ Filter papers	Schleicher and Schiill Gmbh (Germany)
❖ Glass slide	Standard (Germany)
❖ Incubator	Fi sher Scientific (USA)
❖ Magnetic stirrer with hot plate	Classico (India)
❖ Micropipettes – automatic (٥-٥٠, ١٠٠-١٠٠٠) µL	SLamed (Germany)
❖ pH – meter	HANNA (Portugal)
❖ Spectro photometer	Spectronic ٢١,Milton Roy (USA)
❖ Stirring sticks	Standard (Germany)
❖ Tape measure	Japan
❖ Vortex (Electronic)	Vib rofix,VF-١ Janke and Kunkel (Germany)
❖ Water bath	Schutzart Din ٤٠٠٥٠-IP ٢٠ Memmert GmbH,Schwabach FRG (Germany)

٢.٢ Methods :-

٢.٢.١ Body Mass Index (BMI) :-

BMI has been proposed as an alternative to the traditionally used height-weight tables in assessing obesity. BMI measures weight corrected for height and is significantly correlated with total body fat content. BMI was calculated as weight (in kilograms) divided by height (in meters) squared.

BMI= Weight (kg) / Height^{٢} (m^{٢})

BMI was classified into :

-Underweight when BMI < ١٨.٥ kg/m^{٢}.

-Normal when BMI between ١٨.٥-٢٤.٩ kg/m^{٢}.

-Overweight when BMI between ٢٥.٠-٢٩.٩ kg/m^{٢}.

-Obesity when BMI between ٣٠.٠-٣٩.٩ kg/m^{٢}.

-Extreme obesity when BMI ≥ ٤٠.٠ kg/m^{٢}.

(Grodner *et al.*,٢٠٠٠).

٢.٢.٢ Determination of Serum Glucose :-

For the determination of glucose in the sera of tested individuals, enzymatic colorimetric test kit was used.

- **Method :**

Enzymatic colorimetric test on basis of Trinder-Reaction.

- **Principle :**

The test was based on the coupling of the enzymatic oxidation of glucose by glucose oxidase resulting in hydrogen peroxide, subsequently used for the generation of a colored product by peroxidase. In the Trinder method,

the carcinogenic ortho-dianisidine used in earlier formulations have been replaced by phenol and ٤-amino-antipyrine. Enzymatic colorimetric test on basis of Trinder-Reaction as in the following equations:

$$Glucose + O_2 + H_2O \xrightarrow{Glucose\ oxidase} Gluconic\ acid + H_2O_2$$

$$2H_2O_2 + phenol + 4-aminantipyrine \xrightarrow{peroxidase} Red\ Chinonimin + 4H_2O$$

- **Reagent Concentration:**

R١:

Phosphate buffer pH (٧.٥) ١٥٠ mmol/L

Phenole ٧.٥ mmol/L

R٢:

Glucose oxidase (GOD) ١٢٠٠٠ U/L

Peroxidase (POD) ٦٦٠ U/L

٤-aminoantipyrine ٠.٤٠ mmol/L

R٤:

Glucose ١٠٠ mg/dl (٥.٥٥ mmol/L)

- **Preparation :**

Dilute contents of enzyme reagent /R٢ with the corresponding volume of buffer /R١.

The working solution R٢ solved in R١ is stable for :

٣ months at +٢°C to +٨°C

٤ weeks at +٢٠°C to +٢٥°C

keep protected from light

R٤: Ready for use.

The standard is refrigerated stable up to expiry date.

- **Procedure:**

Test tubes are prepared for sample, blank, and standard and follow the instructions below:

Reagents	Sample (µL)	Blank (µL)	Standard (µL)
Working Reagent	١٠٠٠	١٠٠٠	١٠٠٠
Standard R٤			١٠
Serum	١٠		

-Mix, incubating for ١٥ minutes at +٣٧°C or for ٣٠ minutes at +٢٥°C.

-Within ٦٠ minutes read absorbance (A) at ٥٤٦ nm of the sample, reagent blank and standard.

- **Calculation:**

Determine the absorbance change as :

$\Delta A_{Sample} = (A_{sample} - A_{blank})$

$\Delta A_{Standard} = (A_{standard} - A_{blank})$

and use this for the calculation of serum glucose.

$$Glucose\ Concentration = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times Standard\ Concentration$$

The concentration of the supplied standard / calibrator R٤ :

(١٠٠ mg/dl) or (٥.٥٥mmol/L).

٢.٢.٣ Measurement of Serum C-reactive protein (CRP):-

The presence of CRP in the sera of the tasted individuals was evaluated by using CRP latex reagent kit , which is a rapid test for the qualitative and semi quantitative measurement of CRP in serum by agglutination of latex particles on the slide.

- **Method:**

Latex agglutination test.

- **Principle:**

The CRP reagent kit is based on an immunological reaction between CRP antisera bound to biologically inert latex particles and CRP in the test specimen. When serum contains greater than ٨ mg/L, CRP is mixed with the latex reagent, and visible agglutination occurs.

- **Reagents:**

Store the reagent kit at ٢-٨°C.

١- CRP latex reagent :

A suspension of uniform polystyrene particles was coated with monospecific antihuman CRP (goat) in glycine buffer, pH ٧.٥ ± ٠.٥; reagent sensitivity adjusted to approximately ٨ mg/L.

٢- CRP positive control serum :

A stabilized prediluted human serum containing greater than ٨ mg/L.

٣- CRP negative control serum :

A stabilized prediluted human serum.

٤- Glycine – Saline buffer pH ٨.٢ ± ٠.١:

This buffer is required for dilution but not provided in the kit, so preparation of it done by the following way :

- Glycine solution ($NH_٢CH_٢COOH$) (٠.١ M) :

٠.١٨٧٦٧٥ gm of glycine dissolved in ٢٥ ml distilled water.

- Sodium chloride solution (NaCl) (٠.١٥ M) :

٠.٢١٩١٥ gm of NaCl dissolved in ٢٥ ml distilled water.

- Glycine – Saline buffer prepared by mixing both the prepared glycine and sodium chloride solutions (pH ٨.٢ ±٠.١).

- **<u>Procedure:</u>**

A-Qualitative test :

١- Bring reagents and specimens to room temperature before use.

٢- Place (٤٠ µL) of the serum / controls into test wells on the glass slide. Use fresh pipette tip for each sample.

٣- Add an equal volume (٤٠ µL) of the CRP latex reagent to serum / controls.

٤- Mix well with the provided stirring sticks.

٥- Rotate the slide. If the serum contains approximately ٨ mg/L of CRP, a clear agglutination will appear within two minutes.

B-Semi-Quantitative test :

١- Setup at least five test tubes : ١:٢,١:٤,١:٨,١:١٦,١:٣٢,etc …

٢- Dilute sample according to dilution factor on each test tube with glycine diluent.

٣- Place (٤٠ µL) of each of positive and negative controls on to the slide ring. Place (٤٠ µL) of each dilution on successive fields of the reaction slides.

٤- Gently resuspend the CRP latex reagent and add an equal volume (٤٠ µL) to each test field.

٥- Mix well with the provided stirring sticks.

٦- Rotate the slide for ٢ minutes. The approximate concentration will be equal to the reciprocal of the highest dilution that would present with a clearly visible agglutination multiplied by ٨. Results are expressed in mg/L based on the WHO International Standard for human CRP.

٢.٢.٤ Determination of Serum Total Vitamin C(Ascorbic Acid):

- **Method:**

٢,٤–Dinitrophenylhydrazin (٢,٤-DNPH) photometric method (Lleyd *et al .,* ١٩٤٥ ; Burtis and Ashwood , ١٩٩٩).

- **Principle:**

In the ٢,٤- dinitrophenylhydrazin (٢,٤- DNPH) method, ascorbic acid is oxidized by $Cu^{+٢}$ to dehydroascorbicacid and diketogulonicacid. When treated with ٢,٤-DNPH, the ٢,٤-dinitrophenylosazone product forms, which in the presence of sulfuric acid, forms an orange – red complex that absorbs at ٥٢٠ nm. Thus this method measures both the oxidized (dehydroascorbicacid) and the reduced ascorbic acid forms of vitamin C.

- **Preparation of reagents:**
  - Metaphosphoric acid (m-HPO_3) (٠.٧٥ M): ٣٠ gm of m-HPO_3 are dissolved in a final volume of ٥٠٠ ml of distilled water.(Stable for١ week).
  - Sulfuric acid (H_2SO_4) (٤.٥ M): Carefully ٢٥٠ ml of concentrated H_2SO_4 are added to ٥٠٠ ml of cold distilled water. When the solution has cooled to room temperature, distilled water is added to ١ liter, with mixing. (Stable for ٢ years).
  - Sulfuric acid (H_2SO_4) (١٢ M): Carefully ٦٥٠ ml of concentrated H_2SO_4 are added to ٣٠٠ ml of cold distilled water and brought to a final volume of ١ liter. (Stable for ٢ years).
  - ٢,٤-Dinitrophenylhydrazin (٢,٤-DNPH) (٠.٠١ M): ١٠ gm of ٢,٤-DNPH are dissolved in ٤٠٠ ml of ٤.٥ M H_2SO_4 , and brought to a final volume of ٥٠٠ ml with ٤.٥ M H_2SO_4, then refrigerated over night, and filtered. (Stable for at least ١ week at refrigerated temperature).
  - Thiourea (٠.٦٦ M): ٥ gm of thiourea are dissolved in a final volume of ١٠٠ ml of distilled water.(Stable for ١ month at ٤^{o}C).
  - Copper sulfate (٠.٠٢٧ M): ٠.٦ gm of anhydrous copper sulfate is dissolved in a final volume of ١٠٠ ml of distilled water. (Stable for ١ year at room temperature).
  - DTCS reagent: ٥ ml of thiourea, ٥ ml of copper sulfate, and ١٠٠ ml of the ٢,٤-DNPH reagent are combined. (Store in bottle at ٤^{o}C for a maximum of ١ week).
  - Ascorbic acid standards: Stock standard solution (٢.٨ mM) is prepared by dissolving ٥٠ mg of ascorbic acid in a final volume of ١٠٠ ml of m-HPO_3 . Dilutions are made in m-HPO_3 to ٢.٥, ٥, ١٠ and ٢٠ mg/L (٠.٠١٤, ٠.٠٢٨,

٠.٠٥٦٧ and ٠.١١ mM) respectively. These are the working standards. (All working standards should be prepared daily).

- **Procedure:**

The procedure for the determination of total vitamin C in serum by ٢,٤-DNPH method is summarized as follows:

Sample, blank, and standard test tubes are prepared, then pipetted into test tubes,

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
m-HPO_3	٨٠٠		
Serum	٢٠٠		

The tubes are mixed in vortex mixture, then centrifuged at ٢٥٠٠ xg for ١٠ minutes

Reagents	Sample (µL)	Reagent Blank(µL)	Standard(µL)
Supernatant	٦٠٠		
Standards			٦٠٠
m-HPO_3		٦٠٠	
DTCS reagent	٢٠٠	٢٠٠	٢٠٠

The tubes are capped and mixed in vortex mixture, then incubated in a water bath at ٣٧°C for three hours. The tubes are removed from the water bath and chilled for ١٠ minutes in an ice bath, slowly mixed.

Reagents	Sample(µL)	Reagent Blank(µL)	Standard(µL)
Cold H_2SO_4 (١٢M)	١٠٠٠	١٠٠٠	١٠٠٠

The tubes are mixed in vortex mixture and returned immediately to the ice bath. The spectrophotometer is adjusted with blank to read zero absorbance (A) at ٥٢٠ nm, and the absorbance of standards and sample is read.

- **<u>Calculation of Serum Total Vitamin C:</u>**

The concentration of the samples is obtained from the calibration curve (Figure ٢.١) and is multiplied by ٥ (to correct for dilution of the serum by m-HPO_3) to give the concentration of vitamin C (ascorbic acid) per liter of serum.

The concentration of ascorbic acid can be determined directly from a standard as follows:

$$\frac{A_{sample}}{C_{sample}} = \frac{A_{standard}}{C_{standard}}$$

$$C_{sample} = C_{standard} \times \frac{A_{sample}}{A_{standard}} \times ٥$$

where:

C = concentration(mg/l) of ascorbic acid in sample and standard.

A = absorbance at ٥٢٠ nm for sample and standard.

٥ = factor is added to correct for the dilution of sample.

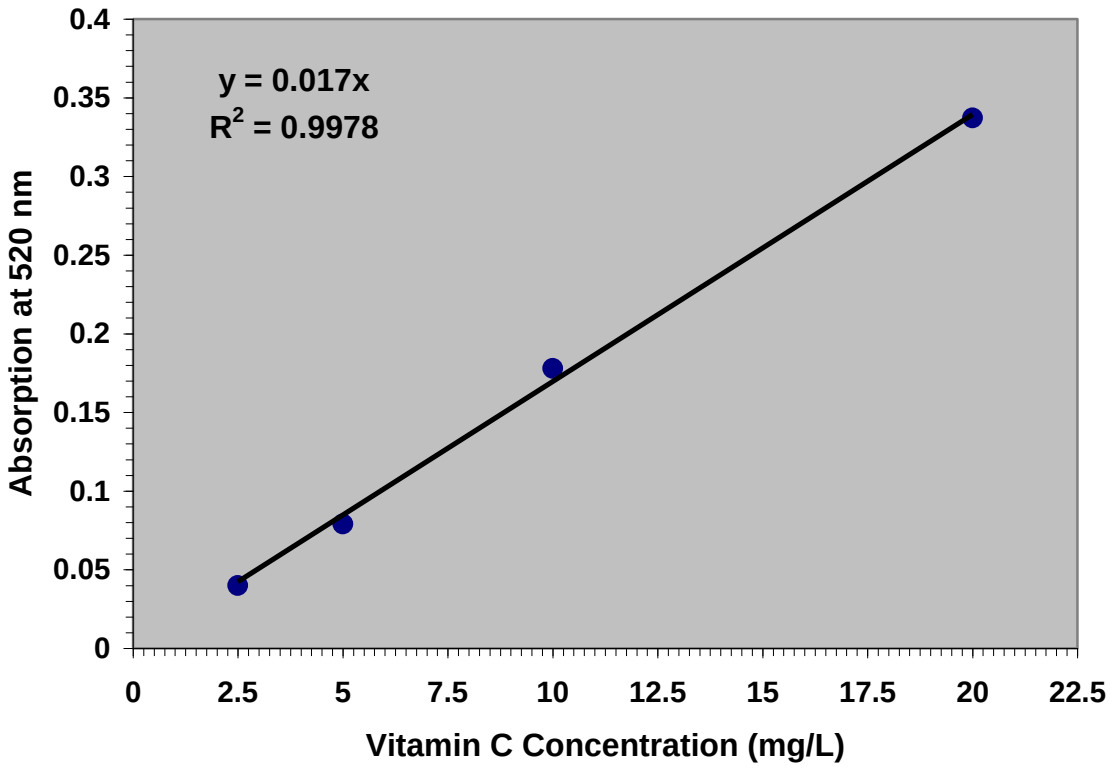

Figure (٢.١): Standard curve for (٢,٤ –DNPH) method of ascorbic acid.

٢.٢.٥ Calculation of Serum Reduced Vitamin C (Ascorbic Acid):-

In the present study, reduced ascorbic acid was calculated from a correlation study between the ٢,٤-DNPH method (used to measure total vitamin C concentration) and ٢,٦-dichloroindophenol (٢,٦-DCIP) method (used

to measure reduced vitamin C concentration) conducted on ٦١١ patients samples, and having show good agreement (Lleyd *et al.*, ١٩٤٥).

DNPH = ١.٠٨٨ (DCIP) + ٠.١٢ (r=٠.٩٥)

Therefore,

$$\text{Reduced Ascorbic Acid} = \frac{\text{Total ascorbic acid (DNPH)} - ٠.١٢}{١.٠٨٨}$$

٢.٢.٦ Determination of Serum Reduced Glutathione (GSH):-

- **Method:**

DTNB photometric method (Ellman's method)
(Ellman,١٩٥٩; Alta'ee, ٢٠٠٣).

- **Principle:**

٥,٥'-Dithiobis (٢-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl group of GSH to an intensely yellow compound. The absorbance of the reduced chromogen is measured at ٤١٢ nm and is directly proportional to the GSH concentration.

- **Preparation of reagents:**

١- Precipitating solution. Trichloro acetic acid (TCA) ٥٠٪ : ٥٠ gm of TCA are dissolved in a final volume of ١٠٠ ml of distilled water.

٢- Ethylenediamine tetra acetic acid-disodium ($EDTANa_٢$) (٠.٢M): ٣٧.٢٢٤ gm of $EDTANa_٢$ are dissolved in a final volume of ٥٠٠ ml of distilled water.

٣- Tris-EDTA buffer pH ٨.٩: ٤٨.٤٥٨ gm of Tris are dissolved in ٨٠٠ ml of distilled water,١٠٠ ml of (٠.٢ M) $EDTANa_٢$ solution are added and brought

to a final volume of ١ liter with distilled water. The pH was adjusted to ٨.٩ by the addition of ١M of HCl.(Stable for at least ١٠ days).

٤- DTNB reagent (٠.٠١M): ٠.٠٩٩ gm of DTNB is dissolved in absolute methanol, and brought to a final volume of ٢٥ ml.(Stable for at least ١٣ week at ٤^{o}C).

٥- GSH standards: Stock standards solution (٠.٠٠١ M)is prepared by dissolving ٠.٠٣٠٧ gm of GSH in a final volume of ١٠٠ ml of (٠.٢ M) $EDTANa_{٢}$ solution. Dilutions are made in $EDTANa_{٢}$ solution to ٥,١٠,٢٠,٣٠ and ٥٠ µM.(This working standard should be prepared daily).

- **Procedure:**

Serum GSH was determined by using a modified procedure using Ellman's reagents (DTNB), and summarized as follows:

Sample, blank, and standard test tubes are prepared then pipetted into test tubes.

Reagents	Sample (µL)	Reagent Blank (µL)	Standard (µL)
Serum	١٠٠		
Standard			١٠٠
Distilled water	٨٠٠	٩٠٠	٨٠٠
TCA	١٠٠	١٠٠	١٠٠

The tubes are mixed in vortex mixture intermittently for ١٠-١٥ minute, and centrifuged for ١٥ minute at ٣٠٠٠ xg, then pipetted into test tubes.

Reagents	Sample (µL)	Reagent Blank (µL)	Standard (µL)
Supernatant	٤٠٠	٤٠٠	٤٠٠
Tris-EDTANa٢ buffer	٨٠٠	٨٠٠	٨٠٠
DTNB reagent	٢٠	٢٠	٢٠

The tubes are mixed in vortex mixture. The spectrophotometer is adjusted with reagent blank to read zero absorbance (A) at ٤١٢ nm, and the absorbance of standards and sample is read within ٥ minutes of the addition of DTNB.

- **<u>Calculation of Serum GSH:</u>**

The concentration of serum GSH is obtained from the calibration curve in µM (Figure ٢.٢).

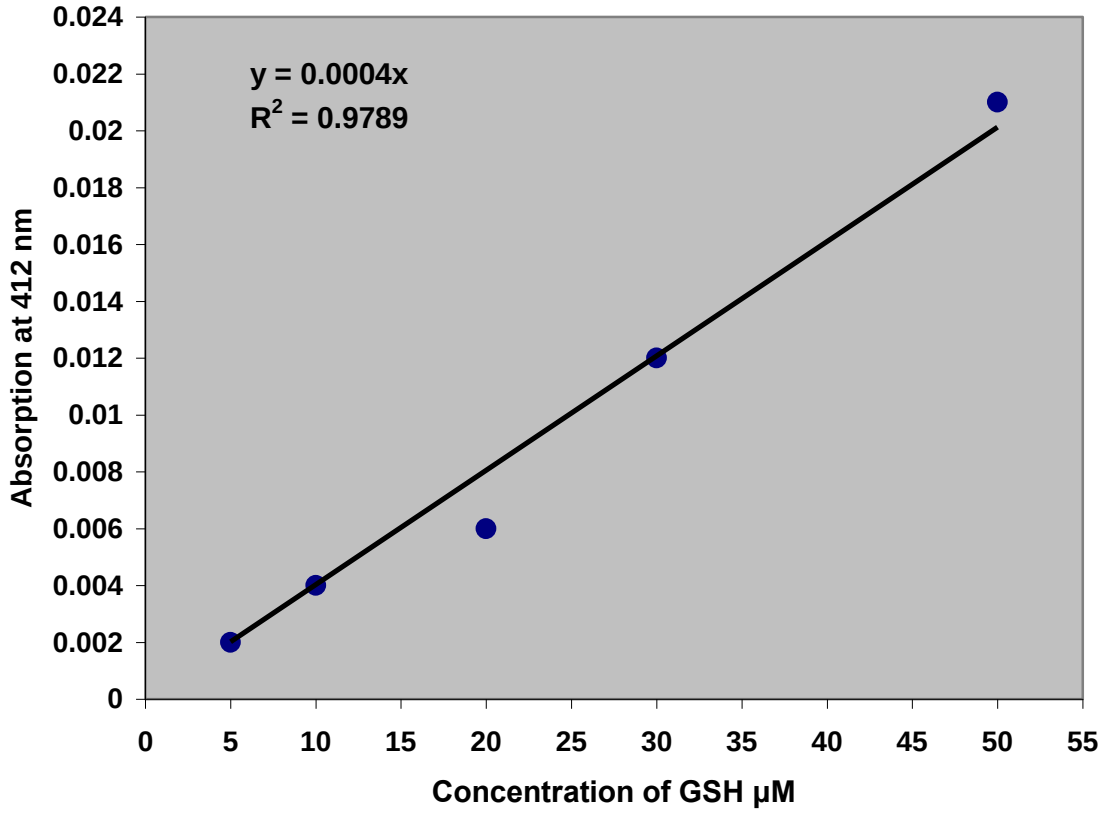

Figure (٢.٢):Standard curve of reduced glutathione concentration.

٢.٢.٧ Statistical Analyses :-

Statistical analyses were performed using SPSS program version ١٠.٠, all values were expressed as mean ± standard deviation (SD) or percentage (%).

Multiple comparisons between diabetic and control groups and between the diabetic groups (type ١ and ٢ DM) were made, using one way-analysis of variance (ANOVA).The person rank correlation test and simple linear regression analysis were used to assess the relationship between variables. Results were considered significant when the probability (p) was less than ٠.٠٥ ($p<٠.٠٥$).

٣.١ Demographic, Clinical, and Biochemical Characteristics of the Study :-

The demographic (sex,age), clinical, and biochemical characteristics of healthy control subjects, type ١ DM, and type ٢ DM of the present study are shown in (Table ٣.١) expressed by mean ± SD or percentage (%).

Type ١ DM patients constitute ٣١٪ (٢٧:٨٧) of the total number of diabetic patients, whereas ٦٩٪ (٦٠:٨٧) of them have type ٢ DM (Figure ٣.١).

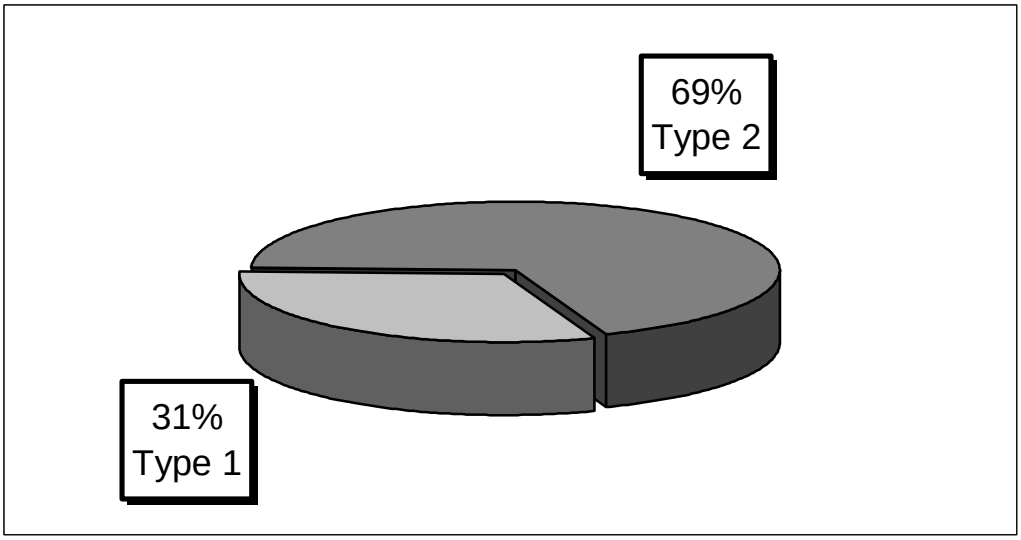

Figure (٣.١): The percentage of type١ and type٢ DM patients.

Regarding the sex factor of DM patients, type١ DM involve (١٧ females and ١٠ males), whereas type٢ DM involve (٣٣ females and ٢٧ males).

The age group distribution in type ٢ DM patients shows a peak level in the age between (٥٠-٥٩) years old represented by (٢٧) patients. (Figure ٣.٢); whereas for type ١ DM patients the age group is equally distributed from (١٠-٣٩) years old represented by (٥) patients for each age group. The age group between (٥٠-٥٩) years old has the same number of patients (Figure ٣.٣).

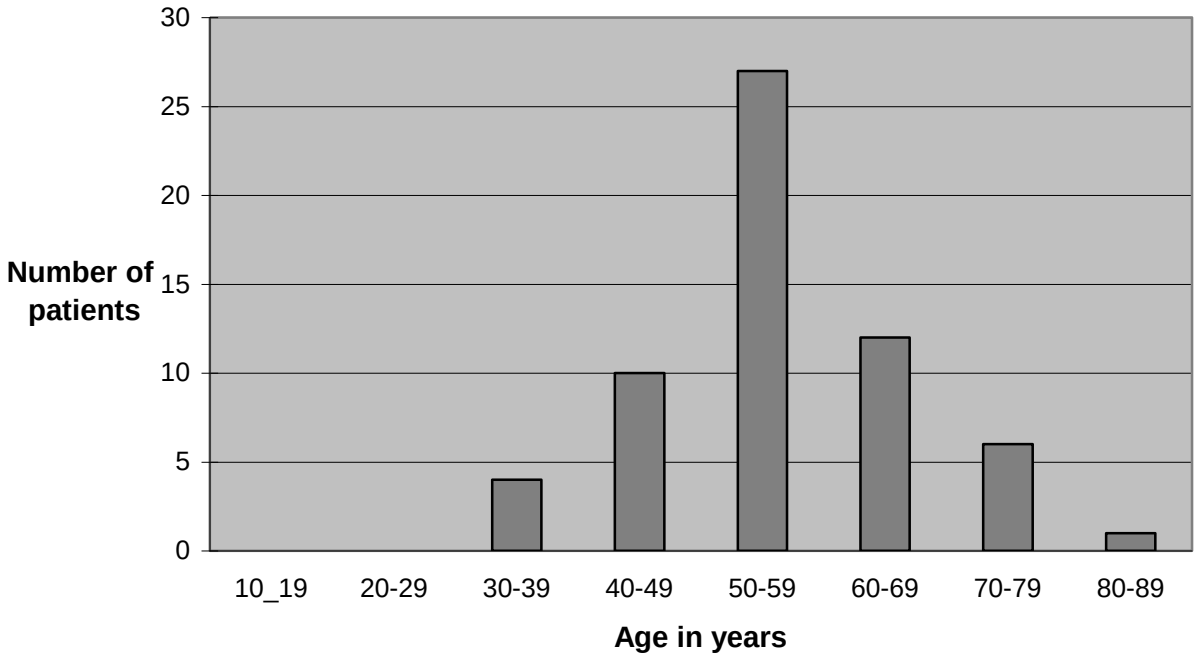

Figure (٣.٢): The age group distribution of type ٢ DM patients.

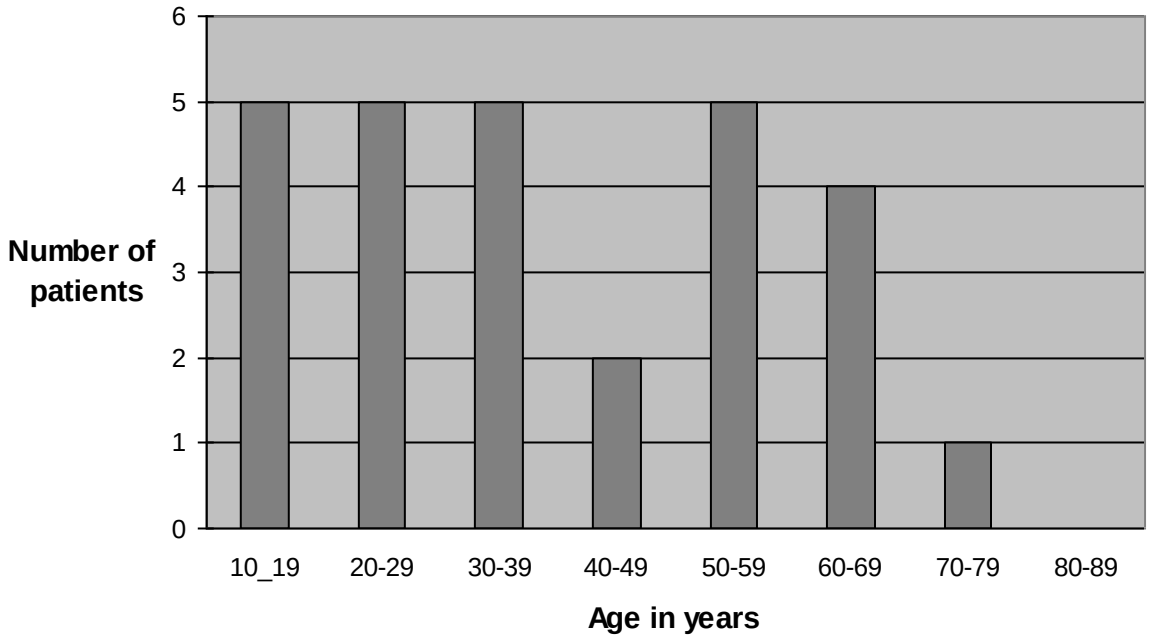

Figure (٣.٣): The age group distribution of type ١ DM patients.

Figures (٣.٢) and (٣.٣), excluding the duration of DM, also show that in type ١ DM the distribution of age group starts from (١٠) years, whereas in type ٢ DM the distribution starts from (٣٠) years.

Smoking status of the diabetic patients is divided into three groups: those who never smoke, past smokers, and current smokers diabetic patients. Never smoke patients represent (٨١.٤٨٪) for type ١ DM and (٧١.٦٦٪) for type ٢ DM, these results show that most of the diabetic patients in the present study are non smokers. Past smokers represent (٣.٧٠٪) for type ١ DM and (١٦.٦٦٪) for type ٢ DM. The current smokers represent (١٤.٨١٪) for type ١ DM and (١١.٦٦٪) for type ٢ DM.

The body mass index (BMI kg/m^{٢}) of diabetic patients is classified as: underweight (<١٨.٥), normal (١٨.٥ – ٢٤.٩), overweight (٢٥.٠ – ٢٩.٩),

obesity (٣٠.٠ – ٣٩.٩), and extreme obesity (≥ ٤٠.٠) (Grodner *et al.*, ٢٠٠٠). (Figure ٣.٤ and ٣.٥).

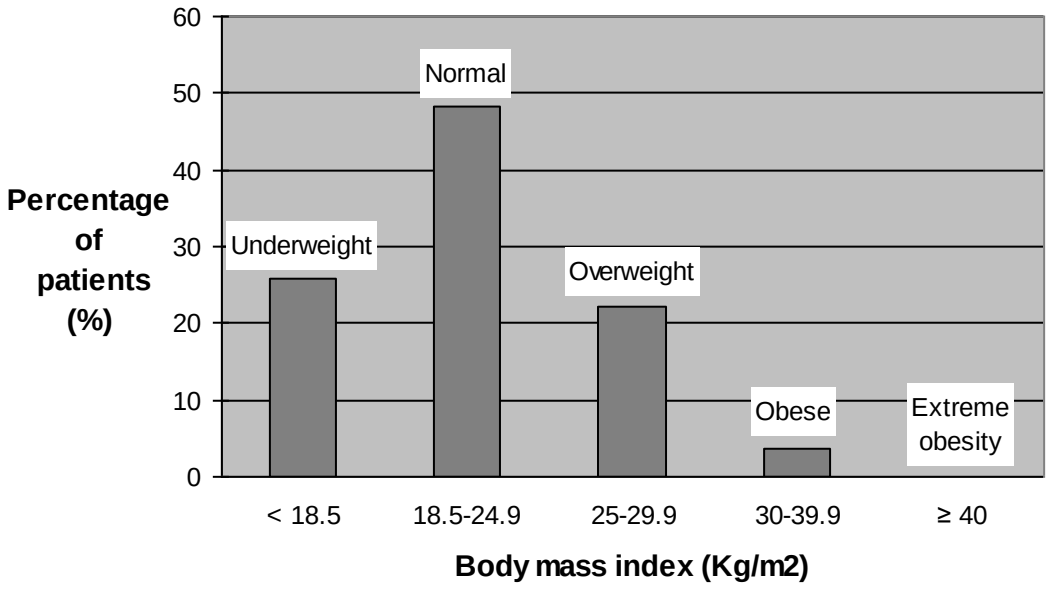

Figure (٣.٤): Classification of body mass index in type ١ DM patients.

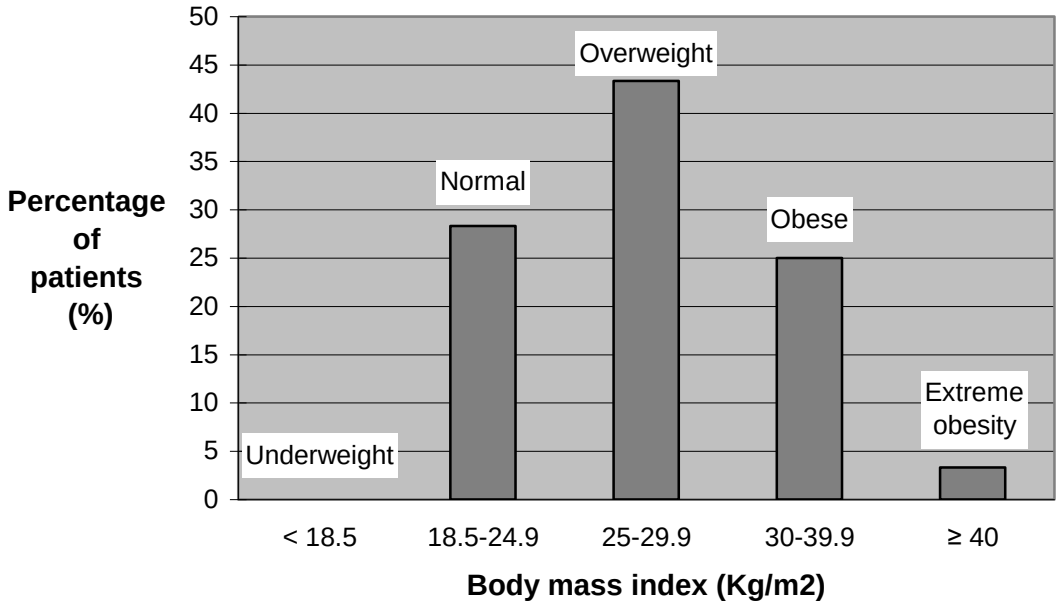

Figure (٣.٥): Classification of body mass index in type ٢ DM patients.

Patients of type ١ DM who have BMI < ٢٥.٠ kg/m^{٢} (i.e. underweight and normal weight) constitute ٧٤٪ of the total number of type ١ DM patients (٢٠:٢٧) (Figure ٣.٦). On the other hand, in type ٢ DM patients who have BMI ≥ ٢٥.٠ kg/m^{٢} (i.e. overweight, obese, and extreme obesity) constitute ٧٢٪ of the total number of type ٢ DM patients (٤٣ : ٦٠) (Figure ٣.٧).

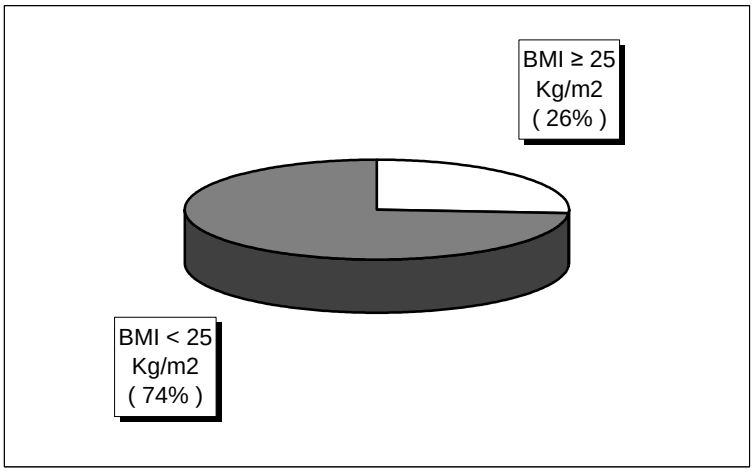

Figure (٣.٦):The percentage of obese and non obese patients in type ١ DM .

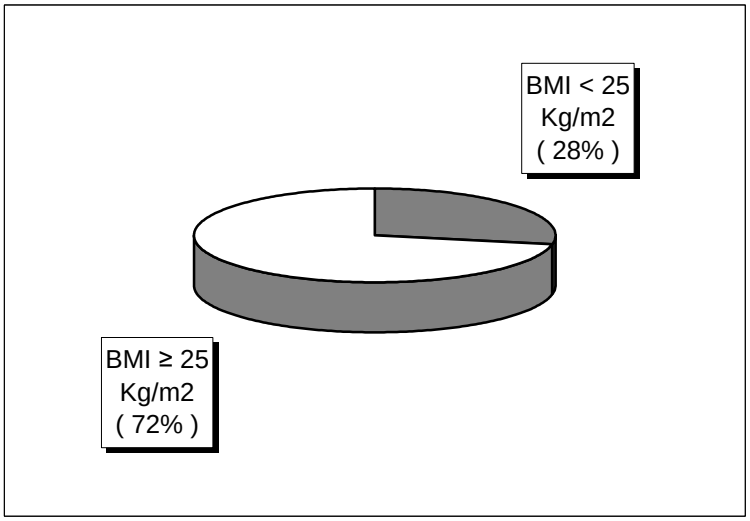

Figure (٣.٧):The percentage of obese and non obese patients in type ٢ DM .

Weight loss is common in type ١ DM patients, though they could be normal weight (Hasslett, ١٩٩٩). Obesity is very common in type ٢ DM. It acts as a diabetogenic factor because the accompanying insulin resistance increases the need for insulin. Adipocytes secrete a number of biologic products (leptin , tumor necrosis factor α (TNF-α), free fatty acids , resistin , and adiponectin) that modulate insulin secretion, insulin action , and body weight and may contribute to the insulin resistance (Kasper *et al.*, ٢٠٠٥).

The mean duration of diabetes mellitus of type ١ and type ٢ DM are nearly equal (٩.٦٨ , ٨.٠٨) years respectively.

Regarding the history of ischaemic heart disease, (١٤.٨١٪) of type ١ DM patients have a positive history of ischaemic heart disease, whereas in type ٢ DM patients, the percentage is (٢١.٦٦٪).

Biochemical parameters random serum glucose (RSG), total vitamin C (TvitC), reduced vitamin C (RvitC), reduced glutathione (GSH), and C-reactive protein (CRP) expressed by mean ± SD are measured in the sera of healthy control subjects, type ١ DM, and type ٢ DM patients (Table ٣.١).

Table (٣.١): Demographic , clinical , and biochemical characteristics of healthy control subjects and diabetic patients.

Variables	Healthy Control	Type ١ DM	Type ٢ DM
-№	٣٩	٢٧	٦٠
-sex (M/F)	١٦/٢٣	١٠/١٧	٢٧/٣٣
-Age (year)	٤٦.١٢±١٤.٢٢	٣٨.٤٨±١٨.٧٧	٥٤.٩٣±١٠.٩٥
-Smoking status :			
Never	٨٧.١٧ %	٨١.٤٨ %	٧١.٦٦ %
Past	٢.٥٦ %	٣.٧٠ %	١٦.٦٦ %
Current	١٠.٢٥ %	١٤.٨١ %	١١.٦٦٪
-BMI (Kg/m٢)	٢٦.٠٤±٣.٧٦	٢٢.١٩±٤.٢٨	٢٧.٥٣±٥.٧٥
-Classification of BMI:			
Underweight%	٠ %	٢٥.٩٢ %	٠٪
Normal%	٤١.٠٢ %	٤٨.١٤ %	٢٨.٣٣ %
Overweight%	٤٣.٥٨ %	٢٢.٢٢ %	٤٣.٣٣ %
Obesity%	١٥.٣٨ %	٣.٧٠ %	٢٥ %
Extreme Obesity%	٠ %	٠ %	٣.٣٣ %
-Duration of DM (year)	-	٩.٦٨±١٠.٢٥	٨.٠٨±٨.٢٨
-Ischaemic heart disease%	٠ %	١٤.٨١ %	٢١.٦٦ %
-RSG (mmol/L)	٤.٨٤±٠.٧٧	١٦.٠٢±٦.١٠	١٣.٢٧±٥.٣٢
-TvitC (mg/L)	١٤.١٩±٤.٥٦	٧.٢٩±٣.٣٥	٧.٢٦±٣.٣٤
-RvitC (mg/L)	١٢.٩٣±٤.١٩	٦.٥٩±٣.٠٨	٦.٥٧±٣.٠٧
-GSH (μ M)	٢٩.١٦±١٤.٧٤	٧.٩٦±٤.٧٥	١٠.٦٢±٤.٩٩
-CRP :			
Positive test%	٠ %	١٨.٥١ %	١١.٦٦ %
Conc. of CRP in positive test (mg/L)	-	٣٥.٢٠±١٣.٣٨	٣٢.٠٠±١٦.٠٠

٣.٢ Multiple Comparisons between the Studied Groups' Serum Antioxidants (Reduced Vitamin C and Reduced Glutathione) :-

Multiple comparisons have been made between the studied groups' serum antioxidants (reduced vitamin C and reduced glutathione) ($p<٠.٠٥$) are shown in Table (٣.٢).

Table (٣.٢): Multiple comparisons between the studied groups' serum antioxidants (reduced vitamin C and reduced glutathione).

Variables	Mean difference (I – J)	Sig.	Mean difference (I – K)	Sig.	Mean difference (J – K)	Sig.
RvitC(mg/L)	٦.٣٣٦	S	٦.٣٦٣	S	٢.٦٥٣	NS
GSH(μ M)	٢١.٢٠٣	S	١٨.٥٤١	S	-٢.٦٦٢	NS

(I=mean of healthy control group, J=mean of type ١ DM, K=mean of type ٢ DM, S=significant (when $p<٠.٠٥$), NS=not significant).

The mean of reduced vitamin C (RvitC mg/L) shows a decrease in its level in type ١ DM patients in comparison with that of the Control Group and the difference is significant between them. The mean of RvitC also shows a decrease in its level in type ٢ DM patients in comparison with that of the Control Group and the difference is significant between them. But no

significant difference is found between the mean of RvitC of type ١ DM patients and that of type ٢ DM patients.

The decrease in the antioxidant vitamin C in diabetes may be due to vitamin C consumption by ROS , or it can be consumed due to its role in the reduction of the formation of advanced glycation end products (AGE), or its role in slowing the conversion of glucose to sorbitol (Smith, ٢٠٠٣). This reveals that vitamin C requirements increases in diabetes. It can also be consumed due to the regeneration of vitamin E in which the later is also largely consumed by ROS (Bachi and Puri, ١٩٩٨). Hyperglycaemia is also known to enhance renal ascorbic acid losses by inhibiting renal ascorbic acid reabsorption (Cunningham, ١٩٩٨).

The mean of reduced glutathione (GSH μM) shows a decrease in its level in type ١ DM patients in comparison with that of the Control Group and the difference is significant between them. In type ٢ DM patients the mean of GSH shows a decrease in its level in comparison with that of the Control Group and the difference is significant between them. But no significant difference is found between the mean of GSH of type ١ DM patients and that of type ٢ DM patients.

In diabetes, the decrease in the antioxidant GSH level may be an outcome of greater GSH consumption by ROS. It is also largely consumed, mainly because of the regeneration of vitamin C, which is largely oxidized in diabetic patients. Lower amounts of GSH may also be explained by the glycation of the enzyme γ–glutamyl cysteine synthetase (an enzyme catalyze GSH synthesis) , which generates decreased amounts of GSH synthesis in diabetes (Shurtz-Swirski*etal.*,٢٠٠١).

In addition, under hyperglycaemic conditions, as much as ٣٠٪ of the glucose is channeled into the polyol pathway, causing a substantial depletion of NADPH, in which the latter is used as a co-factor by aldose reductase (AR) to reduce glucose to sorbitol, and by that it competes with glutathione reductase (GR) for their co-factor NADPH which is also required to regenerate GSH. The depletion of NADPH consequently leads to a significant decrease in the GSH level (Figure ٣.٨) (Chung *et al.*, ٢٠٠٣).

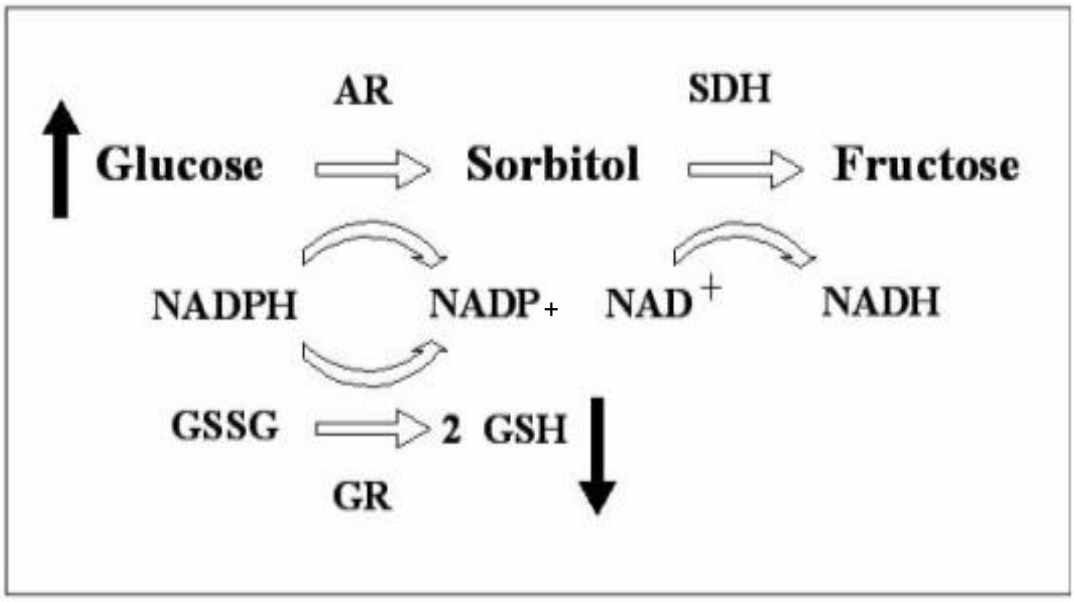

Figure (٣.٨):Contribution of polyol pathway to GSH decrease in DM.

٣.٣ Analysis of the Raised CRP Levels in the Studied Groups:-

CRP concentrations are measured in the sera of the studied groups using latex agglutination test (Table ٣.٣).CRP levels above the normal value(>٨ mg/L) constitute ١٣.٧٩٪ (١٢:٨٧) of the total number of diabetic patients, whereas ٨٦.٢٠٪ (٧٥:٨٧) of them are within normal CRP levels; however, all of the healthy control subjects have normal CRP levels.

The percentage of patients with raised CRP levels in type١ DM is ١٨.٥١٪ (٥:٢٧), whereas in type٢ DM patients, the percentage is ١١.٦٦٪ (٧:٦٠). This reveals that the percentage of patients of type ١ DM with raised CRP level, (all of them are females), is higher than that of type ٢ DM patients with raised CRP level, (٥ males, ٢ females), and this reveals that raised CRP levels are higher in female sex. This result is supported by other studies (Kilpatrick *et al.*, ٢٠٠٠; Kluft and de Maat, ٢٠٠١).

Table (٣.٣): C-reactive protein concentration in the studied groups using latex agglutination test.

CRP Concentration (mg/L)	DM			Healthy Controls
	Type-١	Type-٢	Total	
	№:Total (%)	№:Total (%)	№:Total (%)	№:Total (%)
< ٨	٢٢ : ٢٧ (٨١.٤٨ %)	٥٣ : ٦٠ (٨٨.٣٣ %)	٧٥ : ٨٧ (٨٦.٢٠ %)	٣٩ : ٣٩ (١٠٠ %)
٨	٠ : ٢٧ (٠ %)	٠ : ٦٠ (٠ %)	٠ : ٨٧ (٠ %)	٠ : ٣٩ (٠ %)
١٦	١ : ٢٧ (٣.٧٠ %)	٣ : ٦٠ (٥ %)	٤ : ٨٧ (٤.٥٩ %)	٠ : ٣٩ (٠ %)

٣٢	٢ : ٢٧ (٧.٤٠ %)	١ : ٦٠ (١.٦٦ %)	٣ : ٨٧ (٣.٤٤ %)	٠ : ٣٩ (٠ %)
٤٨	٢ : ٢٧ (٧.٤٠ %)	٣ : ٦٠ (٥ %)	٥ : ٨٧ (٥.٧٤ %)	٠ : ٣٩ (٠ %)

Patients with raised CRP level in both types ١ and ٢ DM are found to be either having:

١-Diabetic complications [diabetic ketoacidosis DKA ٨.٣٣% (١:١٢),diabetic foot ulcer DFU ٢٥٪ (٣:١٢) or acute myocardial infarction AMI ٨.٣٣٪ (١:١٢)], or

٢-Infection [urinary tract infection UTI ١٦.٦٦٪ (٢:١٢) , respiratory tract infection RTI ٢٥٪ (٣:١٢)] , or being

٣-Newly diagnosed diabetes ١٦.٦٦٪ (٢:١٢) , (٢weeks, ١week) for type١ and ٢ DM patients respectively (Table ٣.٤).

These durations are the shortest ones noticed in diabetic patients whether of type ١ or ٢ DM. This proves that CRP is involved in the development of type ١ and ٢ DM. All of the above conditions show to have raised CRP level in many other studies (Craig *et al.*, ٢٠٠٢; Jain *et al.*, ٢٠٠٣; Ford , ١٩٩٩; Pagana and Pagana, ٢٠٠١; Fischbach , ٢٠٠٠; Chase *et al.*, ٢٠٠٤; Thorand *et al.*, ٢٠٠٣).

Chronic hyperglycaemia is not sufficient to induce inflammation. This result is supported by other studies (Pickup, ٢٠٠٤; Chase *et al.*, ٢٠٠٤), can be clarified by two points:

a- Not all diabetic patients with hyperglycaemia whether of type ١ or ٢ DM have a raised CRP level.

b- There is a non significant correlation between the raised CRP concentration and serum glucose concentration in diabetic patients of both types ١ and ٢ ($p > ٠.٠٥$).

Table (٣.٤): The conditions that related to CRP elevation in type ١ and ٢ DM.

Conditions related to CRP elevation	DM		
	Type ١	Type ٢	Total
	№ : Total (%)	№ : Total (%)	№ : Total (%)
Diabetic complications			
DKA	١:٥ (٢٠٪)	٠:٧ (٠٪)	١:١٢ (٨.٣٣٪)
DFU	١:٥ (٢٠٪)	٢:٧ (٢٨.٥٧٪)	٣:١٢ (٢٥٪)
AMI	٠:٥ (٠٪)	١:٧ (١٤.٢٨٪)	١:١٢ (٨.٣٣٪)
Infections			
UTI	٠:٥	٢:٧	٢:١٢

	(٠٪)	(٢٨.٥٧٪)	(١٦.٦٦ %)
RTI	٢:٥ (٤٠٪)	١:٧ (١٤.٢٨٪)	٣:١٢ (٢٥٪)
Newly diagnosed diabetes	١:٥ (٢٠٪)	١:٧ (١٤.٢٨٪)	٢:١٢ (١٦.٦٦ %)

٣.٤ Contribution of Hyperglycaemia in Raising CRP Level through Oxidative Stress :-

Hyperglycaemia may contribute to raise CRP level indirectly by increasing reactive oxygen species (ROS), in which glucose present in excess undergoes auto-oxidation, yielding ROS and intracellular precursors of advanced glycation end products (AGE), in which the latter stimulates proinflammatory cytokine production. Hyperglycaemia also alters the cellular redox state by increasing the NADH / NAD^+ ratio and decreasing NADPH / $NADP^+$. The reduction of glucose by NADPH in polyol pathway decreases the cytoplasmic ratio of NADPH / $NADP^+$, whereas the oxidation of sorbitol by NAD^+ increases the cytoplasmic ratio of NADH / NAD^+, in addition to that, the inhibition of glycolysis and lipogenesis and the stimulation of glycogenolysis, lipolysis, ketogenesis, and gluconeogenesis can affect the cellular redox state. Normally, the pentose phosphate pathway maintain a cytoplasmic ratio of NADPH / $NADP^+$ ≈ ١٠٠. Interestingly, because NAD^+ is required for glycolysis, the ratio of NADH / NAD^+ in the cytoplasm is nearly the inverse, less than ٠.٠١ . The ROS creates oxidative stress, which damages molecules and activates a number of signaling molecules such as protein kinase C or the transcription factor NFκB (NFκB is a key determinant of inflammatory response), and thus CRP elevation occurs (Baynes and Dominiczak, ٢٠٠٥; Bloomgarden, ٢٠٠٤; King *et al.*, ٢٠٠٣).

In type ١ DM patients, there is an inverse significant correlation between the concentration of CRP (mg/L) and GSH (μM), r (correlation coefficient) = -٠.٩٦٠, p=٠.٠٠٩ (p significant when < ٠.٠٥) (Figure ٣.٩).

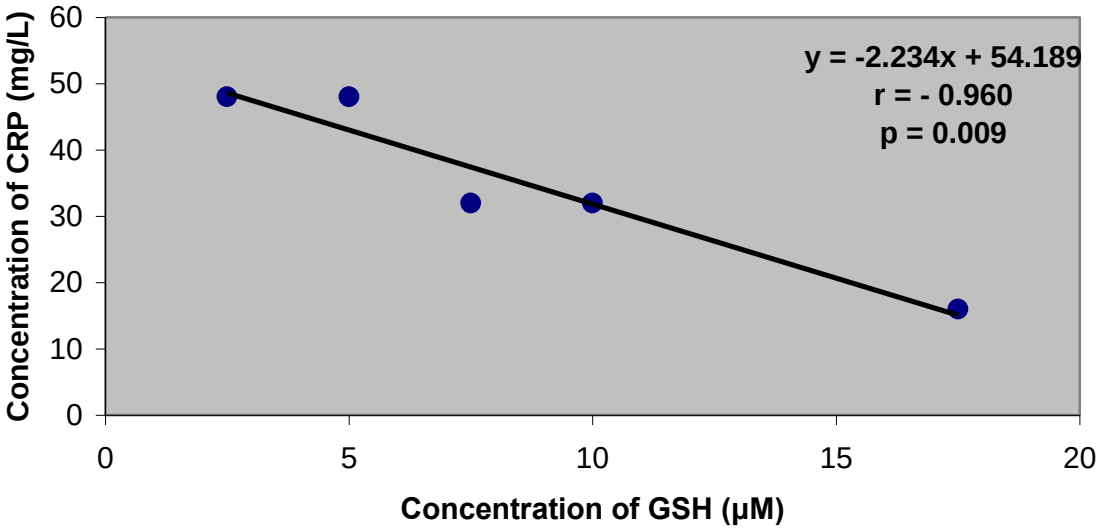

Figure (٣.٩) : The relationship between the concentration of CRP and GSH in type ١ DM patients.

Considering RvitC, there is an inverse significant correlation between the concentration of CRP (mg/L) and RvitC (mg/L) , r = -٠.٩٣٢ , p = ٠.٠٢١ (Figure ٣.١٠).

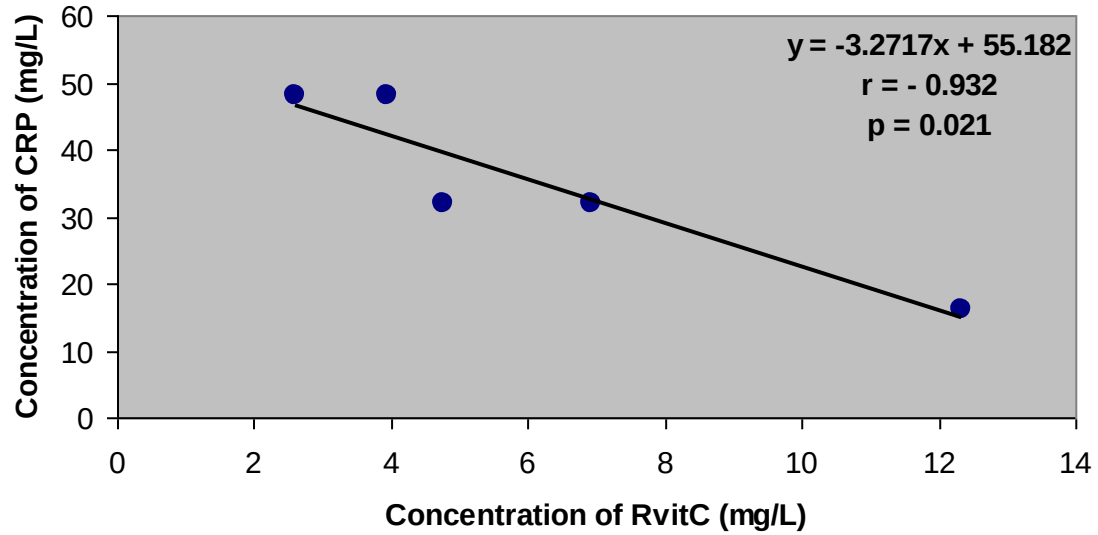

Figure (٣.١٠): The relationship between the concentration of CRP and RvitC in type ١ DM patients.

In type ٢ DM patients, there is an inverse significant correlation between the concentration of CRP (mg/L) and GSH (µM), r = -٠.٨٥٢ , p = ٠.٠١٥ (Figure ٣.١١).

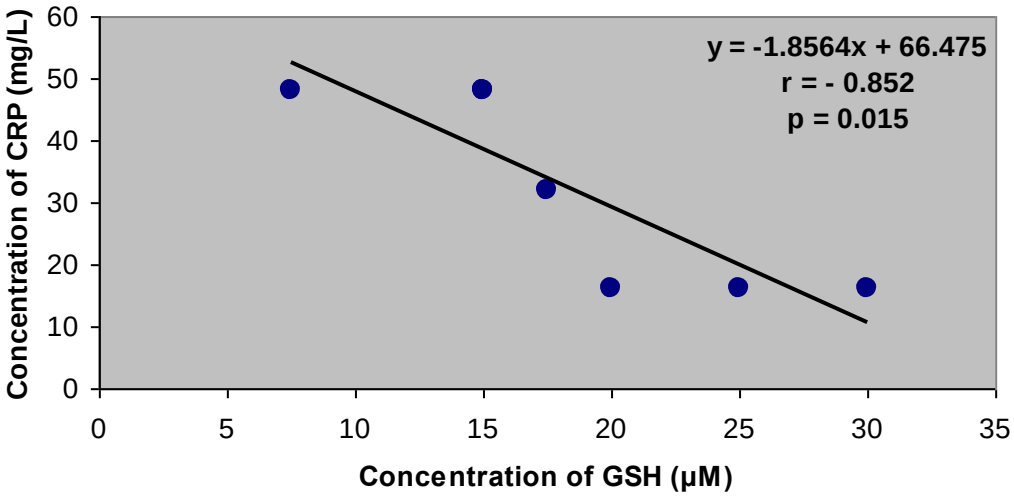

Figure (٣.١١) :The relationship between the concentration of CRP and GSH in type ٢ DM patients.

Considering RvitC, there is an inverse significant correlation between the concentration of CRP (mg/L) and RvitC (mg/L), r = -٠.٧٩٣, p = ٠.٠٣٣ (Figure ٣.١٢).

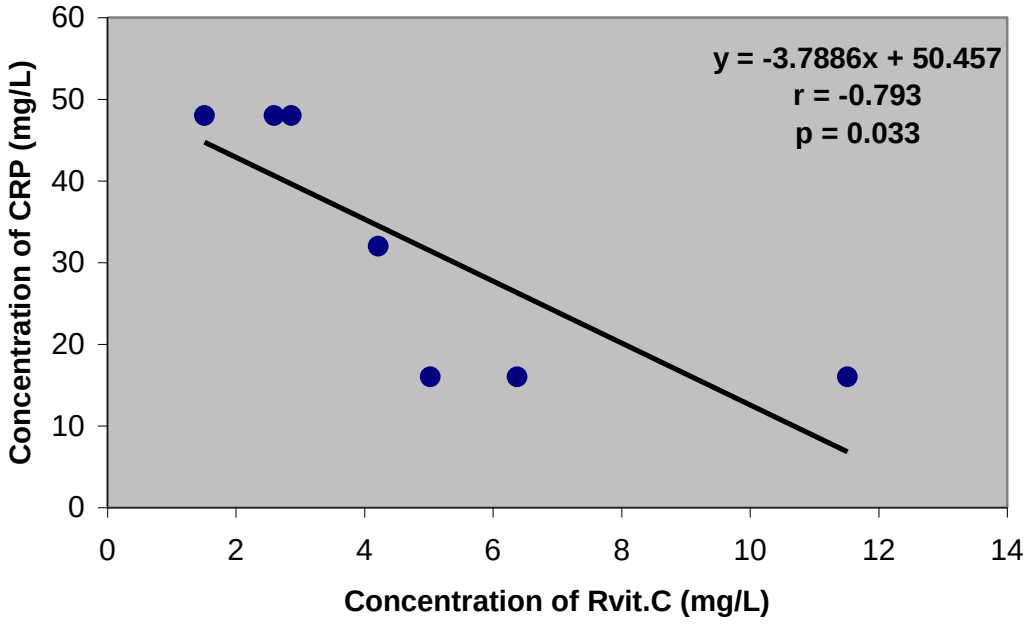

Figure (٣.١٢) :The relationship between the concentration of CRP and RvitC in type ٢ DM patients.

In general, in both types of diabetic patients, there is an inverse significant correlation between the concentration of CRP and the antioxidants (GSH, RvitC), but the correlation in type ١ diabetic patients is stronger and more significant than that in type ٢ diabetic patients.

The stronger and more significant correlation between CRP and antioxidants (GSH, RvitC) in type ١ DM patients than that in type ٢ DM patients is attributed to significantly higher hyperglycaemic level of type ١ DM patients with raised CRP level in comparison with that of type ٢ DM patients with raised CRP level. This result is observed from statistical analyses done on the mean serum glucose level (mmol/L), in which mean serum glucose of type ١ DM patients with raised CRP level shows a significant increase in its level in comparison with the mean of serum glucose of type ٢ DM patients with raised CRP level. Both types ١ and ٢ DM patients with raised CRP level show a

significant increase in their mean serum glucose levels in comparison with the mean serum glucose level of the healthy Control Group (Table ٣.٥).

Table (٣.٥) :The comparison of mean serum glucose level between type ١ and type ٢ DM patients with raised CRP level, and also between them and healthy control group.

Variable	Mean Difference (I - J)	Sig.	Mean Difference (I - K)	Sig.	Mean Difference (J - K)	Sig.
RSG (mmol/L)	- ١٤.٨١٧	S	- ٦.٥٧٥	S	٨.٢٤١	S

(I= mean serum glucose of healthy control group, J= mean serum glucose of type ١ DM patients with raised CRP levels, K= mean serum glucose of type ٢ DM patients with raised CRP levels, S= significant (p<٠.٠٥), NS= not significant).

The measurement of the antioxidants (GSH, RvitC) in both types ١ and ٢ DM patients with raised CRP level are used as indicators of oxidative status and by using statistical analyses, they reveal the existence of oxidative stress, in which a significant decrease of the antioxidants (GSH, RvitC) in both types ١ and ٢ DM patients with raised CRP level in comparison with that of the healthy Control Group. Although the mean of GSH level in type ٢ DM patients with raised CRP level shows no significant difference in comparison with the mean of GSH level of the healthy Control Group, it is close to a significant level p = ٠.٠٦ (p<٠.٠٥ consider significant) (Table ٣.٦).

Table (٣.٦) : The comparison of mean serum antioxidants (GSH , RvitC)

between type ١ , type ٢ DM patients with raised CRP level and healthy control group.

Variables	Mean Difference (I – J)	Sig.	Mean Difference (I – K)	Sig.
GSH (µ M)	٢٠.٦٦٦	S	١٠.٥٩٥	NS
RvitC (mg/L)	٦.٨٢٧	S	٨.٠٦٣	S

(I= mean of healthy control group, J= mean of type ١ DM patients with raised CRP levels, K= mean of type ٢ DM patients with raised CRP levels, S = significant (when p<٠.٠٥) , NS= not significant).

From the above analyses done on the relationship between hyperglycaemia, oxidative stress and raised CRP level, all these results demonstrate the indirect contribution of hyperglycaemia in raising CRP level through oxidative stress in people with established diabetes of both types١and ٢.

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