Relationship Between The Levels of Circulating Modified LDL And The Extent of Coronary Artery Disease In Type 2 Diabetic Patients

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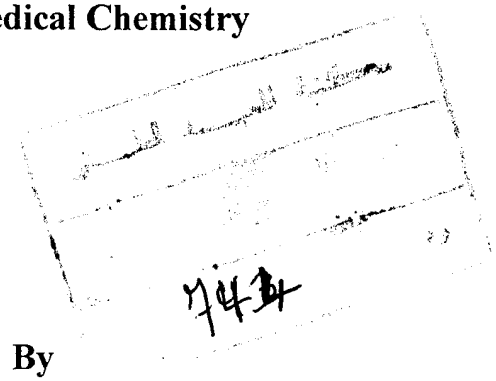

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ABTS	2,2–Azino–di–[3–ethylbenzthiazoline sulphate]
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
Apo-A	Apolipoprotein-A
Apo-B	Apolipoprotein-B
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
CAD	Coronary artery disease
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CETP	Cholesteryl ester transfer protein
CML	N^{ϵ}-(carboxymethyl) lysine
DAP	Dihydroxy acetate phosphate
DNA	Deoxyribonucleic acid
DTNB	5,5'–dithiobis–(2-nitro benzoic acid)
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FPG	Fasting plasma glucose
FRs	Free radicals
GDM	Gestational diabetes mellitus
GSH	Reduced glutathione
GSHpx	Glutathione peroxidase
GSSG	Oxidized glutathione
GST	Glutathione S-transferases
HbA1c	Glycated hemoglobin
HDL	High-density lipoproteins
HOPE	Heart Outcomes Prevention Evaluation
HSL	Hormone-sensitive lipase
ICAM-1	Intercellular adhesion molecule 1
IDL	Intermediate-density lipoprotein
IMT	Intima-media thickness
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoproteins
LP(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LysoPtdCho	Lysophosphatidylcholine

MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MDA	Malondialdehyde
MM-LDL	Minimally oxidized LDL
MMP-9	Metalloproteinase 9
MODY	Maturity-onset diabetes of young
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide dinucleotide
NADPH	Nicotinamide dinucleotide phosphate
NOS	Nitric oxide synthase
OX-LDL	Oxidized LDL
PAI-1	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
PGI_2	Prostacyclines
pHA	*p*-hydroxyphenyl acetaldehyde
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard Error
SGPx	Selenium-dependent glutathione peroxidase
SOD	Superoxide dismutase
TAS	Total antioxidant status
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substance
TBN	5-thio-nitrobenzoic acid
TC	Total cholesterol
TF	Tissue factor
TG	Triglycerides
TIMP-1	Tissue inhibitor Metalloproteinase
TMB	Tetramethylbenzidine
TMP	Tetramethoxy prepare
tPA	Tissue-type plasminogen activator
TRAP	Total radical trapping antioxidant parameter
VCAM-1	Vascular adhesion molecule 1
VLDL	Very low-density lipoproteins

INTRODUCTION

INTRODUCTION

DIABETES MELLITUS

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both[1]. It is estimated that the worldwide burden of this disease in adults is around 173 million in the year 2002[2].The basis of the metabolic abnormalities in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action [3]. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the B- cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action[3].

The classification of diabetes is based on aetiological types[4,5] and it is usually classified into 4 main categories:

Type 1 diabetes: involves the process of B-cell destruction that may ultimately lead to absolute insulin deficiency, and in which exogenous insulin is required for survival. Pathogenesis of type 1 diabetes begins with a genetic susceptibility to the disease, and some environmental events that initiate the process in such susceptible individual[6]. This type may be further subdivided into:

1- Immune mediated diabetes; previously known as insulin- dependent or juvenile-onset diabetes, results from a cellular mediated autoimmune destruction of the B-cells of the pancreas[7]. It commonly occurs in childhood and adolescence, but it can occur at any age[3].

2- Idiopathic diabetes; some forms of type 1 diabetes have no known etiology. Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity [5].

Type 2 diabetes: (ranging from predominantly insulin resistance with relative insulin deficiency to predominantly insulin secretory defect with insulin resistance). This form of diabetes, previously referred to as non-insulin-dependent diabetes, or adult-onset diabetes, applies to individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency. At least initially, and often throughout their lifetime, these individuals do not need insulin treatment to survive [8]. In contrast to type 1 diabetes, plasma insulin levels are normal to high in absolute terms, although they are lower than predicted for the level of the plasma glucose; i.e. relative insulin deficiently is present[6].

Most patients with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance [9]. Patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region[10]. The risk of developing this form of diabetes increases with age, and lack of physical activity[11]. It occurs more frequently in women with prior gestational

diabetes mellitus (GDM) and in individuals with hypertension or dyslipidemia, and it frequency varies in different racial/ethnic subgroups[12]. It is often associated with a strong genetic predisposition, more so than is the autoimmune form of type 1 diabetes [13].

Gestational diabetes mellitus (GDM):

Gestational diabetes encompasses any degree of glucose intolerance with onset or first recognition during pregnancy[5,14]. When GDM complicates the pregnancy it may develop other problems including altered duration of pregnancy, placental failure, hypertension/ preeclampsia or high birth weight of the newborn.

Other specific types of diabetes:

i) Genetic defects of B-cell: These forms of diabetes are characterized by onset of hyperglycemia at early age. They are referred to as maturity-onset diabetes of young (MODY) and are characterized by impaired insulin secretion with minimal or no effects in insulin action [15].

ii) Genetic defects in insulin action: These are unusual causes of diabetes that result from genetically determined abnormalities of insulin action (mutation in insulin receptor gene). The associated metabolic abnormalities may range from hyperinsulinemia and modest hyperglycemia to severe diabetes[16].

iii) Disease of exocrine pancreas: Any process that diffusely injures the pancreas can cause diabetes, including pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma. With the exception of cancer, damage to the pancreas must be extensive for diabetes to occur[17].

iv) Endocrinopathies: Excess secretion of several hormones (e.g. growth hormone, cortisol, glucagons, epinephrine) which antagonize insulin action, can cause diabetes, the hyperglycemia typically resolves when the hormone excess is removed[5].

v) Drug- or chemical-induced diabetes: Many drugs can impair insulin secretion. These drugs may not cause diabetes by themselves, but they may precipitate diabetes in individuals with insulin resistance[18].

vi) Infections: Certain viruses have been associated with B-cell destruction, for example diabetes occurs in patients with congenital rubella[19].

vii) Other forms of immunologically- and genetically- mediated diabetes: Anti-insulin receptor antibodies can cause diabetes by binding to the insulin receptor, thereby blocking the binding of insulin to its receptor in the target tissues[16]. Many genetic syndromes are accompanied by an increased incidence of diabetes mellitus[5].

Complication of Diabetes mellitus:

Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction[3]. Patients with diabetes have an increased incidence of atherosclerotic, cardiovascular, peripheral

arterial and cerebrovascular disease [20]. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes [3].

Atherosclerosis is a complex process involving the deposition of plasma lipoproteins and the proliferation of cellular elements in the artery wall. The lesion of atherosclerosis occur within the innermost layer of the artery, the intima, and are largely confined to this region of the vessel [21]. This chronic condition advances through a series of stages beginning with fatty streak lesions composed largely of lipid-engorged macrophage foam cells and ultimately progressing to complex plaques consisting of a core of lipid and necrotic cell debris covered by a fibrous cap [22]. These plaques provide a barrier to arterial blood flow and may precipitate clinical events, particularly under conditions that favor plaque rupture and thrombus formation [23].

The atherosclerotic process is indistinguishable from that affecting the non-diabetic population but begins earlier and may be severe [21]. The mechanisms by which diabetes accelerates atherosclerosis are not well understood. The customary clusters of risk factors for coronary artery disease (CAD), which are more common in patients with diabetes, are not sufficient to explain this phenomenon[24]. Oxidative stress is widely invoked as a pathogenic mechanism for atherosclerosis. Among the sequelae of hyperglycemia, oxidative stress has been suggested as a potential mechanism for accelerated atherosclerosis[25,26].

OXIDATIVE STRESS

All forms of aerobic life are constantly subjected to oxidant pressure from molecular oxygen (O_2) and reactive oxygen metabolites produced both during the biochemical utilization of O_2 and by prooxidants[27]. Compounds and reactions disposing of the toxic oxygen species, scavenging them, suppressing their formation or opposing their action are called antioxidants[28]. The susceptibility of a given organ system to oxidative stress is a function of the balance between pro-oxidant factors and antioxidants. Oxidative damage can therefore be a consequence of raised free radical production, insufficient antioxidant potential, or both[29].

A free radical can be defined as a chemical species possessing an unpaired electron. It can also be considered as a fragment of a molecule. As such, free radicals can be formed in three ways: (i) by the homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons; (ii) by the loss of a single electron from a normal molecule and (iii) by the addition of a single electron to a normal molecule. The latter, electron transfer, is a far more common process in biological systems than is homolytic fission [132].

Types of Free Radicals (FRs) :

A) Oxygen – centered;

These include, molecular oxygen, either triplet ($^3O_2$) or singlet ($^1O_2$), superoxide radical ($O_2^{-}\cdot$), hydroxyl radical ($OH\cdot$) which is highly destructive and alkoxyl ($RO\cdot$) and peroxyl ($ROO\cdot$) radicals[31].

B) Non-oxygen– centered;

These encompass different types including: carbon-centered such as lipid (L•) and alkoxy radicals (R•), sulfur-centered such as thiyl radicals (R-S•), hydrogen-centered such as hydrogen atom (H•), and iron –centered such as Perferryl radical

Of importance are also the non-radical toxic metabolites, which are active biologically. These cytotoxic compounds include: ozone (O_3), hydroperoxides such as hydrogen peroxide (H_2O_2) and lipid hydroperoxide (LOOH), hypochlorous acid (HOCl) and chloramines (R′ RNCl) [31].

Formation of Free Radicals and Reactive Oxygen Species

The most important free radicals in biological system are radical derivatives of oxygen. Their formation often begins with a 1-electron reduction of molecular oxygen to superoxide free radical anion (O_2^{-}•) by various oxidases[32]. A two-electron reduction of oxygen by certain oxidases would yield hydrogen peroxide (H_2O_2) [30].It is often generated in a biological system via the reaction of two superoxide molecules [32]. Hydrogen peroxide is not a free radical but falls into the category of reactive oxygen species (ROS) that also includes non-radical oxygen derivatives involved in oxygen radical production[30]. Hydrogen peroxide is an important compound in free radical biochemistry because it can rather easily break down, particularly in the presence of transition metal ions, to produced the most reactive and damaging of the oxygen free radicals, the hydroxyl radical (OH•) [33].

Biological production of free radicals

Free radicals are generally produced in cells by electron transfer reactions. These can be mediated by the action of enzymes or non-enzymatically, often though the redox chemistry of transition metal ions [30,34]. Free radical production in animal cells can either be accidental or deliberate. Some enzymes utilize a free radical at their active site in the process of catalysis; for example ribonucleotide reductase[35].Under normal circumstances, the major source of free radicals in cells is electron 'leakage' from electron transport chains, such as those in mitochondria and in the endoplasmic reticulum, to molecular oxygen, generating superoxide[30]. Other potential sources of free radicals exist within cell membranes. Metabolism of arachidonic acid by cyclooxygenase to produce prostaglandins and by lipooxygenase to produce leukotrienes involves the formation of intermediate peroxy compounds and hydroxyl radicals [31].

The primary biochemical source of reactive oxygen species in the vasculature appears to be the membrane-associated nicotinamide dinucleotide (phosphate) (NADH/NAD(P)H) oxidase enzyme complex. [36]. This system catalyses the reduction of molecular oxygen to generate superoxide. NADH/NAD(P)H oxidases are also functional in membranes of vascular endothelial and smooth muscle cells, and fibroblasts providing a constitutive source of superoxide anion[37]. Other sources of vascular superoxide include xanthine oxidoreductase enzyme system that catalyses the oxidation of hypoxanthine to xanthine during purine metabolism[36] and endothelial nitric oxide synthase ,which is a cytochrome P450 reductase-like enzyme[37].

Free radical production in cells can be greatly increased by certain toxic foreign compounds. The classic example is carbon tetrachloride[38]. Many toxic compounds, exerting their toxicity via the production of free radicals, are 'redox-cycling' compounds that readily accept an electron to form a free radical and then transfer it to oxygen, generating superoxide radical and hydrogen peroxide[39] .

Biochemical mechanism of free radical-mediated injury:

Free radicals can attack any biochemical component of the cell, but proteins, nucleic acids, and lipids are particularly important targets.

Oxidative damage to proteins:

Oxidative attack on proteins results in site-specific amino acid modification and cross-linking of the peptide chains, aggregation of cross-linked reaction products, altered electrical charge and increase susceptibility to proteolysis [40]. In general, sulfur containing amino acids and thiol groups specifically are very susceptible sites[41]. Proteins containing the amino acids tryptophan, tyrosine, phenylalanine, histidine and cysteine undergo free radical-mediated modification resulting in inhibition of enzymes dependent on these amino acids for reactivity [42].

Oxidative Damage to Nucleic Acid

DNA is readily attacked by oxidizing radicals if they are formed in its vicinity as in case of exposure to radiation. It must be either 'site-specific' such that damage is focused and of high intensity, leading to strand breaks, or must elude the repair systems before replication occurs, leading to mutations[43,44].

Lipid peroxidation

The cell injury produced by lipid peroxidation of cell membranes can range from increased permeability to cell lysis [31]. Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation [45]. The whole process can be depicted as follows:

(1) Initiation phase, which starts with the abstraction of hydrogen atom from target polyunsaturated fatty acid (PUFA) by any initiating oxidizing radical (X·) to form fatty acid radical (R·) [46]. The lipid free radical produced undergoes molecular re-arrangement to produce a conjugated diene.

$$ROOH + \text{metal}^{(n)+} \rightarrow ROO\cdot + \text{metal}^{(n-1)+} + H+$$

$$X\cdot + RH \rightarrow R\cdot + XH$$

(2) Propagation phase, the conjugated diene can be converted into a lipid peroxide (ROO·) if the environment contains free molecular oxygen in appropriate amounts. The lipid peroxide can then either abstract a hydrogen ion form an adjacent molecule (forming a further free radical) to become a stable hydroperoxide (ROOH) or it can undergo transformation to an unstable cyclic endoperoxide that undergoes reductive cleavage to produce a number of compounds which include the 3 carbon aldelyde [47].

$$R\cdot + O_2 \rightarrow ROO\cdot$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot, \text{ etc}$$

$$ROOH \rightarrow RO\cdot, ROO\cdot, \text{aldehydes}$$

Many of the formed Aldehydes are biologically active, particularly malondialdelyde and other members of hydroxyalkenals, whose best known member is 4-hydroxynonenal. These compounds can diffuse from the original site of attack and spread the damage to other parts of the cell [48].

DEFENSE AGAINST FREE RADICALS

The two main categories of antioxidants are those whose role is to prevent the generation of free radicals and those that intercept, or 'scavenge' any that are generated. They exist in both the aqueous and membrane compartments of cells and can be enzymes or non-enzymes as summarized in table (1) [49].

Table (1): Summary of the endogenous antioxidant defenses present in biological system.

Extracelluler	Intracellular	Membrane
• Low molecular weight chain breaking molecules: Ascorbic acid (vitamin C) α-tocopherol (vitamin E) Urate Bilirubin Glutathione Thiol compounds • Preventive (metal-binding) antioxidants: Transferrin Haptoglobin Ceruloplasmin Hemopexin Lactoferrin	Ascorbic acid (vitamin C) Metal-binding proteins • Enzymes: Superoxide dismutase (SOD) Catalase Glutathione peroxidase Glutathione reductase DNA repair system Proteolytic system	α-tocopherol (vitamin E) β-carotene (vitamin A) Ubiquinol

Adapted from Maxwell, 1993 [49].

Enzymatic defense mechanisms:

The mitochondria cytochrome oxidase system consumes most of the available oxygen in the cell, preventing 95% to 99% of molecular oxygen from ever forming toxic metabolites. This mechanism, which is so important

Superoxide dismutase (SOD): catalyzes the dismutation of the superoxide anion free radical ($O_2^{-}\cdot$) to hydrogen peroxide and molecular oxygen[50]. As a result, no superoxide anion is available to react with hydrogen peroxide to form the hydroxyl radical through the iron-catalyzed reactions. This enzyme exists in several forms and is present within the mitochondrial matrix, the cytoplasm, and the extracellular fluid [31].

Catalase: catalyzes the breakdown of toxic hydrogen peroxide directly to water, also prevents the secondary generation of toxic intermediates such as the hydroxyl radical [51]. While this reaction is particularly important when hydrogen peroxide concentrations are elevated, at lower concentrations other peroxidases catalyze the breakdown of hydroperoxides to less reactive alcohols and water. The most important of these is glutathione peroxidase [31].

Glutathione peroxidase: oxidizes two molecules of glutathione to glutathione disulfide, thereby reducing the hydroperoxides. Reduced glutathione can then be regenerated by a glutathione reductase in the presence of NADPH. NADPH is subsequently regenerated, with the consumption of energy, by the pentose phosphate shunt. These pathways

therefore represent another example of redox cycling, serving to detoxify, rather than to amplify, the influence of reactive oxygen metabolites. There are two forms of glutathione peroxidase. The selenium-containing enzyme can catalyze the breakdown for both hydrogen peroxide and lipid hydroperoxides, while the non-selenium glutathione peroxidase cannot catalyze the detoxification of hydrogen peroxide [51].

Glutathione peroxidases are located mainly in the cytosol, but also occur intramitochondrially[52].

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

The selenium-dependent glutathione peroxidase (sGPx) is a tetrameric protein with each subunit containing one atom of the element selenium at its active site as selenocysteine.[53] Part of the glutathione peroxidase activity is non-selenium dependent and is found associated with glutathione S-transferase isoenzymes[54]. The catalytic mechanism proposed for reduction of hydroperoxides involves oxidation of the active site selenolate (Se) to selenenic acid (SeOH). Upon addition of one molecule of GSH, the selenenic acid is transformed to a selenenylsulfide adduct either glutathione (Se-SG), which can be regenerated to the active slenolate and glutathione disulfide (GSSG) by addition of a second molecule of GSH [55].

Non-enzymatic defense mechanisms:

A number of nonenzymatic endogenous antioxidant mechanisms also exist within the normal cell and functions free-radical scavengers both in cytosol and within membranes. Since many oxidants are generated first in

the aqueous phase, aqueous antioxidants may provide a first line of defense against oxidant injury. On the other hand, the lipid-phase antioxidants are, in general, more effective, particularly with respect to the prevention of lipid peroxidation [56]. Other aqueous scavengers, including urate, cysteine, ceruloplasmin, transferrin, and even albumin, all appear to act as primary scavengers, reacting directly with toxic oxygen metabolites to produce more stable compounds. Transferrin, like ferritin, also binds iron, thereby preventing it's acting as a catalyst to generate more toxic secondary species [56].

Vitamin E:

Vitamin E consists of two groups of lipid-soluble compounds (tocopherols and tocotrienols) with four structurally related forms in each group. In humans, α–tocopherol is a powerful lipid-soluble antioxidant predominates and is generally considered the most active form of vitamin E[57].

Vitamin E is absorbed in the intestine and enters the circulation via the lymphatic system. It is absorbed together with lipids, packed into chylomicrons, and transported to the liver[58]. Most of the ingested β-, γ-, and δ-tocopherol is secreted into bile or not taken up and excreted in the feces [59].

α-Tocopherol intercalated within all cellular and organelle membranes and functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions[60,61]. The antioxidant activity of vitamin E gives rise to a well-defined period of strong inhibition of lipid

peroxidation, termed the 'lag time'. After complete consumption of the vitamin, the rate of lipid peroxidation increases rapidly, and increasing its content results in enhanced inhibition of lipid peroxidation, reflected by a prolonged lag time[62].

Vitamin E up-regulates the activities of cytosolic phospholipase A2[63] and cyclooxygenase[64]. The enhanced activity of these two rate-limiting enzymes in the arachidonic acid cascade provides a mechanism for the observation that vitamin E dose-dependently enhances release of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation[65,66].

Vitamin A

Vitamin A is a general term that refers to fat-soluble compounds that are similar in structure and biologic activity to retinol [67]. The precursors of vitamin A (retinol) are the carotenoids (most commonly beta-carotene). The term retinoid refers to any compound that is structurally similar to retinal (aldehyde), retinol (alcohol), or any other substance that exhibits vitamin A activity. Most compounds within the vitamin A family are soluble in fat and essential to numerous processes within the body. There have been several water-soluble retinoids, extracted from plasma, bile, and other tissue[68].

Vitamin A is essential for numerous intrinsic processes. The 11-cis retinal form of vitamin A is essential for the neural transmission of light into vision. Vitamin A also has antioxidant properties. However, beta-carotene has been noted as having pro-oxidant properties. Despite these

discrepancies vitamin A is known to help repair damaged tissue and therefore may be beneficial in counter-acting free radical damage [69].

β-Carotene may play a role in trapping peroxy free radicals in tissues at low partial pressures of oxygen. The ability of β-carotene to act as an antioxidant is due to the stabilization of organic peroxide free radicals within its conjugated alkyl structure. Since β-carotene is effective at low oxygen concentrations, it complements the antioxidant properties of vitamin E, which is effective at higher oxygen concentrations. LDL is the major carrier of β-carotene [70].

Vitamin C

In the aqueous phase, ascorbic acid (vitamin C) is an important antioxidant both within cells and in the plasma [71]. It has the potential to protect both cytosolic and membrane components of cells from oxidant damage. In the cytosol, ascorbate acts as a primary antioxidant to scavenge free radical species that are generated as by-products of cellular metabolism. For cellular membranes, it may play an indirect antioxidant role to reduce the α-tocopheroxyl radical to α-tocopherol. Recycling of α-tocopherol by ascorbate has been demonstrated in liposomes and cellular organelles [72][73]. Evidence suggests that ascorbate also spares and probably recycles α-tocopherol in erythrocyte membranes [74] and intact erythrocytes [75]. It can also donate electrons to a trans-plasma membrane electron transfer activity in erythrocytes [76][77] and nucleated cells [78].

Upon interaction with ROS, ascorbate is oxidized to dehydroascorbate via the intermediate ascorbyl free radical. Dehydroascorbate is recycled back to the reduced form by dehydroascorbate reductase[79]. Ascorbate recycling in human cells is accomplished by several mechanisms in which GSH is believed to be a major factor [80].

Vitamin C can also scavenge reactive nitrogen species efficiently preventing the nitrosation of target molecules[81]. Under certain *in vitro* conditions, ascorbate displays pro-oxidant properties through reduction of transition metal ions (e.g. Fe^{3+}). However, *in vivo*, ascorbate does not promote oxidative lipid damage but rather acts as an antioxidant even in the presence of iron overload[82].

Selenium:

Selenium is a trace element essential to human health and functions through selenoproteins[83]. There are about 20 known selenium-containing proteins in mammals[84]. In all major domains of life, selenium is co-translationally inserted into protein as the amino acid selenocysteine (Sec). Such occurrence of this element in protein is responsible for the majority of biological effects of selenium. Significant health benefits have been attributed to selenium. It is increasingly recognized as one of the more promising cancer chemopreventive agents[85], and there are strong indications that it has a role in reducing viral expression[86], in preventing heart disease and other cardiovascular and muscle disorders[87]. Additional

evidence suggests that selenium may have a role in mammalian development[88], in immune function[89], in male reproduction[90], and in slowing the aging process[89].

Glutathione peroxidase, of which selenium is an integral component, provides a second line of defense against hydroperoxides before they can damage membranes and other cell components. Thus, tocopherol and selenium reinforce each other in their actions against lipid peroxides. In addition, selenium is required for normal pancreatic function, which is necessary for the digestion and absorption of lipids, including vitamin E. Conversely, vitamin E reduces selenium requirements by preventing loss of selenium from the body or maintaining it in an active form [91].

Glutathione

The term glutathione is typically used as a collective term to refer to the tripeptide L-gamma-glutamyl-L-cysteinylglycine in both its reduced and dimeric forms. Monomeric glutathione is also known as reduced glutathione and its dimer is also known as oxidized glutathione, glutathione disulfide and diglutathione [92].

Reduced glutathione (GSH) synthesis involves two closely linked, enzymatically-controlled reactions that utilize ATP. First cysteine and glutamate are combined, by gamma-glutamyl cysteinyl synthetase. Second, GSH synthetase combines gamma-glutamylcysteine with glycine to generate GSH[93]. GSH has a thiol (SH) group on the cysteinyl portion, which accounts for its strong electron-donating character. Its high electron-donating capacity

(high negative redox potential) combined with high intracellular concentration generate a great reducing power[94]. As electrons are lost the GSH molecule becomes oxidized, and two such molecules become linked (dimerized) by a disulfide bridge to form glutathione disulfide or oxidized glutathione (GSSG). This linkage is reversible upon reduction. GSH is under tight homeostatic control both intracellularly and extracellularly. A dynamic balance is maintained between GSH synthesis, its recycling from GSSG, and its utilization. GSH recycling is catalyzed by glutathione disulfide reductase, which uses reducing equivalents from NADPH to reconvert GSSG to 2GSH. The reducing power of ascorbate helps conserve systemic GSH[95].

GSH is an essential cofactor for many enzymes which require thiol-reducing equivalents, and helps to keep redox-sensitive active sites on enzymes in the necessary reduced state[96].GSH is used as a cofactor by (1) multiple peroxidase enzymes, to detoxify peroxides generated from oxygen radical attack on biological molecules; (2) transhydrogenases, to reduce oxidized centers on DNA, proteins, and other biomolecules; and (3) glutathione S-transferases (GST) to conjugate GSH with endogenous substances (e.g., estrogens) and to exogenous electrophiles (e.g., unsaturated carbonyls), and diverse xenobiotics[97].

GSH is an extremely important cell protectant. It directly quenches reactive hydroxyl free radicals, other oxygen-centered free radicals, and radical centers on DNA and other biomolecules [94]. GSH is a primary protectant of skin, lens, cornea, and retina against radiation damage, and

the biochemical foundation of P450 detoxication in the liver, kidneys, lungs, intestinal epithelia, and other organs [98]. Inside the cell GSH helps re-reduce oxidized forms of other antioxidants such as ascorbate and alpha-tocopherol. GSH and its metabolites also interface with energetics and neurotransmitter synthesis, through several prominent metabolic pathways. GSH availability down-regulates the pro-inflammatory potential of leukotrienes and other eicosanoids [99].

LIPOPROTEINS

Chemically, lipids are either compounds that yield fatty acids on hydrolysis or complex alcohols that can combine with fatty acids to form esters[100]. Lipids synthesized in the liver and the intestine have to be transported to the various tissues to accomplish their metabolic functions. They are transported in the plasma in macromolecular complexes called lipoproteins. Lipoproteins are spherical particles with non-polar lipids (triglycerides and cholesterol esters) in their core and more polar lipids (phospholipids and free cholesterol) oriented near the surface. They also contain one or more specific proteins, called apolipoproteins that are located on their surface[101].

According to physical and chemical properties, four groups of lipoproteins have been identified that are important physiologically and for clinical diagnosis. These are: (1) chylomicrons, derived from intestinal absorption of triglycerides, (2) very low density lipoproteins (VLDL, or pre β–lipoproteins), derived from the liver for the export of triacylglycerol;

(3) low-density lipoproteins (LDL, or β-lipoproteins), representing a final stage in the catabolism of VLDL; and (4) high density lipoproteins (HDL, or α-lipoproteins), involved in VLDL and chylomicron metabolism and also in cholesterol transport [102]. Lipoprotein (a) [Lp(a)] is a distinct class of lipoprotein that is structurally related to LDL. However unlike LDL, Lp(a) contains a carbohydrate-rich protein apo(a)[103]. Triacylglycerol is the predominant lipid in chylomicrons and VLDL, whereas cholesterol and phospholipids are the predominant lipids in LDL and HDL, respectively[102].

Apolipoproteins:

Apolipoproteins are the protein components of lipoprotein. Each class of lipoprotein has a variety of apolipoproteins in differing proportions, with the exception of LDL, which contains only apo B-100. Apo A-I is the major protein in HDL. Apo C-I,-II, III and E are present in various proportions in all lipoproteins but not LDL [101].

Apolipoproteins collectively have three major physiological functions. They are involved in (1) activating important enzymes in the lipoprotein metabolic pathways, (2) maintaining the structural integrity of the lipoprotein complex, and (3) facilitating the uptake of lipoprotein into cells through their recognition by specific cell surface receptors [104].

Apolipoprotein B

Apolipoprotein B exists in two forms: apoB-100 and apoB-48. The two proteins are know to be translation products of a single structural gene [104]. Apo B-100, a single large polypeptide chain of 4536 amino acids,

is the full-length translation product of the apo B gene. [105]. In humans, apoB-100 is made in the liver and secreted into plasma as part of VLDL. Each VLDL particle contains one molecule of apo B-100. In the fasting state, most of the apo B in plasma is apo B-100. Unlike the other apolipoproteins, however, apo B-100 cannot move from one lipoprotein particle to another and VLDL apo B-100 remains with lipoprotein as it is catabolized to LDL. Both apo B-100 and B-48 play important roles in the secretion of VLDL and chylomicrons, respectively [100]. Apo B-100 is recognized by the LDL receptor in hepatic and peripheral tissues and allows the LDL receptor-mediated internalization of LDL [106].

Low density lipoprotein composition:

LDL contains, by weight, 80% lipid and 20% protein. Consistent with this increased protein content, LDL is smaller (has a diameter between 22 and 28 nm) and is of higher hydrated density (1.006 to 1.063 g/ml) than are VLDL and chylomicrons. About 60% of LDL is cholesterol [107][108].

LDL is a spherical particle with molecular weight of about 2.5 million Da. A central core contains about 1600 cholesteryl ester molecules and 170 molecules of triglyceride; which are surrounded by an outer monolayer shell composed of about 700 phospholipid molecules , consisting primarily of lecithin, small amounts of sphingomyelin and lysolecithin, and 600 molecules of free cholesterol .Apolipoprotein B is embedded in the outer monolayer[109,110].

LDL particle contains total number of fatty acids bound in the different lipid classes averaging 2600, roughly half of which are polyunsaturated fatty acids (PUFAS), mainly linoleic acid (18:2) with minor amounts of arachidonic acid (20:4) and docosahexaenoic acid [111]. These PUFAs are protected against free radical attack and oxidation by antioxidants, primarily α-tocopherol (about six molecules per LDL particle), with minor amounts of γ-tocopherol, carotenoids, cryptoxanthin, and ubiquinol-10 [112]. The amount of PUFAs and antioxidants varies significantly within individuals, resulting in a great variation in LDL oxidation susceptibility [113].

Most individuals can be classified on the basis of the particle diameter of their predominant LDL subclass (using nondenaturing gradient gel electrophoresis) into one of two LDL subclass patterns[114]. LDL subclass pattern A is characterized by a predominance of large, more buoyant LDL. LDL subclass pattern B is characterized by a predominance of small, denser LDL [115].

Lipoprotein Metabolism

The pathways of lipoprotein metabolism are complex[101] and can be divided conceptually into exogenous and endogenous pathways based on whether they carry lipids of dietary or hepatic origin; the intracellular LDL receptor pathway; and HDL reverse cholesterol transport pathway [100].

Exogenous pathway

Nascent chylomicrons are assembled from dietary triglyceride and cholesterol and acquire the C apolipoprotein and apo E from circulating HDL[110]. Apo C-II activates lipoprotein lipase (LPL), which rapidly hydrolyzes the triglycerides to free fatty acids and chylomicron remmant that can be recognized by specific hepatic remnants receptors and internalized by endocytosis, and cholesteryl esters and triglycerides are hydrolyzed and metabolized [116].

Endgenous pathway

Hepatocytes have the ability to synthesize triglycerides and cholesterol, which are packaged in secretory vesicles, transported by exocytosis into the extracellular space, and introduced into circulation in the form of nascent VLDL. This triglyceride-rich particle (55% by mass) contains apo B-100, apo E, and small amount of C apolipoproteins on its surface. Additional C apolipoproteins are transferred after secretion from circulating HDL[117]. VLDL is metabolized initially by lipoprotein lipase, which leads to the hydrolysis of triglyceride, and the C apolipoproteins are transferred back to HDL. VLDL remnants possess apo E, which mediates uptake of approximately half of the VLDL remnants into the liver, and the rest converted to smaller, denser particles called intermediate-density lipoprotein (IDL)[105].

Surface materials from IDL, including some phospholipids, free cholesterol, and apolipoproteins, are transferred to HDL, or form HDL *de*

novo in the circulation. Cholesterol esters are transferred from HDL to IDL and the net result is the replacement of much of triglyceride core of origin VLDL with cholesteryl esters. IDL undergoes further hydrolysis in which most of the remaining triglycerides are removed and all apolipoproteins, except B-100, are transferred to other lipoproteins. This process ends with ultimate formation of LDL [101].

Low-Density Receptor Pathway

LDL is removed by receptor-mediated endocytosis, which is initiated by binding of apo B-100 to the LDL receptor (apo B-100/ apo E receptor) approximately two-third by the LDL is taken up by the liver, and one third by extrahepatic tissues[105].

LDL can also be taken up by extrahepatic tissues through scavenger receptors or receptor- mediated pinocytosis. Scavenger receptor are upregulated as well, and recognize LDL that has been modified in various ways, they are found in macrophages and other cells[100].

High Density Lipoprotein Reverse Cholesterol Transport Pathway:

Free cholesterol from cell membranes is transferred to the nascent HDL. Cholesterol is estrerified by the action of lecithin cholesterol acyltransferase (LCAT) in the presence of its cofactor apo A-1. The size of the HDL particle depends strongly on the amount of accumulated cholesterly esters and the activity of LCAT. HDL cholestreryl esters are delivered to the liver by one the following mechanisms: (1) cholesteryl esters are selectively taken up from HDL, probably by the hepatic HDL

receptors, and HDL particles are returned to circulation for further transport; (2) cholesteryl esters are transferred from HDL to apo B-100-containing lipoprotein, a process mediated by cholesterol ester transfer protein, then taken up by the liver through receptors for these lipoprotein; or (3) HDL apo E can recognized by the hepatic remnant receptors [118][119]. These processes constitute the reverse cholesterol transport mechanism, by which cellular and lipoprotein cholesterol is delivered back to the liver for reuse or disposal.

LOW DENSITY LIPOPROTEIN MODIFICATION

Cholesterol accumulation in the developing atherosclerotic lesion is probably due to the uptake of some modified form of LDL by way of one or more receptor [120].

It was shown that simply incubating LDL overnight with a monolayer of arterial endothelial cells converted it to a form that was taken up much more rapidly by macrophages and capable of increasing their cellular cholesterol content [121]. Incubation with smooth muscle cells could also modify LDL in much the same way [122]. This cell-mediated modification turned out to be oxidative modification [123]. The addition of antioxidants to the culture medium completely blocked cell-induced modification, and the changes induced by the cells could be duplicated by incubating LDL in the presence of transition metals in cell-free systems. Thus, oxidative modification induced by cells appeared to be a biologically

plausible modification of LDL that could account for foam cell formation and the initiation, or at least acceleration, of the atherosclerotic process[124].

The Nature of "Oxidized LDL"

Oxidation of LDL is a complex process[125,126]. Both the protein moiety as well as each of the components of the lipid moiety can be oxidatively attacked. The extent of the changes in the LDL particle induced by oxidation depends on the prooxidation conditions in the LDL bio-environment. Therefore, there is no unique LDL particle corresponding to oxidized LDL, [108] but there is a broad spectrum of "oxidized LDLs, which differ both structurally and functionally. Even the LDLs subjected to a very mild oxidative stress (minimally oxidized LDL) can acquire important biological properties, including the ability to stimulate release of chemokines from endothelial cells[127,128,129].

A key feature of LDL oxidation is the breakdown of these polyunsaturated fatty acids (PUFAs) to yield a broad array of smaller fragments, (3-9 carbons in length), including aldehydes and ketones that can become conjugated to other lipids, especially amino lipids or to apoB[126]. It is believed that the oxidation of LDL occurs in the arterial walls where there is an increased level of redox-active metal ions and LDL is sequestered away from circulating antioxidants[130].

Initial Oxidation of LDL:

The cells of the artery wall constantly secrete oxidative waste products into their membranes and into the subendothelial space. The

ability of the cells of the artery wall to oxidize LDL is directly related to their ability to "seed" the LDL with reactive oxygen species[131]. At the same time, these microenvironments exclude aqueous antioxidants and allow trapped LDL to undergo oxidation[132]. *De novo* oxidation of intrinsic LDL lipids might occur under a wide variety of normal and pathological conditions contributed by xanthine oxidase[133], peroxynitrite[134], myeloperoxidase[135], and other oxidative processes[136]. When such oxidation is carried out to a minimal degree, the resultant particle might represent the "minimally oxidized LDL" or "MM-LDL" and, as such, might be physically indistinguishable from the native lipoprotein, except for the expected loss of polyunsaturated fatty acids and antioxidants. ApoB-100 is intact and little protein damage or modification has been detected. On the other hand, the lipids, particularly phospholipids are enormously affected[137,138,139].

Enzymes and Tissues Involved in LDL Oxidation *in Vivo*

The ability of many cell types including endothelial cells, smooth muscle cells, monocytes, macrophages, fibroblasts, neutrophils, and others to generate superoxide radicals has long been considered a requirement for the oxidation of LDL[124] [131].

They may do so either by directly generating oxidants such as, H_2O_2, LOOH and nitric oxide or by indirectly generating prooxidant conditions extracellularly[140]. In addition, these cells also participate in the propagation of lipid peroxidation in the medium and may also deplete the

antioxidants in the lipoprotein thus increasing its rate of oxidation. The superoxide radical may be inefficient in catalyzing lipid peroxidation but may be generated by cells as an important means of providing extracellular hydrogen peroxide, which in the presence of redox metal generate more potent oxidants [131]. A number of enzyme systems could in principle play a role in the oxidation of LDL. Analysis of products isolated from atherosclerotic lesions strongly supports the involvement of lipoxygenases [141] and of myeloperoxidase[142].

Lipoxygenases

15-Lipoxygenase, produced by endothelial cells and monocytes/ macrophages, converts polyunsaturated fatty acids into lipid hydroperoxides and thereby oxidizes LDL. Lipoxygenase inhibitors block *in vitro* oxidation of LDL by these cells[143,144]. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice[145]. Disruption of the 12/15-lipoxygenase genes diminishes atherosclerosis in apoE knockout mice in the absence of changes in cholesterol, triglyceride, and lipoprotein levels[146].

Myeloperoxidase

Activated phagocytes secrete myeloperoxidase that generates reactive species including hypochlorous acid (HOCl), chloramines, tyrosyl radicals, and nitrogen dioxide (NO_2). These reactive species oxidize antioxidants, lipids, and protein of LDL [147]. Reactive nitrogen species generated by the myeloperoxidase-H_2O_2-NO_2 system of monocytes convert

LDL into an atherogenic form that is avidly taken up and degraded by macrophages, leading to foam cell formation [148].

Activated human neutrophils generate *p*-hydroxyphenyl acetaldehyde (pHA), the major product of L-tyrosine oxidation by the myeloperoxidase-HOCl-H_2O_2 system. The concentration of pHA-modified phospholipid in LDL isolated from human atherosclerotic lesions is markedly increased compared with circulating LDL[149]. Sugiyama et al.[150] identified granulocyte macrophage colony-stimulating factor as an endogenous regulator of myeloperoxidase expression in human atherosclerosis. Furthermore, increased numbers of myeloperoxidase-expressing macrophages were demonstrated in eroded or ruptured plaques causing acute coronary syndromes, suggesting a role for myeloperoxidase-expressing macrophages in human atheroma complications [151].

Oxidation of LDL by Reactive nitrogen species

Nitric oxide (NO) inhibits copper-mediated oxidation [152] as well as cell-mediated oxidation of LDL [153]. NO is converted under aerobic conditions to nitrite, and low concentrations of nitrite (12 μM compared with physiological concentrations of up to 200 μM) inhibit myeloperoxidase-mediated oxidation of LDL [154]. NO also acts as an antioxidant by scavenging alkoxyl and peroxyl radicals.

The nitric oxide radical ($NO^{\bullet}$) interacts with superoxide anion to form the peroxynitrite anion ($ONOO^-$) that decomposes into the hydroxyl radical ($OH^{\bullet}$), which oxidizes LDL[155]. Peroxynitrite also oxidizes

tetrahydrobiopterin, a critical cofactor for NO synthase (NOS), and thereby decreases NO production[156]. Expression of inducible NOS, associated with increased peroxynitrite production, resulted in increased apoptotic cell death in atheromatous plaques of human coronary arteries [157]. Thus, when NO is in excess of surrounding oxidants, lipid oxidation and monocyte migration into the vascular wall are attenuated, producing anti-atherogenic effects. However, when oxidant defenses become depleted or endogenous tissue rates of oxidant production are accelerated, NO gives rise to secondary oxidizing species that increase membrane and lipoprotein lipid oxidation as well as foam cell formation in the vasculature [158].

Oxidation of LDL by metal ions

In vitro oxidation of LDL by metal ions (e.g., Cu^{2+}) occurs in three phases: an initial lag phase (consumption of endogenous antioxidants), a propagation phase (rapid oxidation of unsaturated fatty acids to lipid hydroperoxides), and a decomposition phase (hydroperoxides are converted to reactive aldehydes, e.g., malondialdehyde, 4-hydroxynonenal). Interaction of these aldehydes with positively charged ε-amino groups of lysine residues renders the LDL more negatively charged, resulting in decreased affinity for the LDL receptor and increased affinity for scavenger receptors [113].

It is unlikely that free metal ions are responsible for *in vivo* LDL oxidation. There is no convincing evidence for free metal ion in plasma or the arterial wall. There is also no significant accumulation of o-tyrosine and

m-tyrosine, typical oxidation products of free metal ion, in fatty streaks or intermediate atherosclerotic lesions [159].

Biological consequences of mildly Oxidized LDL

Exposure of endothelial cells to the polar lipid fraction isolated from mildly oxidized LDL induced monocytes but not neutrophils to adhere to the cells. The adhesion of the monocytes was induced by the induction in the endothelial cells of P-selectin[160] and the monocyte-activating proteins MCP-1, [127] M-CSF, [128] and GRO[161]. In artery wall co-cultures, the monocytes not only adhered to the endothelial cells but migrated along the MCP-1 gradient into the subendothelial space [162]. In the subendothelial space, the monocytes differentiated into macrophages under the influence of M-CSF[128]. The mRNA levels for these proteins were induced by the oxidized lipids as a result of increased transcription, mediated by the activation of an NFκB-like transcription factor, and mRNA stabilization[163]. The oxidized lipids also induced high levels of intracellular cAMP via a G protein-mediated mechanism [164]. The series of events initiated by mildly oxidized LDL would result in the migration of monocytes into the subendothelial space and their conversion into macrophages. This would in turn be expected to enrich the microenvironment in reactive oxygen species that could convert the trapped, mildly oxidized LDL into highly oxidized LDL. As a result, foam cells would be expected to form. The subsequent recruitment of more monocytes, proliferation and retention of monocyte-macrophages, elaboration of growth factors, secretion of other potent biological factors by

the monocyte-macrophages, and eventually death of the foam cells would be expected to result in progression of the lesion[129].

Biochemical composition of oxidized LDL

The lipids and the protein component of Ox-LDL differ substantially from those of the native LDL. In contrast to the single long chain polypeptides (apoB-100) of native LDL, the Ox-LDL has proteolyzed, fragmented, oxidized, cross-linked apolipoprotein with a different amino acid composition. The protein is also covalently modified by lipid peroxidation products that include intact core aldehydes derived from esterified lipid[165]. In addition to the expected oxidized products, the lipid fraction of the Ox-LDL also contains enzymatic breakdown products such as lysophosphatidylcholine (lysoPtdCho). It may also contain phospholipids and cholesteryl esters in which the oxidized fatty acid moiety has been shortened[166,167].

Biological activity of oxidized LDL

The ability of OxLDL to induce cholesterol accumulation in macrophages was the first proatherogenic property of OxLDL to be described [121] and was the basis for the hypothesis that oxidation of LDL might be an important step in the atherogenic process.

Effect Of oxidized LDL on monocyte infiltration

Adhesion and infiltration of macrophages into the arterial wall contributes to fatty streak formation. MM-LDL induces endothelium to

express adhesion molecules for monocytes, intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule (VCAM-1)[168]. MM-LDL also promotes monocyte proliferation and differentiation into macrophages by inducing macrophage colony-stimulating factor (M-CSF) expression by endothelial cells[128]. Oxidized LDL stimulates endothelium to secrete monocyte chemotactic protein 1 (MCP-1), which induces the infiltration of monocytes into the subendothelial space[127].

Effect Of oxidized LDL on smooth muscle cell migration and proliferation

During atherogenesis, smooth muscle cells undergo a phenotypic modification to a synthetic state, allowing them to migrate from the intima to the media where they proliferate and secrete growth factor, extracellular matrix glycoprotein, and metalloproteinases[169,170]. This leads to fibrous plaque formation. Oxidized LDL induces migration of smooth muscle cells by increasing the expression of platelet-derived growth factor (PDGF) by endothelial cells, smooth muscle cells, and macrophages[171,172]. Oxidized LDL also stimulates smooth muscle cell proliferation by inducing expression of basic fibroblast growth factor (bFGF) by endothelial cells and smooth muscle cells[173].

Effect of oxidized LDL on vasoreactivity

Intimal thickening is caused by accumulation of foam cells and by smooth muscle cell migration and proliferation. It results in reduction of arterial lumen, which is exacerbated by impairment of the vasodilator

capacity of the artery. Oxidized LDL may induce vasoconstriction through inhibition of NO production[174] and stimulation of expression of endothelin[175].

Effect of oxidized LDL on apoptosis

Apoptosis in endothelial and smooth muscle cells contributes to plaque rupture[176]. The apoptotic effect of oxidized LDL on endothelial cells could be attributed to oxidation products of phosphatidylcholine or to oxysterols[177].

Effect of oxidized LDL on thrombosis

Endothelial dysfunction is associated with enhanced platelet adhesion, increased procoagulant activity, and impaired fibrinolysis[178]. Oxidized LDL stimulates platelet adhesion and aggregation by decreasing endothelial production of NO, increasing prostacyclin (PGI_2) production[179], and stimulating the synthesis of prostaglandins and prostaglandin precursors [180].

Oxidized LDL enhances the procoagulant activity of endothelium by inducing the release of tissue factor (TF) by endothelial cells and smooth muscle cells. TF is a cofactor of factor VIIa that activates factors IX and X, resulting in thrombin formation[181]. Oxidized LDL reduces the fibrinolytic activity of endothelium by decreasing secretion of tissue-type plasminogen activator (tPA) and increasing release of plasminogen activator inhibitor 1 (PAI-1) [182,183].

Oxidized LDL may also contribute to plaque disruption and/or vascular remodeling by increasing expression of metalloproteinase 9 (MMP-9) and decreasing expression of its tissue inhibitor TIMP-1 by monocytes/macrophages [184].

Other LDL Modifications

A number of other modifications of LDL could help account for the formation of foam cells, some of which result from the complexing of LDL with other macromolecules. For example, some of the proteoglycans in the arterial wall bind tightly to LDL and form insoluble complexes that are taken up avidly by macrophages[185]. Complexes of LDL with itself are taken up more rapidly than native LDL[186].

AIM OF THE WORK

AIM OF THE WORK

The aim of the present study was to evaluate the possible relationship between the circulating levels of the modified derivatives of LDL and the development of angiopathy in type 2 diabetic patients with coronary artery disease. The status of the antioxidant defenses and the role of supplementation with combination of antioxidant, as a free radical scavenger were also studied in these patients.

MATERIAL AND METHODS

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Subjects

This study was conducted on 3 groups:

- **Group I (Control)**: Included 15 apparently healthy individuals (8 males and 7 females).Their ages ranged from 41 to 71 years.
- **Group II:** Included 15 type 2 diabetic patients without complications (8 males and 7 females). Their ages ranged from 39 to 72 years. At the time of the study, the individuals of this group were being treated by diet plus oral hypoglycemic agents. They were among those seen on regular basis by the medical staff in the diabetes out-patient clinic of the Medical Research Institute hospital
- **Group III:** Included 15 type 2 diabetic patients with stable coronary artery disease (8 males and 7 females). They were among the patients diagnosed, treated and followed up in the cardiology unit of the Medical Research Institute hospital. Their ages ranged from 46 to 72 years. At the time of the study, the individuals of this group were being treated by diet plus oral hypoglycemic agents. After establishing the baseline values for different studied parameters, patients in this group received adjunct treatment of antioxidant tablets for three months and the assessed parameters were re-evaluated after three months of supplementation.

Criteria for exclusion from the study included history of ketoacidosis renal or liver dysfunction smoking; use of vitamin or antioxidant supplements, treatment with lipid-lowering agents, or other drugs known to affect serum lipids.

Supplements

The supplements consisted of one daily tablet of antioxidant combination tablets containing: 30 mg Vitamin E, 100 mg Ascorbic acid, 5.54 mg Vitamin A acetate, 50 µg Selenium, and 105 mg Medical yeast.

Complete past history of subjects

- All subjects were interviewed for full clinical examination.
- The duration of diabetes and the previous and current medications they received were recorded.
- Coronary heart disease was documented by history, clinical examination, ECG changes and/or previous coronary angiography.

Clinical Investigation

Carotid artery intima-media thickness (IMT) was measured by carotid ultrasound scanner for patients of group III at baseline and after 3 months of supplementation.

Biochemical Assays

A- Glycemic Control: Fasting plasma glucose (FPG), and Glycated hemoglobin (HbA1c).

B- Lipid Pattern: Triglycerides, Total cholesterol, HDL–cholesterol, LDL–cholesterol, and Apolipoprotein B (Apo B).

C- Modified LDL: Circulating oxidized LDL, Circulating oxidized LDL–antibody, and susceptibility of LDL to Oxidation *in vitro*.

D- Oxidative stress and antioxidant defense: Thiobarbituric acid reactive substance (TBARS), free thiol group, and Total antioxidant status.

- All biochemical parameters were repeated for patient in group III after antioxidant supplementation and a follow-up period of 3 months.

Blood Sampling

- A fasting blood sample was obtained from each subject and divided into 3 aliquots; according to the anticoagulant used. In two aliquots EDTA and sodium fluoride were used to prevent coagulation. In the third aliquot no anticoagulant was added and it was used to prepare serum.
- Blood samples with EDTA anticoagulant were immediately analyzed for HbA1c. The serum stored at –80 °C for further analysis. EDTA plasma was stored at 4°C for isolation of LDL which was performed within 3 days for the determination of MDA – LDL.

Glucose Determination

Plasma glucose levels were determined according to an enzymatic calorimetric method which has been described by Trinder [187].

Principle:

Glucose is oxidized in the presence of glucose oxidase. The hydrogen peroxide formed reacts under catalysis of peroxidase. with phenol and 4–amino phenazone to a red-violet quinoneimine dye. The intensity of the color is proportional to glucose concentration.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4-aminophenazone} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4H_2O$$

Reagents

Enzyme reagent

Phosphate buffer (ph 7.5)	0.1 mol/L
4 – Aminophenazone	0.25 mmol/L
Phenol	0.75 mmol/L
Glucose oxidase	>15 KU/L
Peroxidase	>1.5 KU/L

Standard

Glucose	100 mgldl

Procedure:

- 1 ml of enzyme reagent was mixed in test tubes with 10 µl of plasma sample or glucose standard.
- The mixture was incubated at 37 °C for 5 minutes.
- Reagent blank was run through the same procedure.
- The absorbance of standard (△A standard) and the sample (△A sample) were measured against reagent blank at 540 nm.

Calculation

$$\text{Glucose concentration (mg / dl)} = 100 \times \frac{\Delta A\, sample}{\Delta A\, s\tan dard}$$

Where, concentration of standard was 100 mg / dl.

Determination of Glycated Hemoglobin (HbA1c)

The HbA1c determination was based on the turbidimetric inhibition immunoassay for hemolyzed whole blood [188].

Principle

HbA1c reflects the average blood glucose level during the preceding 2 to 3 months. HbA1c is thus suitable to monitor long-term blood glucose control individuals with diabetes mellitus [189]. HbA1c in the hemolyzed sample reacts with anti-HbA1c antibody to form soluble antigen–antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, complex formation does not take place. Polyhaptens reacts with excess anti– HbA1c antibodies to form an insoluble antibody–polyhapten complex which can be determined turbidimetrically.

Liberated hemoglobin in the hemolyzed sample is converted to a derivative having a characteristic absorbance spectrum which is measured in a second channel.

Reagents

HbA1c reagents

1- Buffer / Antibody reagent (pH 6.2):

MES (2– morpholinoethane sulfuric acid) buffer	0.025 mol/l
TRIS (tris (hydroxymethyl–aminomethane) buffer,	0.015 mol/l
HbA1c antibody	0.5 mg / ml

2- Buffer / Polyhapten reagent (pH 6.2):

MES buffer	0.025 mol/l
TRIS buffer	0.015 mol/l
HbA1c polyhapten	≥8 µg / ml

Hemoglobin Reagent

Phosphate buffer (pH 7.4)	0.02 mol/L

Procedure

- Sample preparation: 10 µl of EDTA – blood was mixed with 1000 ul of hemolyzing reagent by gentle swirling. The hemolysate was used after the solution has changed color from red to brownish-green.
- The assay was run on Hitachi automatic clinical chemistry analyzer and the results of HbA1c were evaluated automatically according the equation.

$$\%\,\text{HbA1c} = \frac{\text{HbA1c}[\text{g/dL}]}{\text{Hb}[\text{g/dL}]} \times 100$$

Triglycerides Determination

The triglycerides level was determined by the enzymatic colorimetric method described by Buccdo and David [190].

Principle

- Glycerol and fatty acids are first formed by the action of lipase on the triglycerides.
- Glycerol is then phosphorylated by adenosine–5–triphosphate (ATP) to produce glyceriol-3–phosphate and ADP in a reaction catalyzed by glycerol kinase.
- Glycerol –3– phosphate is oxidized by glyceryl phosphate oxidase producing dihydroxy acetate phosphate (DAP) and hydrogen peroxide.

- Hydrogen Peroxide reacts with 4–aminoantipyrine and 4-chlorophenol under the catalytic influence of peroxidase to form quinoneimine.

$$\text{Triglycerides} \xrightarrow{\text{lipase}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{glycerol kinase}} \text{glycerol–3–phosphate + ADP}$$

$$\text{Glycerol–3-phosphate + } O_2 \xrightarrow{\text{lglyceryl phosphate oxidase}} \text{DAP+}H_2O_2$$

$$H_2O_2\text{+4–aminoantipyrine+4-Cholorophenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine + HCl} + H_2O$$

Reagents

Enzyme / Buffer reagent:

PIPES buffer (pH 7.5)	50 mmol/L
4 – chloro phenol	5 mmol/L
4 – Aminoantipyrine	0.25 mmol/L
Magnesium ions	4.5 mmol/L
ATP	2 mmol/L
Lipase	≥ 1.3 U/ml
Peroxidase	≥ 0.5 U/ml
Glycerol kinase	≥ 0.4 U/ml
Glycerol – 3 – phosphate oxidase	≥ 1.5 U/ml

Standard

Triglycerides	200 mgldl

Procedure

- 1 ml of enzyme reagent was mixed with 10 µl of serum sample or triglycerides standard in test tubes.
- Reagent blank was run through the same procedure.
- All tubes were incubated at 37°C for 5 minutes.
- The absorbance of standard (△A standard) and the sample (△A sample) were measured against reagent blank at 546 nm.

Calculation

$$\text{Triglycerides concentration (mgldl)} = 200 \times \frac{\Delta A \text{ sample}}{\Delta A \text{ s tan dard}}$$

Where, concentration of standard was 200 mgldl

Determination of Total Cholesterol

Serum total cholesterol level was determined on the basis of an enzymatic colorimetric method described by Allain et al [191].

Principle

Cholesterol esterase hydrolyzes esters and H_2O_2 is formed in the subsequent enzymatic oxidation of cholesterol by cholesterol oxidase according to the following reaction:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestene–3–one} + H_2O_2$$

$$2H_2O_2 + 4\text{aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + H_2O$$

Reagents:

Enzyme reagent:

Phosphate buffer (pH 6.5)	100 mmol/L
4 – Amino phenazone	0.3 mmol/L
Phenol	5 mmol/L
Peroxidase	> 5 KU/L
Cholesterol esterase	> 150 U/L
Cholesterol oxidase	> 100 U/L

Standard:

Cholesterol	200 mgldl

Procedure

1 ml of enzyme reagent was mixed with 10 µl of serum sample or cholesterol standard in test tubes. Reagent blank was run through the same procedure. All the tubes were incubated at 37°C for 5 minutes.

The absorbance of standard (△A standard) and the sample (△A sample) were measured against reagent blank at 546 nm.

Calculation

$$\text{Cholesterol concentration (mg/dl)} = 200 \times \frac{\triangle A \text{ sample}}{\triangle A \text{ standard}}$$

Where, concentration of standard was 200 mgldl.

HDL – Cholesterol Determination

High density lipoprotein (HDL) – cholesterol level was determined according to the method described by Albers et al.[192].

Principle

The chylomicrons VLDL (very low density lipoproteins) and LDL (low density lipoproteins) are precipitated by addition of phosphotungstic acid and magnesium chloride. After centrifugation the supernatent fluid contains the HDL fraction, which is assayed for HDL – cholesterol.

Reagents

Precipitant

Phosphotungstic acid	0.55 mmol/L
Magnesium chloride	25.0 mmol/L

Standard

Cholesterol	50 mgldl

Procedure

Precipitation:

- 500 ul of precipitant was added to 200 ul of serum sample, mixed well and incubated for 10 minutes at room temperature.
- The mixture was then centrifuged for10 minutes at 4000 rpm.
- After centrifugation the clear supernatant was separated from the precipitate.

Cholesterol determination:

- 1 ml of enzyme reagent was mixed with 10 µl of HDL supernatant, cholesterol standard, or distilled water (as blank)
- The mixture was incubated at 37°C for 5 minutes.
- The absorbance (△A) of the sample and the standard were measured against blank at 546 nm.

Calculation

HDL -Cholesterol concentration (mg/dl) = 175 x

Calculation of the LDL cholesterol Concentration

The low density lipoprotein cholesterol concentration (LDL–C) was calculated from the total cholesterol concentration (TC), the HDL cholesterol concentration (HDL–C) and the triglycerides concentration (TG) according to Friedewald et al [193].

$$\text{LDL–C} = \text{TC} - (\text{HDL–C}) - \frac{\text{TG}}{5} \quad [\text{mg/dl}]$$

Determination of Apoprotein B (Apo B)

Serum apolipoprotein B was assayed by immunonephelometry[194] with Behring nephelemeter analyzer using a kit (Dad Behring-Germany).

Principle

In an immunochemical reaction, the apolipoprotein B in the serum sample form immune complex with specific antibody. These complex

scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant Apo B in the sample. The result is evaluated by comparison with a standard of known concentration.

Reagent

- Antiserum to human Apo B.
- Apo B standard sera.
- Diluent.

Procedure

- Serum sample was diluted 1:20 with diluent, and according to the assay protocols for the Behring nephelometer, all steps were performed automatically by the instrument.
- Reference curve was constructed by multi–point calibration. Serial dilutions of the Apo B standard serum were automatically prepared for this purpose using diluent.

Calculation

The results were evaluated automatically by means of a logit – log function.

Determination of plasma Oxidized Low Density Lipoprotein

The *in vitro* quantitative measurement of oxidized low density lipoproteins (oxidized LDL) in human blood plasma was carried out using

the Mercodia Oxidized LDL Enzyme-Linked Immunosorbent Assay (ELISA) kit (Mercodia, Sweden) [195,196].

Principle of the procedure

Mercodia Oxidized LDL ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. During incubation, oxidized LDL in the sample reacts with anti- oxidized LDL antibodies bound to microtitration well. After washing, that removes non-reactive plasma components, a peroxidase conjugated anti-human apolipoprotein B antibody recognizes the oxidized LDL bound to the solid phase. After a second incubation and a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm.

Reagents

1- Coated Plate: mouse monoclonal anti-oxidized LDL.

2- Calibrators: 0, 3, 6,11,22,30 mU/l human oxidized LDL.

3- Enzyme Conjugate: Peroxidase conjugated mouse monoclonal anti-ApoB.

4- Enzyme Conjugate buffer.

5- Assay buffer.

6- Sample buffer.

7- Wash buffer.

8- Substrate TMB.

9- Stop Solution: 0.5 M H_2SO_4

Dilution of samples

Samples must be diluted in two steps to a final dilution of 6561 times as follows:

1- Patient sample	25 µl	
Sample Buffer	2000 µl	(Dilution 1/ 81)
2- 1/81 dilution of sample	25 µl	
Sample Buffer	2000 µl	(Dilution 1/6561)

Test procedure

1- 25 µl of each Calibrator, Control and diluted sample were pipetted into appropriate wells.

2- 100 µl of assay buffer was added to each well.

3- Plate incubated on a plate shaker for 2 hours at room temperature (18–25°C).

4- The wells were washed 6 times with 350 µl diluted washing buffer

5- 10 µl of enzyme conjugate was added to each well.

6- Plate incubated on a plate shaker for 1 hour at room temperature (18–25°C).

7- The wells were washed as described above.

8- 200 µl of substrate TMB was added to each well.

9- Plate incubated for 15 minutes at room temperature, without shaking.

10- 50 µl of stop solution was added to each well.

11- Optical density was read at 450 nm.

Calculation of results

1- The absorbance values obtained for the calibrators were plotted against the Oxidized LDL concentration on a semi-log paper and a calibration curve was constructed (fig. A).

2- The concentration of the unknown samples were read from the calibration curve.

3- The concentration of the unknown samples were multiplied by the dilution factor (× 6561).

Determination of Serum Oxidized Low Density Lipoprotein Antibodies

Serum autoantibodies against OxLDL were determined using the enzyme immunoassay (EIA) kit (Biomedica, USA) [197,198].

Principle

- Cu^{++} oxidized LDL is coated onto micro titer strips as antigen.
- Autoantibodies, if present in the prediluted serum, bind specifically to the antigen.

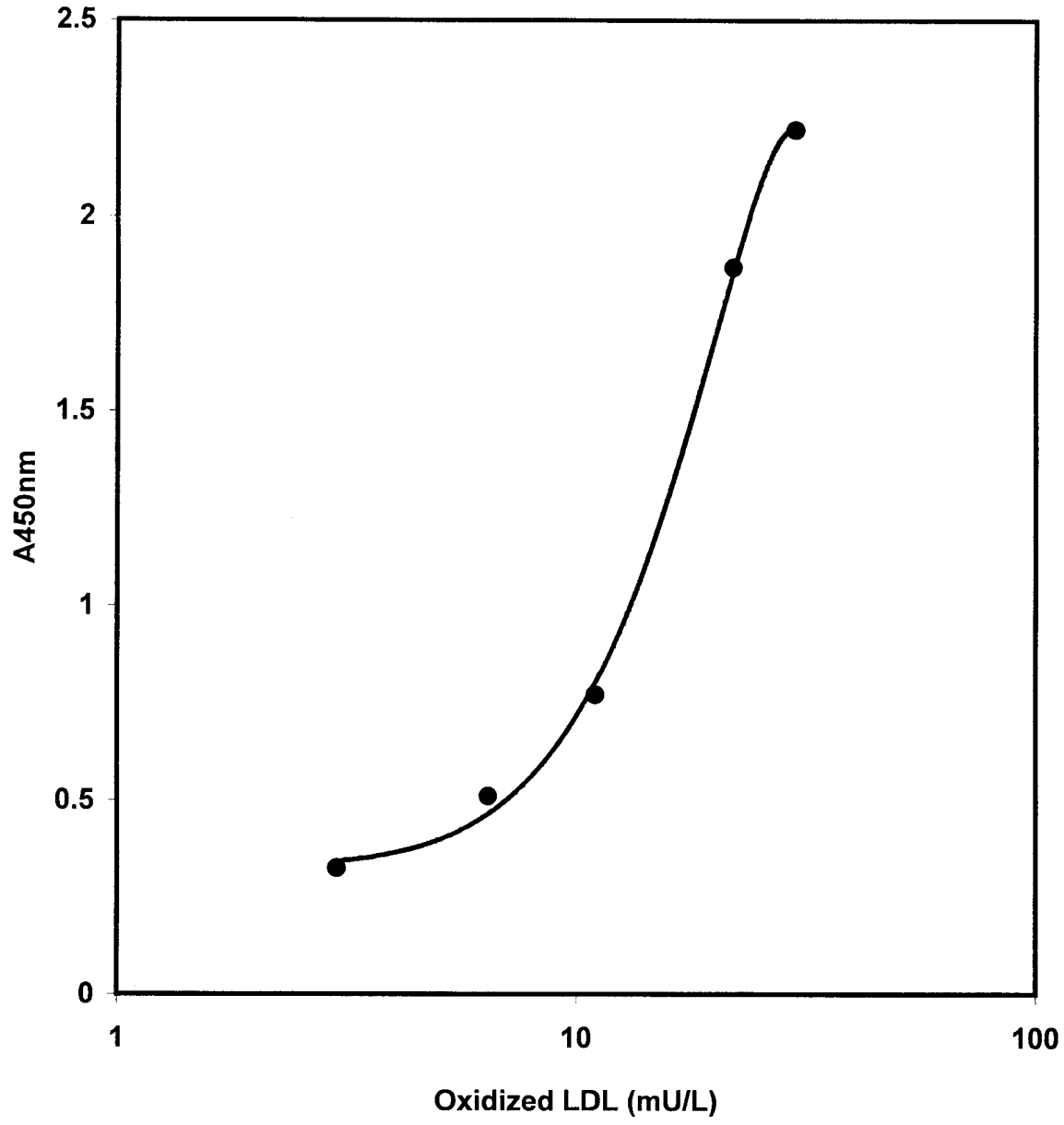

Fig (A) : Oxidized LDL standard curve

- After a washing step, a specific peroxidase conjugated anti human IgG antibody detects the presence of bound autoantibodies.
- After removal of unbound conjugate through washing, tetramethyl benzidine (TMB) is added to the wells as a chromogenic substrate.
- The concentration of specific IgG in the sample is quantitated by an enzyme catalized color change detectable on a standard EIA reader
- The amount of color developed is directly proportional to the concentration of antibodies in the sample.
- The assay is standardized with defined amount of oxidized LDL Ab in a serum matrix.

Reagents

1- Microwell Strips, Coated with Cu^{++} oxidized LDL.

2- Conjugate: mouse monoclonal anti-human IgG to specific horseradish peroxidase conjugate.

3- Substrate: tetramethyl benzidine (TMB).

4- Assay Buffer.

5- Wash buffer: tris buffer saline.

6- Standards: 1200, 600, 300, 150, 75, 37 mU/ml oxidized LDL antibody.

7- Controls : 300 and 1000 mU/ml oxidized LDL-Ab

8- I N Sulphuric acid as a stopping solution.

Procedure

1- Standards, controls, and serum samples were prediluted 1:50 according to the following protocol:

a- 200 µl assay buffer was pipetted into all marked wells of an uncoated microwell plate.

b- 50 µl of standard, control, or sample were pipetted in respective wells of the uncoated plate and shaken.

c- 200 µl of assay buffer was pipetted into all marked wells of the coated microwell plate including blank.

2- 20 µl of the prediluted standards, controls, or samples were transferred to the coated strips.

3- Strips were covered with plastic film and incubated for 90 minutes at 37°C.

4- The wells were washed 4 times with 300 µl diluted washing buffer.

5- 100 µl conjugate was added to all wells.

6- Strips were covered and incubated for 30 minutes at room temperature.

7- The wells were washed 4 times with 300 µl diluted washing buffer.

8- 100 µl of substrate (TMB) was added to all wells.

9- Strips were incubated for 15 minutes at room temperature in dark.

10- 50 µl stop solution was added to all wells.

11-Absorbance was read with ELISA reader at 450 nm

Calculation of Results

- The extinction of the blank was subtracted from all other values.
- A calibration curve was constructed from the standards, (fig. B).
- Results of the sample were read from this calibration curve.

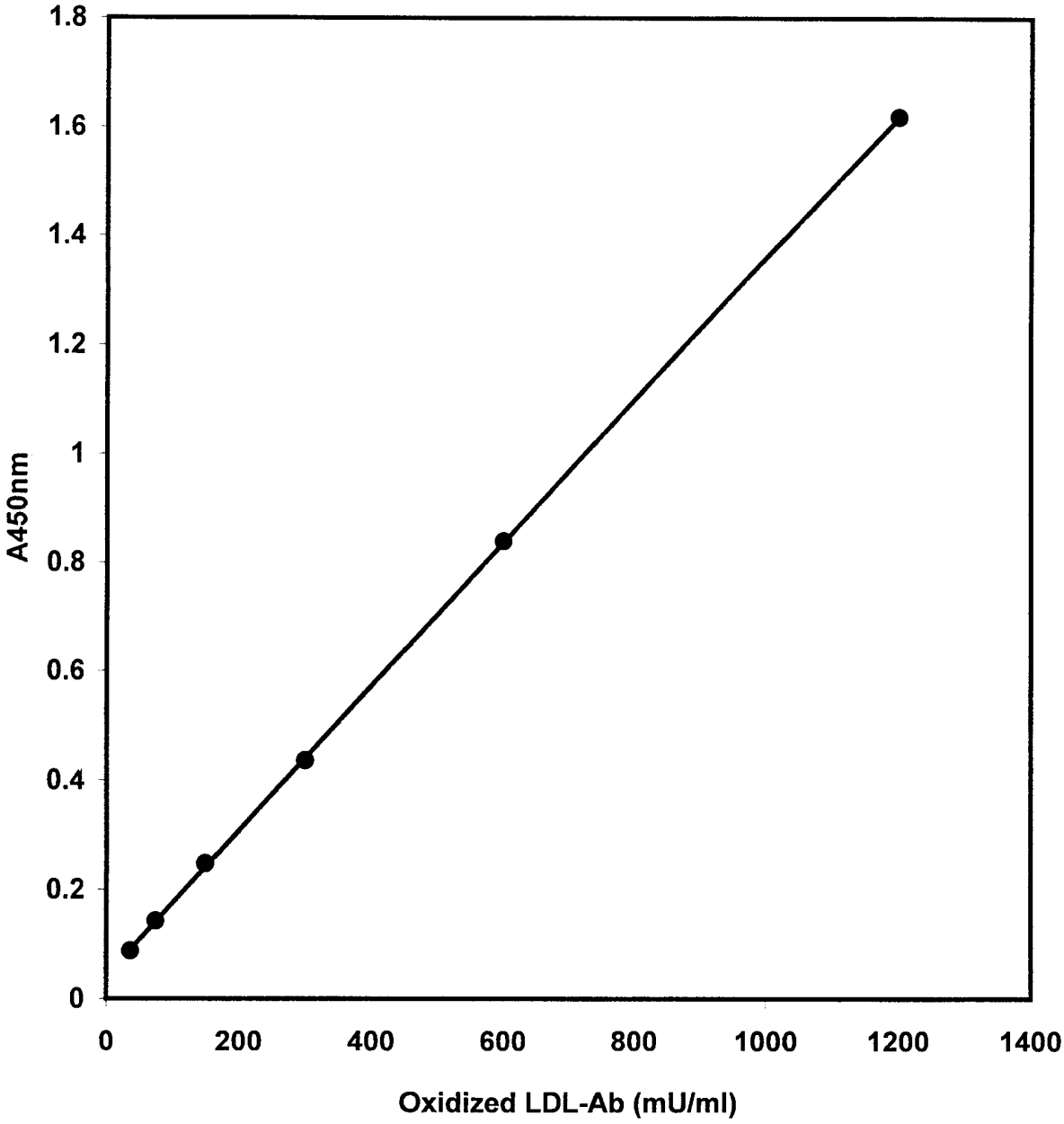

Fig (B) : Oxidized LDL-Ab standard curve

Susceptibility of LDL to Oxidation in Vitro

A- LDL isolation

LDL was selectively precipitated from plasma by addition of precipitating reagent (Randox, UK) [199][200][201].

Principles

Low density lipoproteins are precipitated by heparin at their isoelectric point (pH 5.04).

Reagents

Precipitating Reagent:

Heparin	50, 000 IU/L
Sod. Citrate	0.064 mol/L, pH 5.04

Solublizing solution

0.01 %, triton X100 in 50 g/l NaCl

Procedure

- 100µl plasma sample was mixed with 1 ml precipitating reagent by vortex.
- The mixture was incubated refrigerate for 30 min at 2 – 8 °C, and centrifuged for 15 min at 4000 r.p.m.
- The supernatant was discarded and the precipitate was washed with precipitating reagent.
- The washed precipitate was redissolved in 200 µl of solublizing solution at 37°C and vortexed (resuspended LDL sample) .

B- Determination of total protein content of the resuspended LDL Sample

The method of Lowry et al. [202] was used for the determination of the total protein in the samples

Principle

The colour produced is though to be due to a complex between the alkaline copper-phenol reagent, tyrosine and tryptophan residues of the protein sample.

Reagents

2 % anhydrous sodium carbonate in 0.1 N NaOH.

2 % K / Na tartrate.

1 % copper sulphate.

Lowry C reagent, prepare immediately before use by mixing sodium carbonate, K / Na tartrate and copper sulphate in a ratio of 100:1:1.

Folin-Ciocalteau reagent (sigma chemical co., UK). The working reagent was prepared by diluting the stock reagent 1:1 with distilled water.

Procedure

- 10 µl of the sample was mixed with 2.5 ml of lowry C reagent, and incubated for 10 minutes at room temperature.
- 0.25 ml of working Folin-Ciocallteau reagent was added, mixed and incubated in the dark for 1 hour at room temperature, then the

absorbance was read at 695 nm against blank (distilled water was added instead of the sample).

- The protein concentration in each sample was estimated by referring to a standard curve (Fig.C) which was constructed using bovine serum albumin fraction (v) (Sigma Chemical Co., UK)

C- Oxidation of LDL

Resuspended LDL sample (0.1 mg protein /ml) was incubated with 50 µl of 100 µM Cu^{++} (freshly prepared in phosphate buffered saline solution, pH 7.9) at 37°C for 3 hours.

At the end of incubation period, the lipid peroxidation was stopped by cooling and addition of 30 µl of 1 mM EDTA.

D- Measurement of MDA-LDL levels

The oxidation state of the LDL sample was analyzed after the incubation period by using the thiobarbituric acid reactive substances (TBARS) assay, which is used to measure malondialdehyde equivalents[203].

Results were expressed as nmol MDA / mg LDL protein by dividing the concentration of MDA in the sample by its protein concentration.

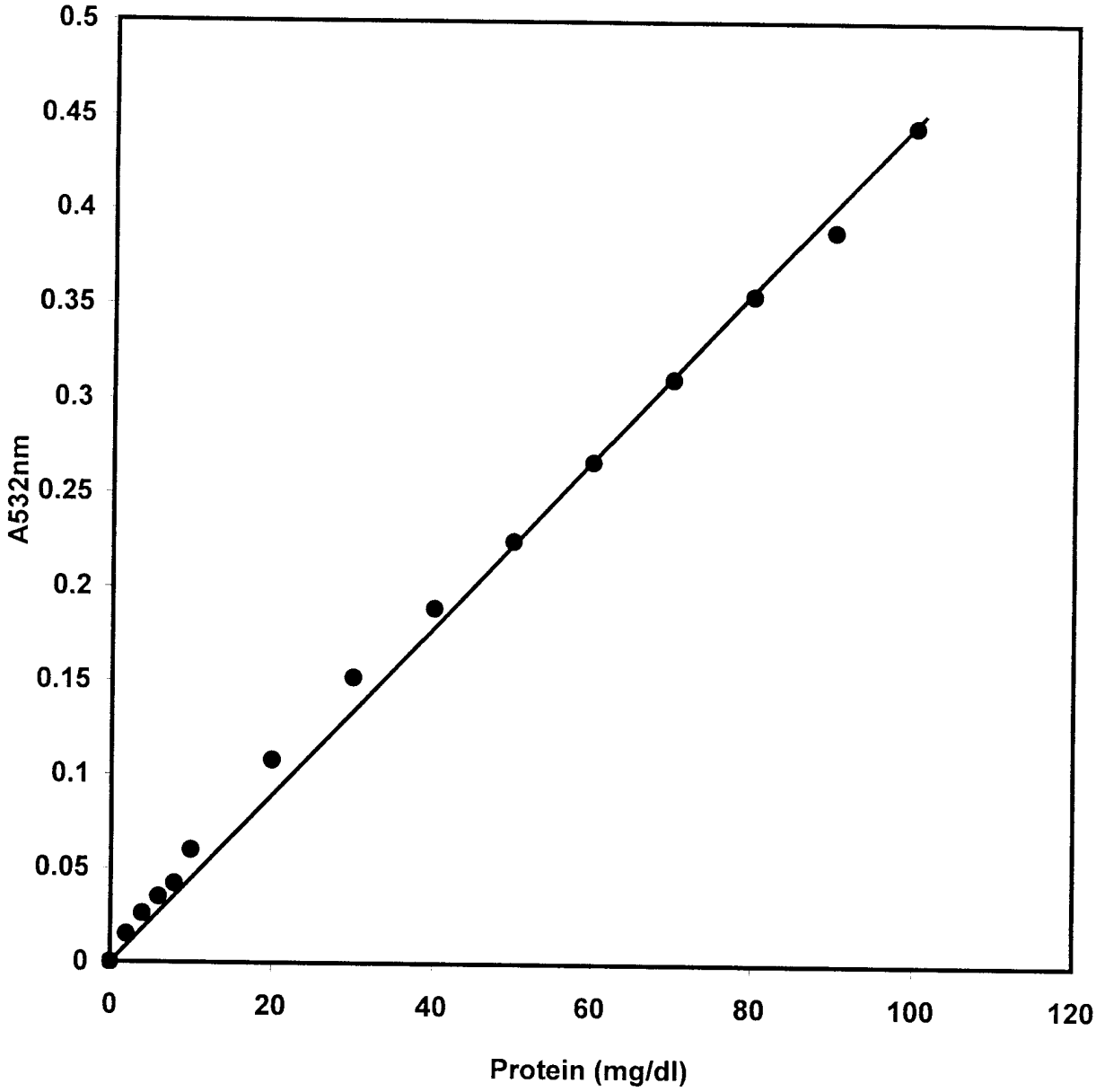

Fig (C) : Standard curve for protein

Determination of Thiobarbituric Acid Reactive Substances

Principle

Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS). In this assay one molecule of malondialdehyde (MDA), the most abundant aldehyde product of lipid peroxidation, reacts with two molecules of thiobarbituric acid (TBA) in acid medium (pH dependent) to yield a pink complex. The resulting chromogen is extracted with n-butyl alcohol and absorbance of organic phase is measured at 532 nm [(203)].

Reagents

- Sodium dodecyl sulfate (SDS), 8.1 %.
- Acetic acid, 20 % (PH was adjusted with IN NaOH to 3.5).
- Thiobarbituric acid (TBA), 0.8 %
- N-butyl alcohol
- 1,1,3,3, tetramethoxy propane (TMP), was used to prepare a standard solution of MDA

Procedure

The following volumes were pipetted into a labeled tube for each sample: 0.1 ml of sample, 0.1 ml of SDS solution, 0.75 ml 20% acetic acid, 0.75 ml TBA solution, and 0.3 ml distalled water.

Each tube was then vortexed and incubated in a boiling water bath for one hour.

After cooling to room temperature, 0.5 ml of distalled water, and 2.5 ml of n-butanol were added to each tube, and vigorously mixed with a vortex for 2 min.

The organic layer was separated by centrifugation at 4000 rpm for 10 min.

Absorbance of the organic layer was read at 532 nm against a blank prepared and treated as the sample but containing PBS instead of the sample.

Calculation

Values of MDA were obtained from the prepared MDA standard curve and expressed in term of nmol/ml.

A standard curve was constructed by preparing serial dilutions of tetramethoxy prepare (TMD) (Aldrich Chemical Co. USA) in ethanol and treating them as the sample, their absorbance values were plotted against corresponding concentrations on a linear paper (Fig D).

Total Antioxidant Status Determination

Total antioxidant status was measured in serum with commercially available kit (Randox, UK)

Principle

Incubation of ABTS (2,2–Azino–di–[3–ethylbenzthiazoline sulphate]) with a peroxidase (metmyoglobin) and hydrogen peroxide to

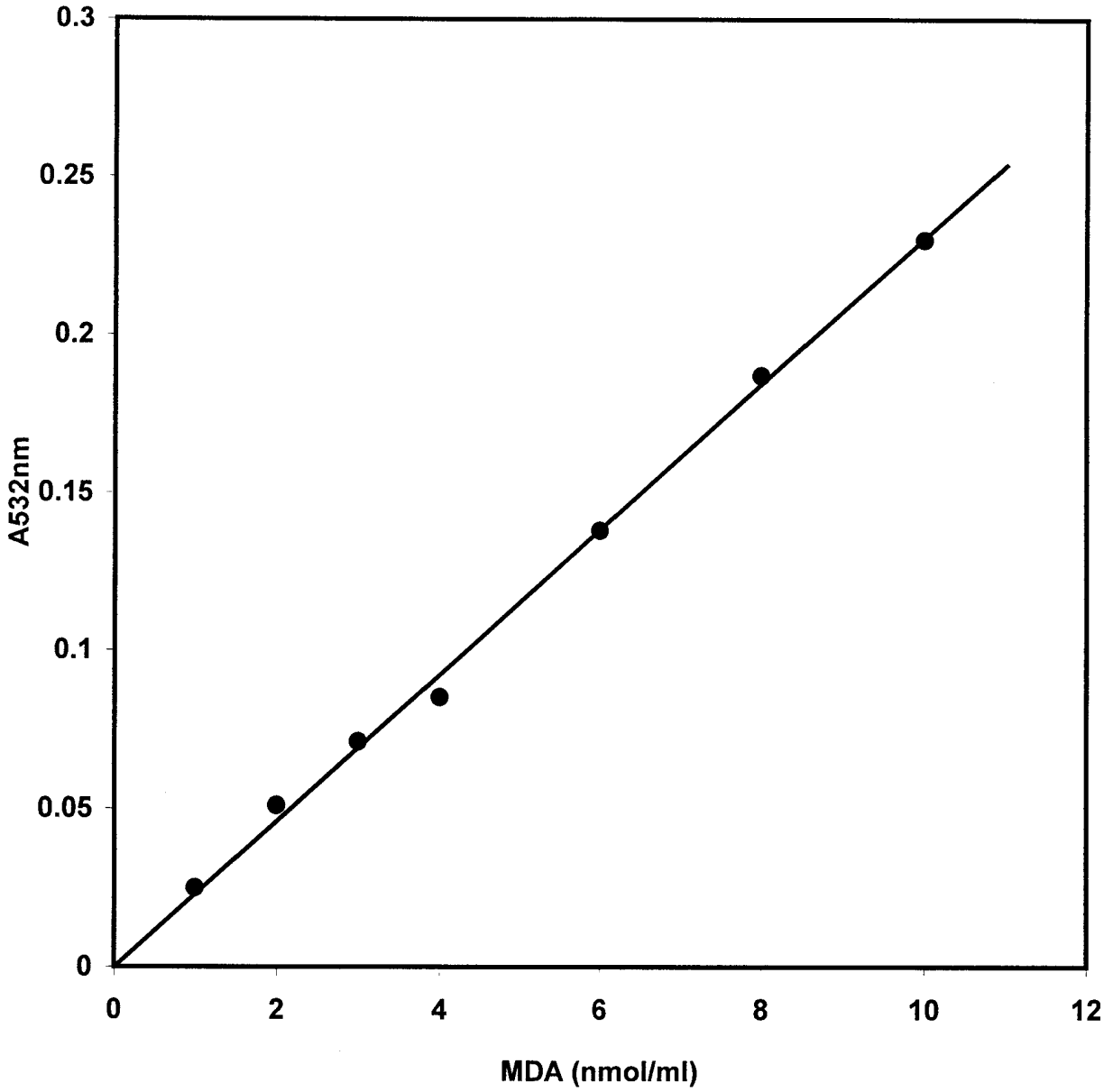

Fig (D) : MDA standard curve

produce the radical cation $ABTS^{\bullet+}$. This has relatively stable blue-green colour, which is measured at 600 nm. Antioxidants in the added sample cause inhibition of this colour production to a degree which is proportional to their concentration[204].

Reagents

Buffer

Phosphate Buffered Saline	80 mmol/l, pH 7.4

Chromogen

Metmyoglobin	6.1 µmol/l
ABTS	610 µmol/l

Substrate

Hydrogen peroxide	250 µmol/l

Standard

6–hydroxyl–2, 5, 7, 8–tetra methylchromon-2–carboxylic acid	1.7 mmol/l

Preparation of reagent:

One vial of chromogen was reconstituted with 10 ml of buffer.

1 ml of substrate was diluted with 1.5 ml buffer.

One vial of standard was reconstituted with 1 ml of deionized water.

Procedure

- 1 ml of chromogen was mixed with 20 µl of serum sample, standard, or deionized water (as blank)

- The mixture was incubated at 37°C for 5 minutes.
- Initial reading (A1) was taken at 600 nm.
- 200 µl of substrate was added to each cuvette, mixed, and then incubated at 37°C.
- Second reading (A2) was taken after exactly 3 minutes.
- △A of sample, standard, or blank = A2 – A1

Calculation

$$\text{Factor} = \frac{\text{Concentration of standard}}{(\Delta A \text{ blank} - \Delta A \text{ standard})}$$

Total antioxidant status [mmol/l] = factor x (△A blank-△A sample)

Assay of glutathione and glutathione disulfide

The enzymatic method described by Griffith [205] was used to measure the total plasma GSH and GSSG content.

DTNB-GSSG reductase recycling assay for total glutathione (tGSH)

Principle

DTNB – GSSG reductase recycling assay for total glutathione (GSH) is a sensitive and specific enzymatic method which depends on the oxidation of GSH by 5,5'–dithiobis–(2-nitro benzoic acid) (DTNB) to yield GSSH and 5-thio-nitrobenzoic acid (TNB). Oxidized GSSG is reduced enzymatically by the action of glutathione reductase and NADPH to regenerate GSH, which reacts again. The rate of TNB formation is

monitored at 412 nm and is proportional to the sum of GSH and GSSG present in the sample.

Procedure

0.1of 6.0 mM DTNB, 0.7 ml of 0.3 mM NADPH, 0.18 ml of distilled water and 10 µl of test sample or standards, were mixed and incubated for 15 minutes at 30°c. The reaction was initiated by the addition of 10 µl of 50 U/ml glutathione reductase. The rate of formation of TNB was monitored by recording the change in the absorbance at 412 nm per minute (ΔA / min).

The total glutathione content in the samples was determined from a GSH standard curve (Fig. E).

DTNB-GSSG reductase recycling assay for oxidized glutathione (GSSG)

The GSSG content is determined by the same assay as total glutathione, but where the reduced glutathione is bound by 2-vinylpyridine.[205]

Procedure:

- Two µl of 2-vinylpyridine was added to 100µl of the sample, with mixing. Six µl of 50 %(v/v) triethanolamine was added to the side of the tube and the solution was vigoursly mixed. The final pH should be 7 to 7.5 .

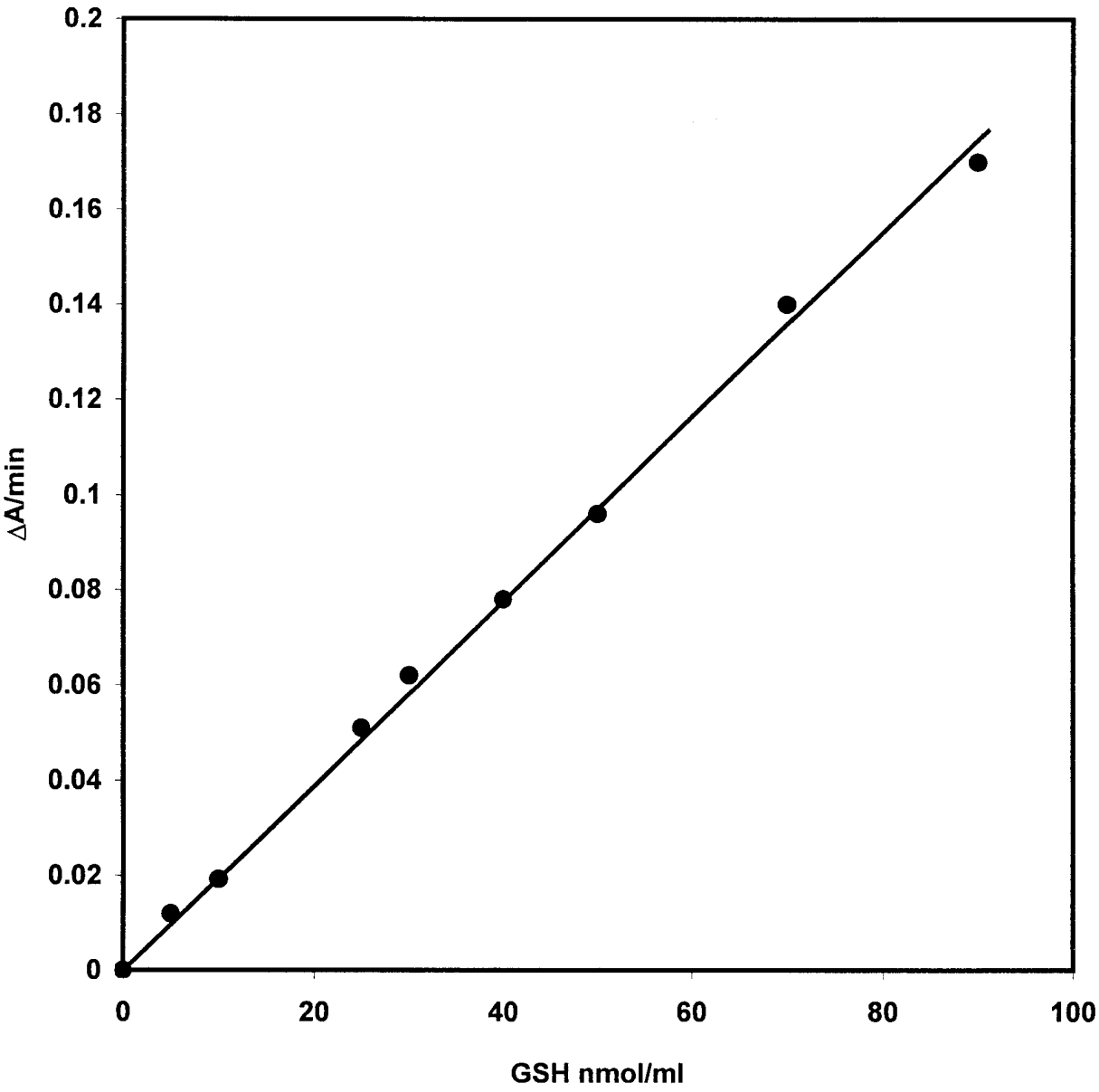

Fig (E) : Standard curve of GSH

- The mixture was allowed to stand for 1 hour at room temperature to derivatize GSH. After incubation the mixture was assayed as in the total glutathione procedure.
- The GSSG content in the samples were determined from a standard curve, (Figure F). Results were subsequently expressed as nmol GSSG/mg protein by dividing the concentration of GSSG in the sample by the protein concentration in the same sample.

Statistical Analysis

For the analysis of the obtained result, the following parameters were calculated:

1- Arithmatic mean (X)[206]

Used as a measured of control tendency.

It was calculated from the formula:

$$\bar{X} = \frac{\Sigma(X)}{n}$$

Where:

$\bar{X}$ = Arithmatic mean

$\Sigma(X)$ = Sum of values recorded

n = Number of observations

2- Standard deviation (SD)[206]

Used as a measured of dispersion

It was calculated as follows:

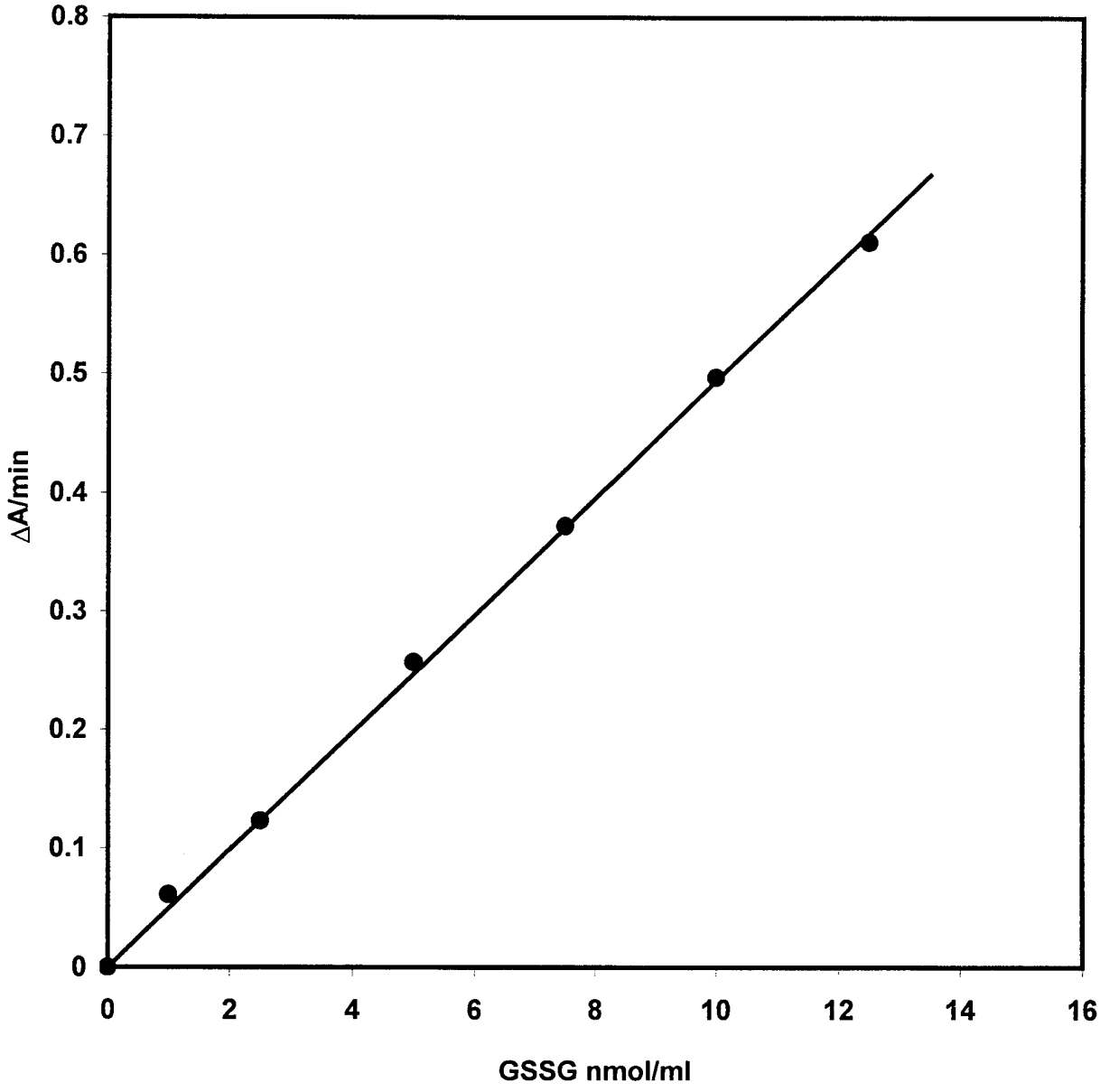

Fig (F) : Standard curve of GSSG

$$SD = + \sqrt{\frac{(\Sigma X^2) - \frac{(\Sigma X)^2}{n}}{n-1}}$$

3- Standard Error (SE)[206]

Used as a measured of precision and statistical reliability of the mean. It was calculated as follows: $SE = \pm \frac{SD}{\sqrt{n}}$

4- One–way ANOVA[207]

An ANOVA tests measures the difference between the means of two or more groups.

5- Paired t test[208]

- This test is concerned with the difference in pair of related observations. It based on the difference in each pair of observations, instead of on the value of the individual observations.
- The test was used to assess the statistical significance of the mean differences between the group of the patients before and after treatment.
- The *t* values were obtained by the application of the following formula.

$$t = \frac{\bar{d}}{SD/\sqrt{n}}$$

Where, $\bar{d}$ is the mean difference between the paired observations.

SD is the standard deviation of the difference between the paired observations

n is the number of paire observations.

6- The Correlation Coefficient (r)[206]

- It is used for measuring the degree of linear relationship between the X and Y variables in the same group.
- The linear correlation coefficient (r) for a collection of n pairs of data is:

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{n(\Sigma x^2) - (\Sigma x)^2}\sqrt{n(\Sigma x^2) - (\Sigma x)^2}}$$

Then to test whether the (r) is significantly at t value is calculated according to this equation:

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$$

- Then the calculated t is compared to the critical value of the t distribution with (n- 2) degrees of freedom at a given level of significance.

RESULTS

RESULTS

Glycemic control

The results of the control of glycemia in the different groups of patients are summarized in Table (R.1). As expected diabetic patients showed higher levels of fasting plasma glucose than the non-diabetic control subjects (Figure 1). At the start of the study the diabetic patients with CAD has slightly higher FPG (10.4%) than those without CAD. Patients compliance for the treatment throughout the study period was reasonably good as could be judged by the levels of glycosylated hemoglobin. Although this parameter was much higher above the control value, it was still below the clinically recommended upper limit of 8 % (Figure 2). Addition of antioxidants to the treatment regimen slightly improved the control of glycemia in diabetics with CAD. This could be seen in the tendency of both FPG and HbA_{1c} to decline during the 3 months follow up period.

Table (R1): Parameters of glycemic control in type 2 diabetic patients without and with coronary artery disease and the effect of three months adjunct treatment with antioxidants[@]

	Non-diabetic control	Type 2 diabetics without CAD	Type 2 diabetics with CAD		
			Time after start of antioxidant treatment		
			0	1 month	3 months
FPG (mg/dl)	88.3 ± 6.63	190.9 ± 50.60[a] (+ 116.2 %)	210.7±48.36[a] (+138.6 %)	204.9±45.75[a] (+132.0 %)	199.9±44.24[a,c] (+126.4 %)
HbA_{1c} (%)	4.73 ± 0.43	7.57 ± 0.85[a] (+ 60.0 %)	7.94±0.80[a] (+67.9 %)	7.89±0.78[a] (+66.8 %)	7.33±0.74[a,c] (+ 55.0 %)

[@] Data presented as mean ± SD

a Significantly different from control group by ANOVA test .

b Significantly different from group II by ANOVA test .

c Significantly different from baseline of group III by paired t-test.

Number between brackets represents the percent of change from control.

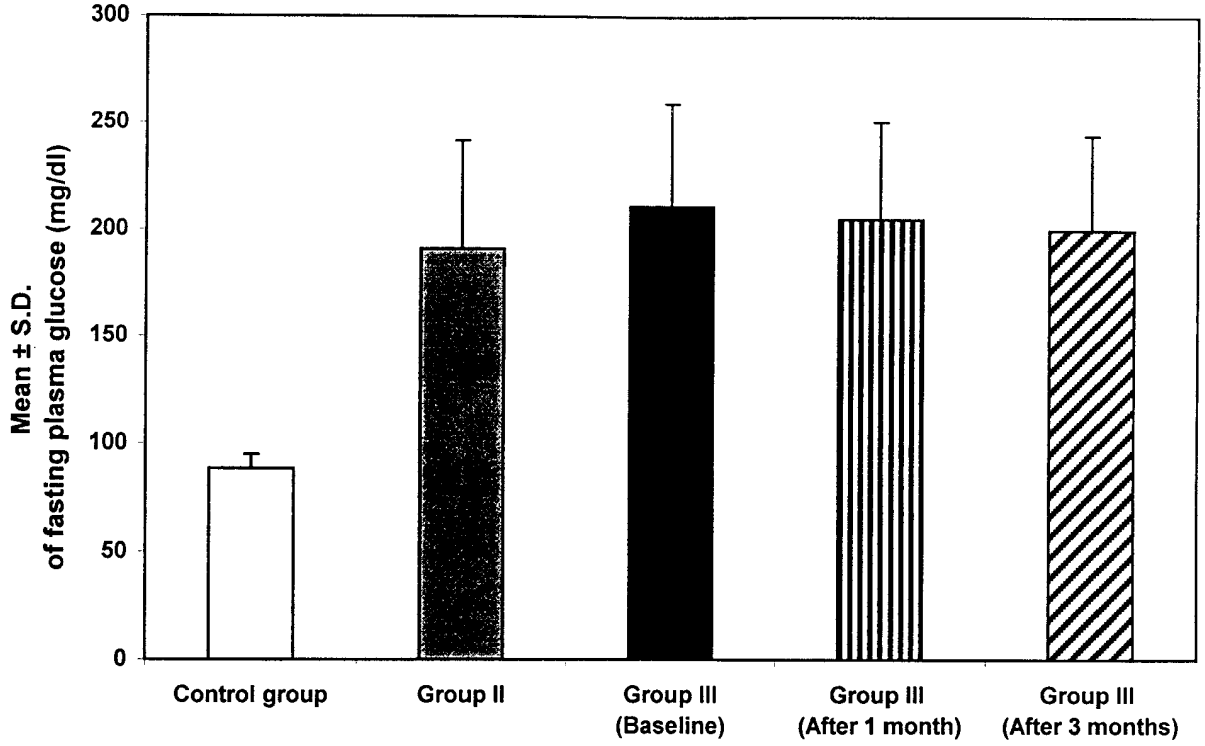

Figure (1) : Mean of fasting plasma glucose (mg/dl) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

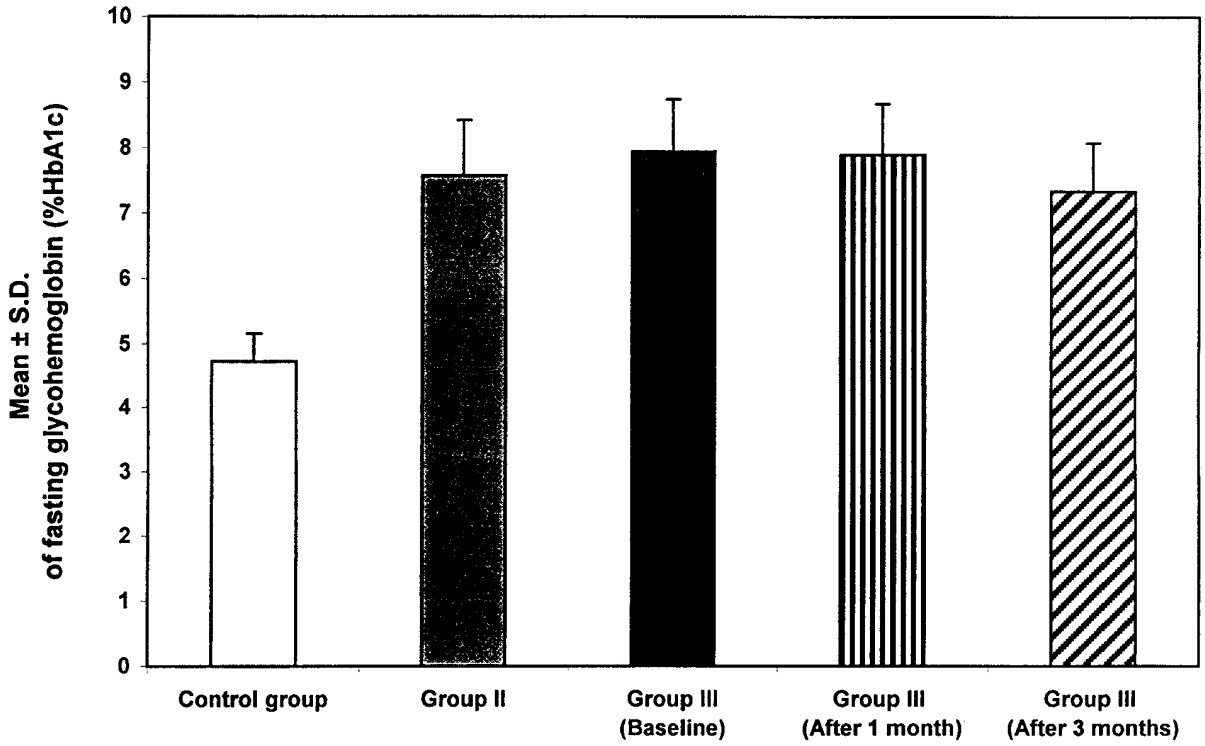

Figure (2) : Mean of glycohemoglobin (%HbA_{1c}) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

Oxidative stress

Diabetes mellitus is a disease known to be associated with oxidative stress, which entails increased production of TBARS, representing lipid peroxidation end products, and disturbance in the balance between reduced and oxidized glutathione. Increased production of TBARS as a reflection of oxidative stress was clear in diabetic patients (Table R-2). It could also be seen that patients with CAD suffered as stronger state of oxidative stress than those without CAD (Figure R3). In patients suffering from type 2 diabetes, but without CAD, the levels of TBARS averaged 43.6% over control, while, such increase was 61.8% in those suffering from CAD. Treatment with antioxidants was effective in alleviating the stressful condition. Even in the short follow up period of 3 months, the level of TBARS slowly but steadily declined to shift toward the normal control. Plasma concentration of TBARS in patients suffering from CAD averaged 51.3% above control after one month and only 25.5% after 3 months. By the end of the follow-up period, the average TBARS level in diabetics with CAD actually declined to be 22.4% below the base line level before treatment with antioxidant and was 12.6% below the average level in patients without CAD (Group II) .

Diabetes was associated with disturbance in glutathione metabolism (Table R-2). Total glutathione levels were much lower in diabetics than in control subjects, especially in patients with CAD (Figure 4). At the beginning of the study the total glutathione level in type 2 diabetic patients

averaged 39.0% below controls. The decrease was more pronounced in diabetics with CAD, as it reached 54.6% below the mean value for control subjects. Antioxidants adjuvant therapy quickly improved this situation, as the mean in diabetic patients with CAD increased by 42.2% above base line value after only one month and by 82.8% after 3 months of adjunct antioxidant therapy. The same pattern was also observed with the reduced form of glutathione. Type 2 diabetic patients had much lower GSH levels than non-diabetic controls (Figure 5). At the start of the experimental period, diabetics without and with CAD had mean serum levels of GSH 60.3% and 80.5% lower than controls respectively. As was seen with total glutathione, antioxidant adjunct therapy had a beneficial effect on GSH level with a steady increase in the short follow up period. After one month of treatment the mean serum GSH in diabetics with CAD practically doubled and after 3 months it was about 3.5 fold the base line value. However, the serum GSH level was still 33.9% lower than the mean control concentration at the end of the follow up period.

The concentration of the oxidized form of glutathione (GSSG) in diabetics without CAD was about 3-fold the control value (Table R-2 and Figure 6). The level was even higher in patients with CAD (3.25 fold). After one month of antioxidant adjuvant therapy the mean GSSG serum concentration did not change despite the observed increase in total glutathione and the level actually decreased by the end of the third month of follow-up by 22.5% when compared to base line value.

Table (R2): Oxidative stress Parameters in type 2 diabetic patients without and with coronary artery disease and the effect of three months adjunct treatment with antioxidants[@]

	Non-diabetic control	Type 2 diabetics without CAD	Type 2 diabetics with CAD		
			Time after start of antioxidant treatment		
			0	1 month	3 months
TBARS (nmol/ml)	3.14±0.39	4.51±0.41 [a] (+43.6%)	5.08±0.55 [a,b] (+61.8%)	4.75±0.54 [a,c] (+51.3%)	3.94±0.49 [a,c] (+25.5%)
tGSH (nmol/ml)	2.82±0.51	1.72±0.34 [a] (-39.0%)	1.28±0.07 [a,b] (-54.6%)	1.82±0.31 [a,c] (-35.5%)	2.34±0.39 [a,c] (-17.0%)
rGSH (nmol/ml)	2.57±0.52	1.02±0.34 [a] (-60.3%)	0.50±0.14 [a,b] (-80.5%)	1.05±0.30 [a,c] (-59.1%)	1.71±0.32 [a,c] (-33.5%)
GSSG (nmol/ml)	0.12±0.02	0.35±0.11 [a] (+191.7%)	0.39±0.07 [a] (+225.0%)	0.39±0.13 [a] (+225.0%)	0.31±0.07 [a,c] (+158.3%)

[@] Data presented as mean ± SD

a Significantly different from control group by ANOVA test .

b Significantly different from group II by ANOVA test .

c Significantly different from baseline of group III by paired t-test.

Number between brackets represents the percent of change from control.

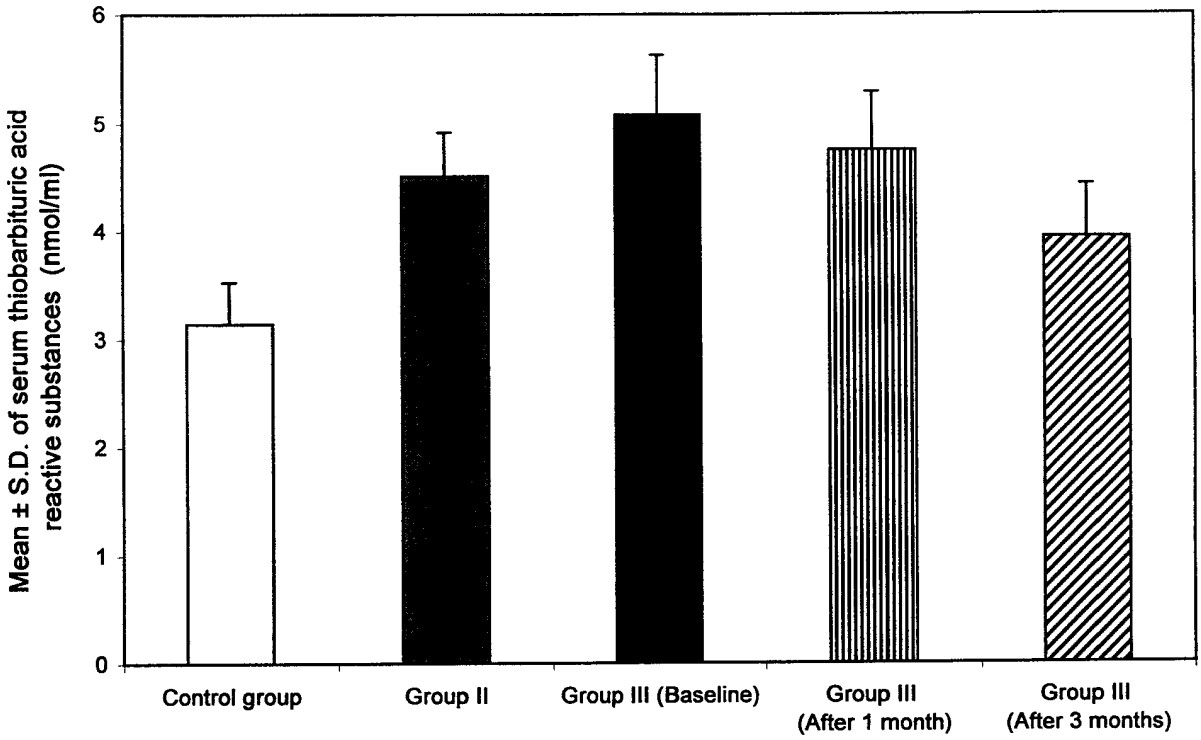

Figure (3) : Mean of serum thiobarbituric acid reactive substances levels (nmol/ml) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

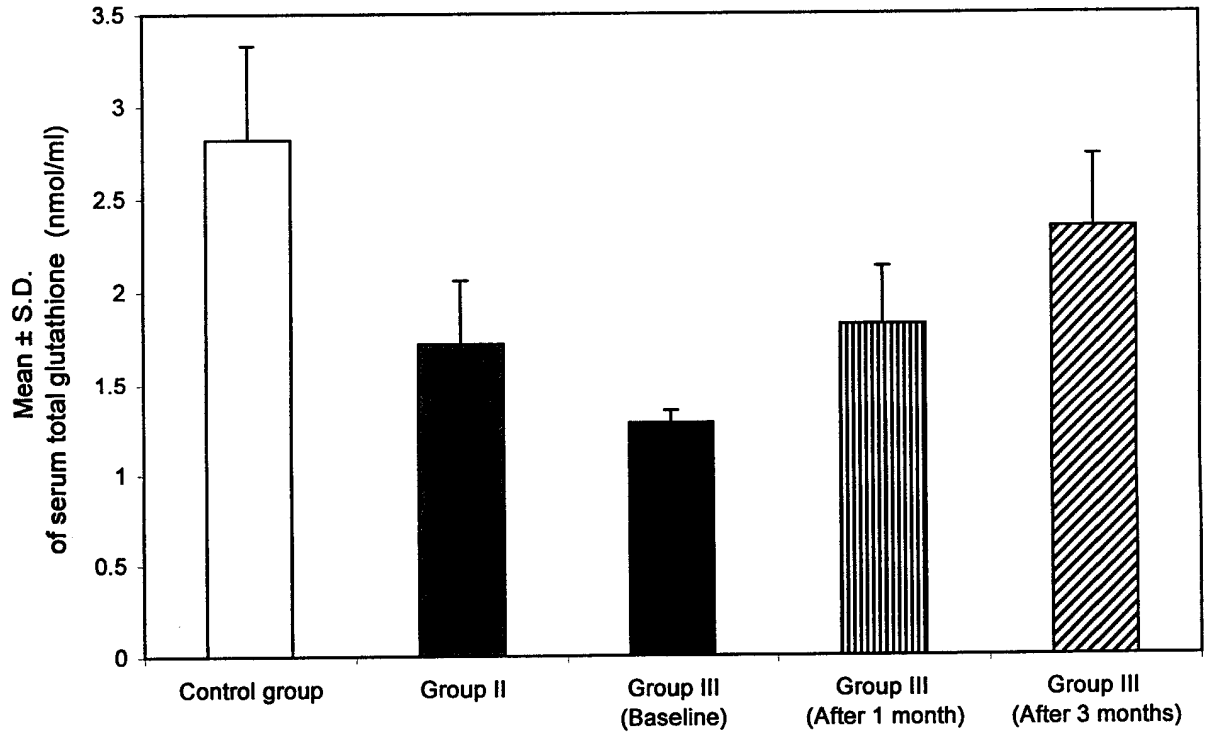

Figure (4) : Mean of serum total glutathione levels (nmol/ml) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

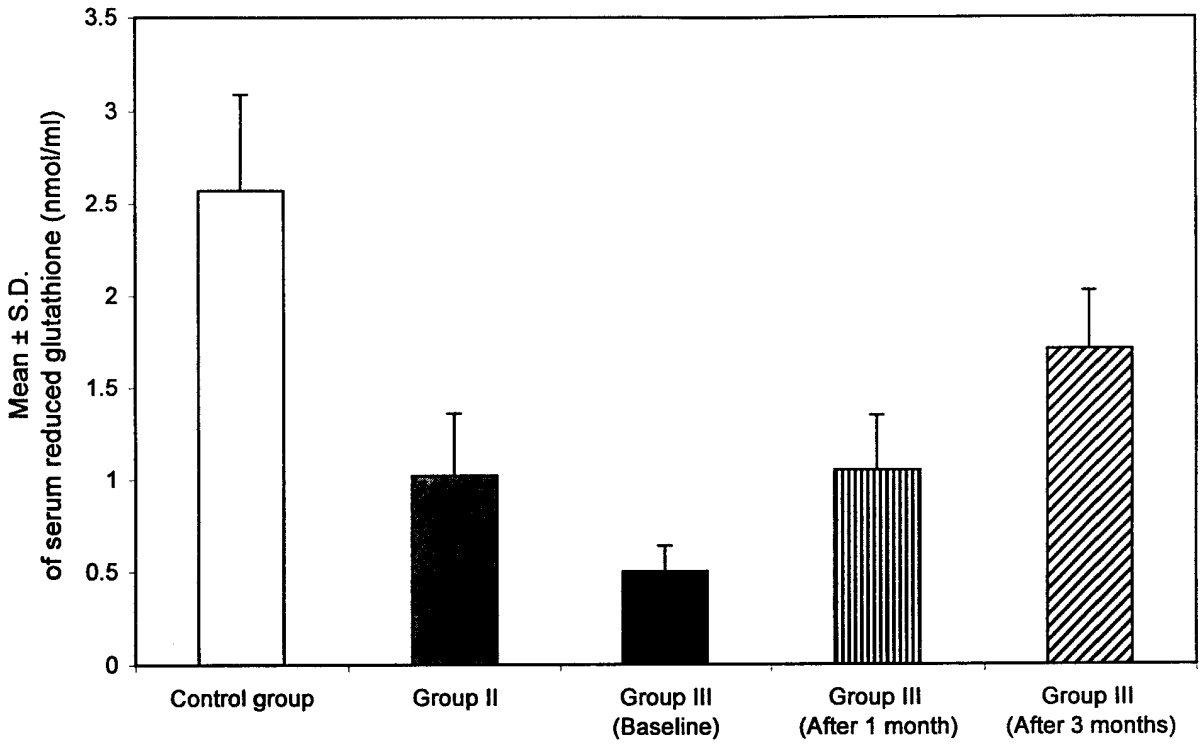

Figure (5) : Mean of serum reduced glutathione levels (nmol/ml) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

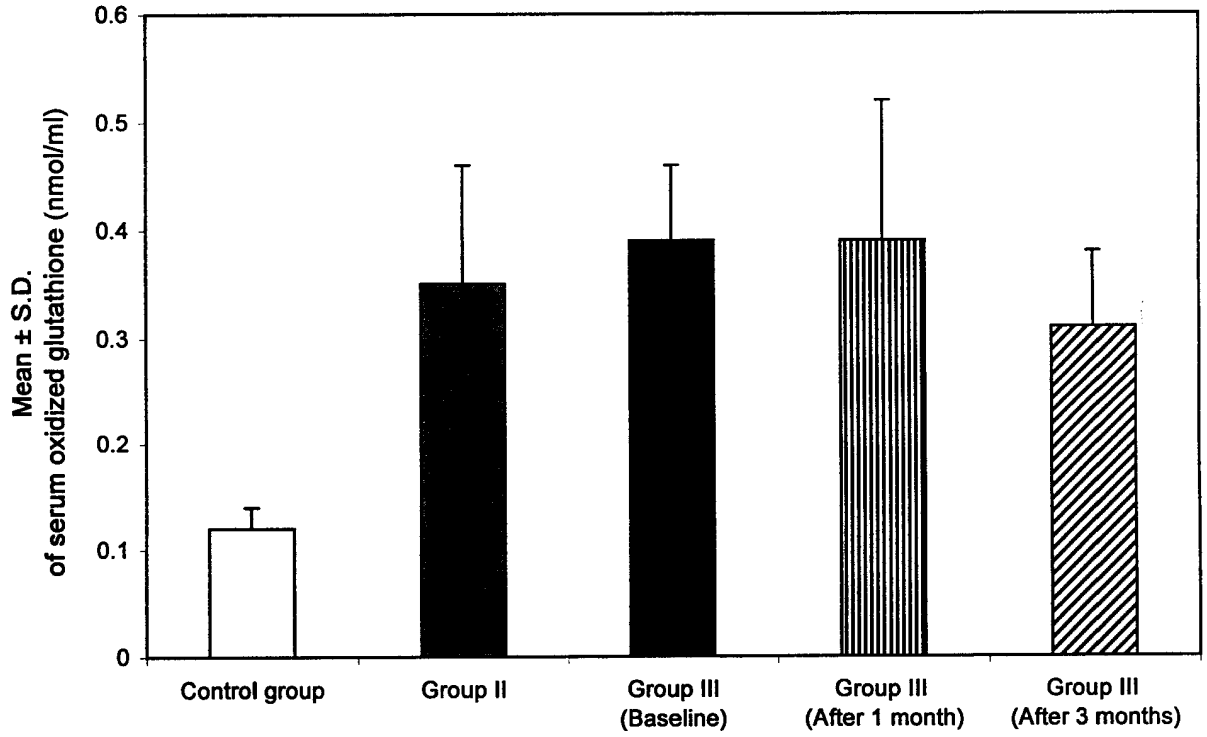

Figure (6) : Mean of serum oxidized glutathione levels (nmol/ml) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

Three parameters were taken to estimate the over all antioxidant capacity of the different groups of subjects who were participated in the present study. Beside the total antioxidant status, which was estimated by practical assay, the ratio of the reduced to the oxidized forms of glutathione (GSH/GSSG ratio) and the redox potential were calculated from the obtained glutathione values. The results of these parameters are presented in Table R-3. All three parameters showed clearly that the antioxidant capacity in the plasma of type 2 diabetic patients was quite low. The assayed total antioxidant status was 23.3% below normal in diabetics without CAD and 27.1% in patients with CAD (Figure 7). With the adjunct antioxidant therapy, the value of the total antioxidant status (TAS) quickly increased to reach levels comparable to that of the non-diabetic control subjects by the end of the 3-month follow-up period (Table R3). The other 2 parameters, which are more directly dependent on the values of the different forms of glutathione, did not show the same dramatic improvement. The calculated redox potential in the plasma of diabetic patients clearly indicated a more oxidative environment than the control non-diabetic subjects (Figure 8). The mean value for the redox potential in the plasma of type 2 diabetics without CAD was 27.5% below control. The plasma of the diabetic patients with CAD represented an even less reductive state, as the redox potential was 41.3% less than control. Following adjunct treatment with antioxidants the redox environment in the plasma improved by 19.0% in the first month and by 41.9% at the end of the third month, to reach a level 16.7% below mean control level. The

ratio of the reduced glutathione to the oxidized form was also decreased in the plasma from diabetic patients (Figure R.9). Such ratio averaged 3.31 in diabetics without CAD, which was much smaller than the ratio in the plasma of non-diabetic controls, which was 21.75. The ratio was even smaller in patients with CAD as it avenged only 1.39. This means that it was more than 15 times smaller than the control. As could be expected, adjunct antioxidants therapy greatly improved the GSH/GSSG ratio, which doubled after the first month and quadrupled after 3 months. However, it was still smaller than the ratio in control plasma. It was about only one fourth of the control ratio. This parameter was the least affected of the parameters used to assess the antioxidant capacity.

Table (R3): Overall assessment of antioxidant capacity in type 2 diabetic patients without and with coronary artery disease and the effect of three months adjunct treatment with antioxidants[@]

	Non-diabetic control	Type 2 diabetic without CAD	Type 2 diabetic with CAD		
			Time after start of antioxidant treatment		
			0	1 month	3 months
TAS (mmol/l)	1.33±0.17	1.02±0.11 [a] (-23.3%)	0.97±0.10 [a] (-27.1%)	1.12±0.11 [a,c] (-15.8%)	1.30±0.10 [c] (-2.3%)
Redox potential (mV)	-138±6.2	-100±12.2 [a] (-27.5%)	-81±8.8 [a,b] (-41.3%)	-100±9.1 [a,c] (-27.5%)	-115±4.5 [a,c] (-16.7%)
GSH/GSSG	21.75±6.45	3.31±1.78 [a]	1.39±0.72 [a]	3.07±1.61 [a,c]	5.68±1.29 [a,c]

[@] Data presented as mean ± SD

a Significantly different from control group by ANOVA test .

b Significantly different from group II by ANOVA test .

c Significantly different from baseline of group III by paired t-test.

Number between bracts represents the percent of change from control.

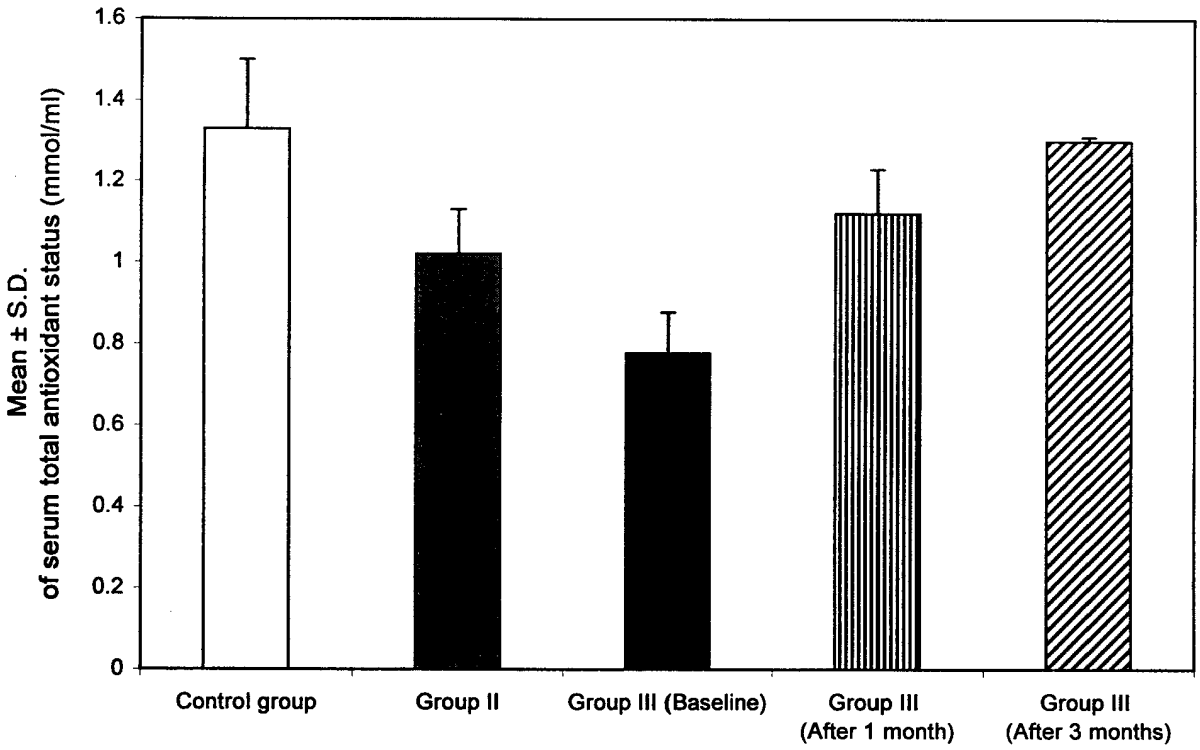

Figure (7) : Mean of serum total antioxidant status levels (mmol/ml) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

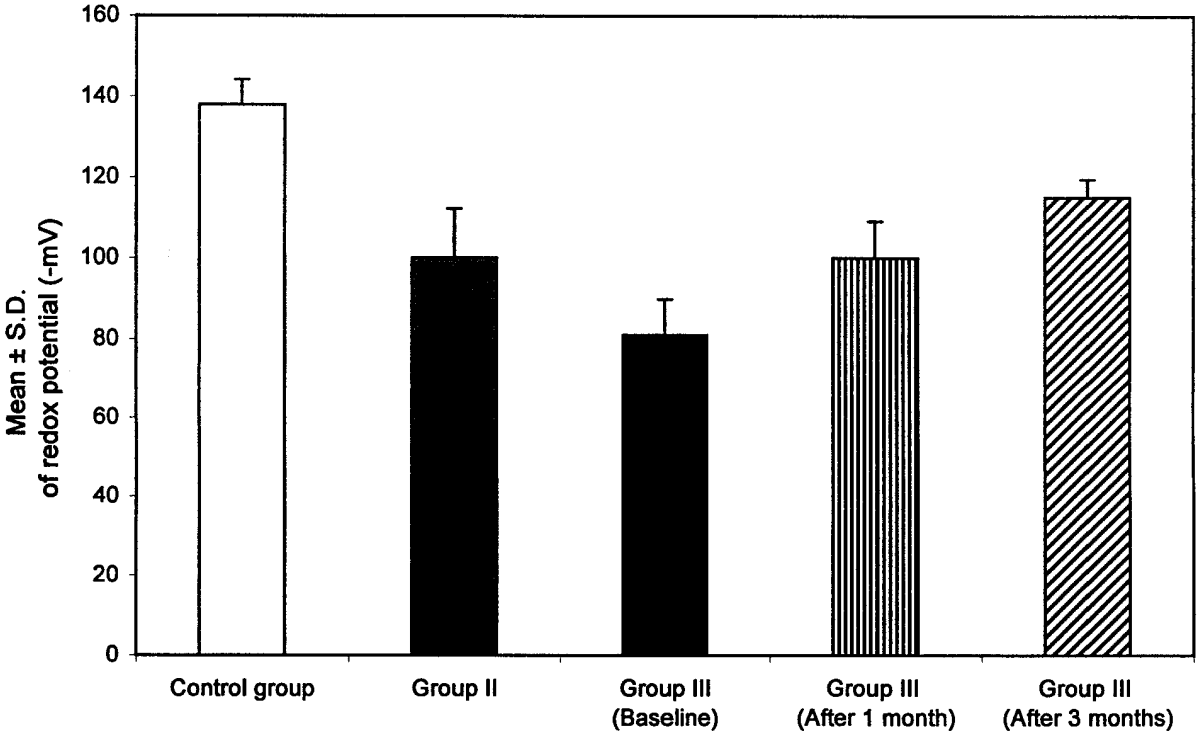

Figure (8) : Mean of redox potential (-mV) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

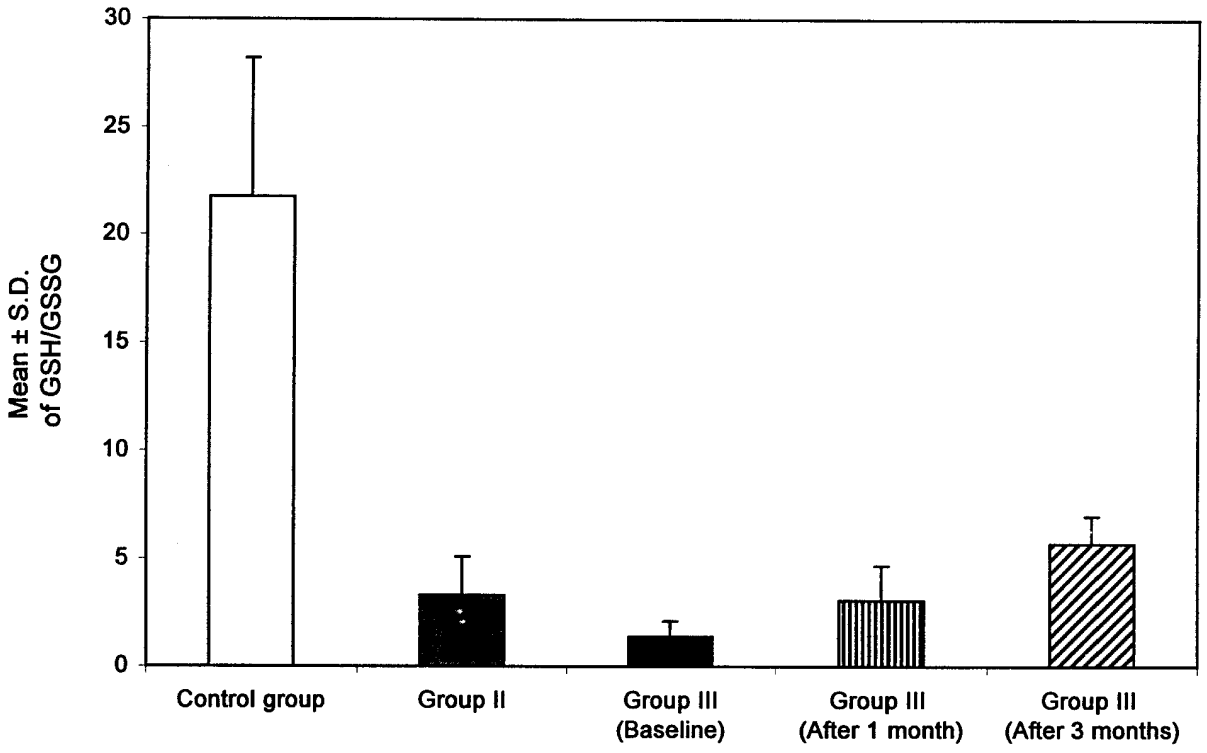

Figure (9) : Mean of GSH/GSSG in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

Lipid pattern

Data on the plasma lipids of the subjects participating in the present study are presented in Table (R.4). The lipid pattern of type 2 diabetics showed deviation from non-diabetic controls in all lipid fractions assessed. Triglycerides showed definite increase (Figure 10). Patients without CAD had a 67.3% increase over controls, while patients suffering from CAD showed even higher levels at the beginning of the study averaging 77.3% above control values. Treatment with adjunct antioxidants did not have any effect on the concentration of triglycerides, as there was practically no change in the average values for these subjects after one month or 3 months of follow-up.

The concentrations of total cholesterol in diabetic patients were, in general, somewhat higher than in non-diabetic controls (Figure 11). There was, however, no detectable significant difference between the diabetic groups with or without CAD. Treating type 2 diabetic patients with antioxidants for 3 months had no effect on total cholesterol values in these patients. It should be noted that the average levels of total cholesterol were all within the accepted clinical normal values, as they were below 200 mg/dl.

The percentage differences in total cholesterol among the different groups studied were almost matched by the differences in LDL cholesterol. Statistically there was no significant difference among the means and adjunct treatment with antioxidants for 3 months did not produce any

Change in the mean values for the type 2 diabetic group with CAD (Figure 12). This was, however, accompanied by a small decrease in HDL-cholesterol below the value for the non-diabetic group (Figure 13). As with all of the cholesterol fractions, antioxidant adjunct treatment for 3 months was without statistically significant effect on the mean level of HDL-cholesterol.

It should be noted, however, that despite the absence of significant alterations in both LDL- and HDL-cholesterol fractions, both ratios of LDL to HDL and total cholesterol to HDL showed some interesting changes. The LDL/HDL ratios of type 2 diabetic patients with or without CAD were about 1.4 fold that of non-diabetic control subjects. The ratio of diabetics with CAD slowly declined after 1 months and 3 months of antioxidant therapy to reach a value (2.57±0.63) statistically different from base-line ratio of these patients (Figure 14).

The Total cholesterol to HDL ratio showed the same pattern with the same relations among the different groups (Figure 15). The total cholesterol (TC) to HDL ratio of type 2 diabetics with or without CAD was approximately 1.4 fold than of non-adiabatic controls. Again, following treatment with antioxidant adjunct therapy, the ratio slowly declined below the baseline level value with a tendency for a shift towards non-diabetic control value, despite a still existing definite obvious difference.

Table (R4): Lipid pattern in the plasma of type 2 diabetic patients without and with coronary artery disease and the effect of three months adjunct treatment with antioxidants[@]

	Non-diabetic control	Type 2 diabetics without CAD	Type 2 diabetics with CAD		
			Time after start of antioxidant treatment		
			0	1 month	3 months
Triglycerides (mg/dl)	126.33±33.9	211.6±46.5 [a] (+67.3%)	224.3±39.8 [a] (+77.1%)	222.9±44.9 [a] (+76.2%)	218.6±39.9 [a] (+72.8%)
Cholesterol (mg/dl)	176.4±18.2	191.7±17.4 [a] (+8.7%)	194.5±19.0 [a] (+10.3%)	193.5±15.0 [a] (+9.7%)	191.5±15.9 [a] (+8.6%)
LDL-C (mg/dl)	99.8±16.8	107.8±12.3 (+8.0%)	111.1±20.3 (+11.3%)	107.8±17.6 (+8.0%)	107.0±17.7 (+7.2%)
HDL-C (mg/dl)	51.3±5.71	39.6±5.11 [a] (-22.8%)	39.3±4.22 [a] (-23.4%)	41.2±5.01 [a] (-19.7%)	41.6±4.28 [a] (-18.9%)
LDL/HDL	1.98±0.44	2.77±0.52 [a]	2.83±0.26 [a]	2.68±0.64 [a]	2.57±0.63 [a,c]
TC/HDL	3.48±0.48	4.91±0.76 [a]	4.94±0.66 [a]	4.78±0.78 [a]	4.65±0.65 [a,c]
Apo-B (mg/dl)	94.5±18.5	120.7±32.6 [a] (+27.7%)	126.6±34.7 [a] (+34.0%)	122.0±35.3 [a] (+29.1%)	121.9±28.7 [a] (+29.0%)

[@] Data presented as mean ± SD

a Significantly different from control group by ANOVA test .

b Significantly different from group II by ANOVA test .

c Significantly different from baseline of group III by paired t-test.

Number between bracts represents the percent of change from control.

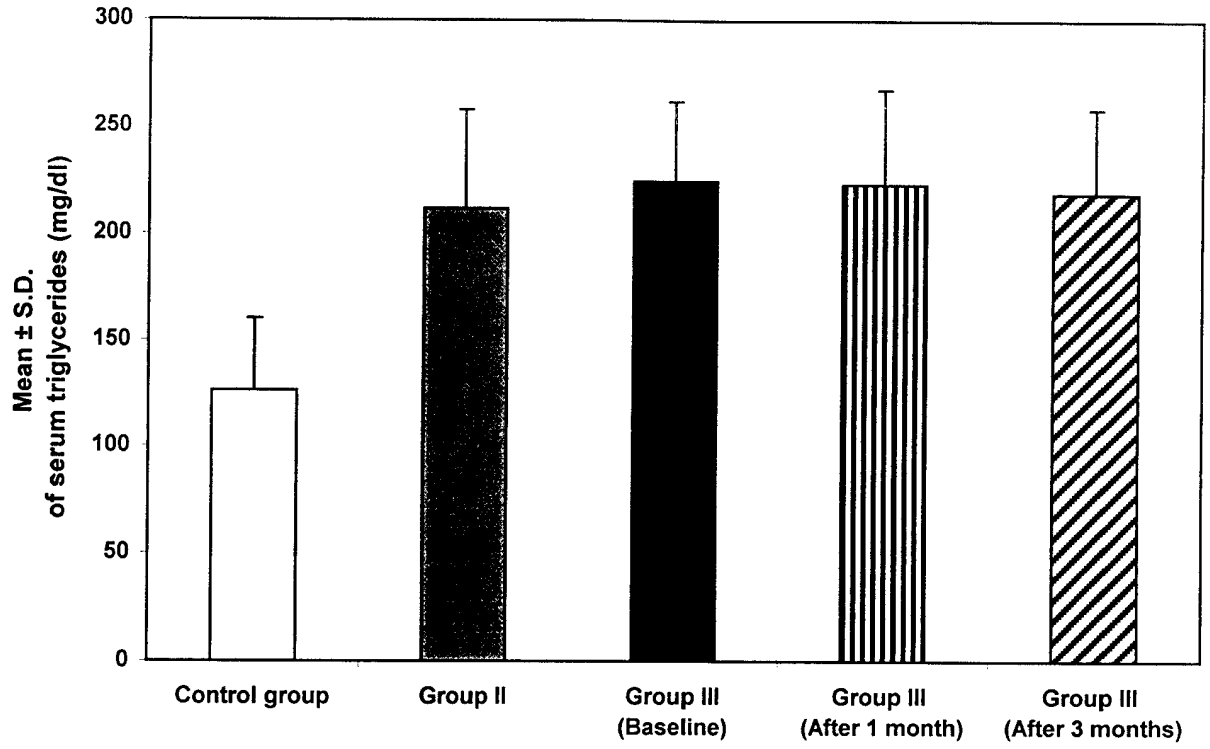

Figure (10) : Mean of serum triglycerides (mg/dl) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

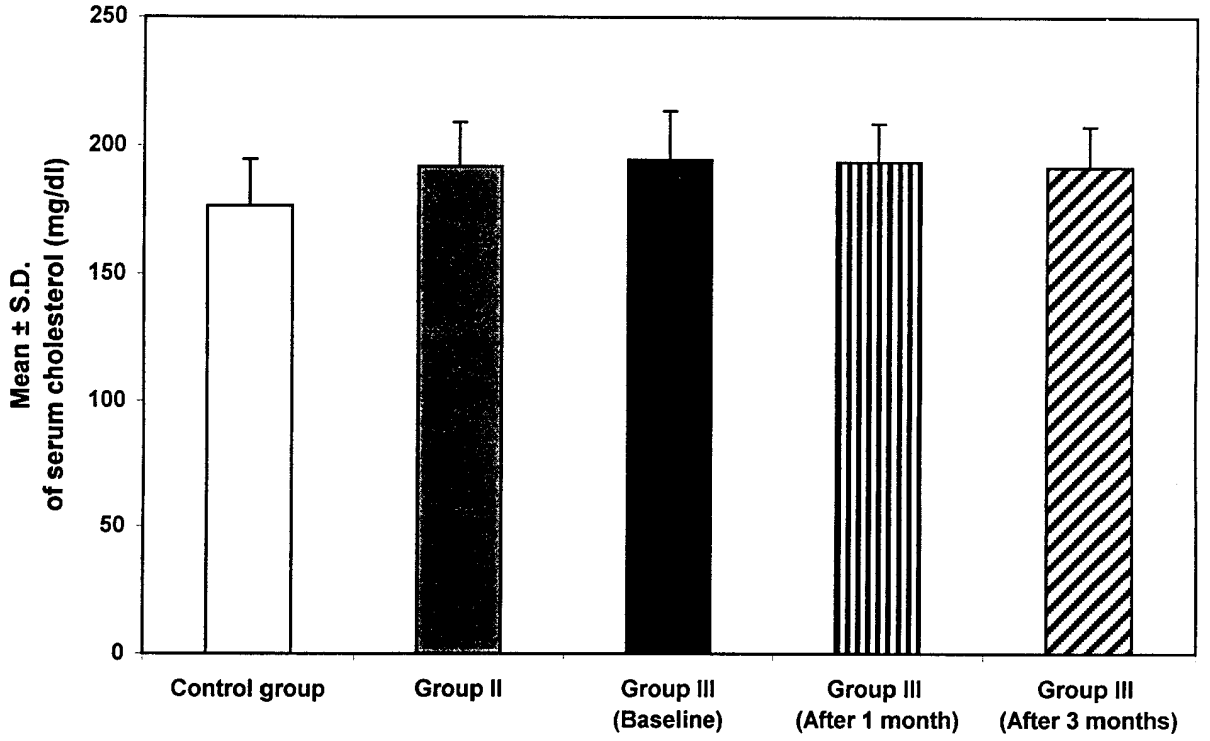

Figure (11) : Mean of serum cholesterol (mg/dl) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

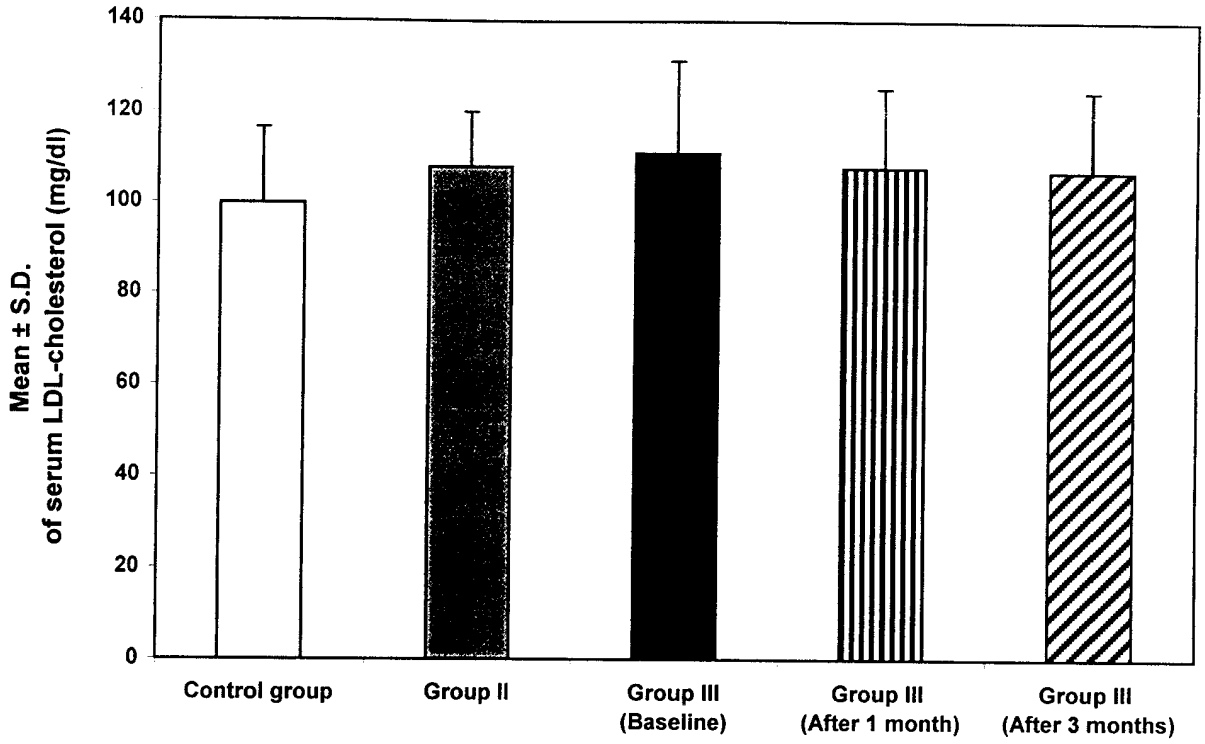

Figure (12) : Mean of serum LDL-cholesterol (mg/dl) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

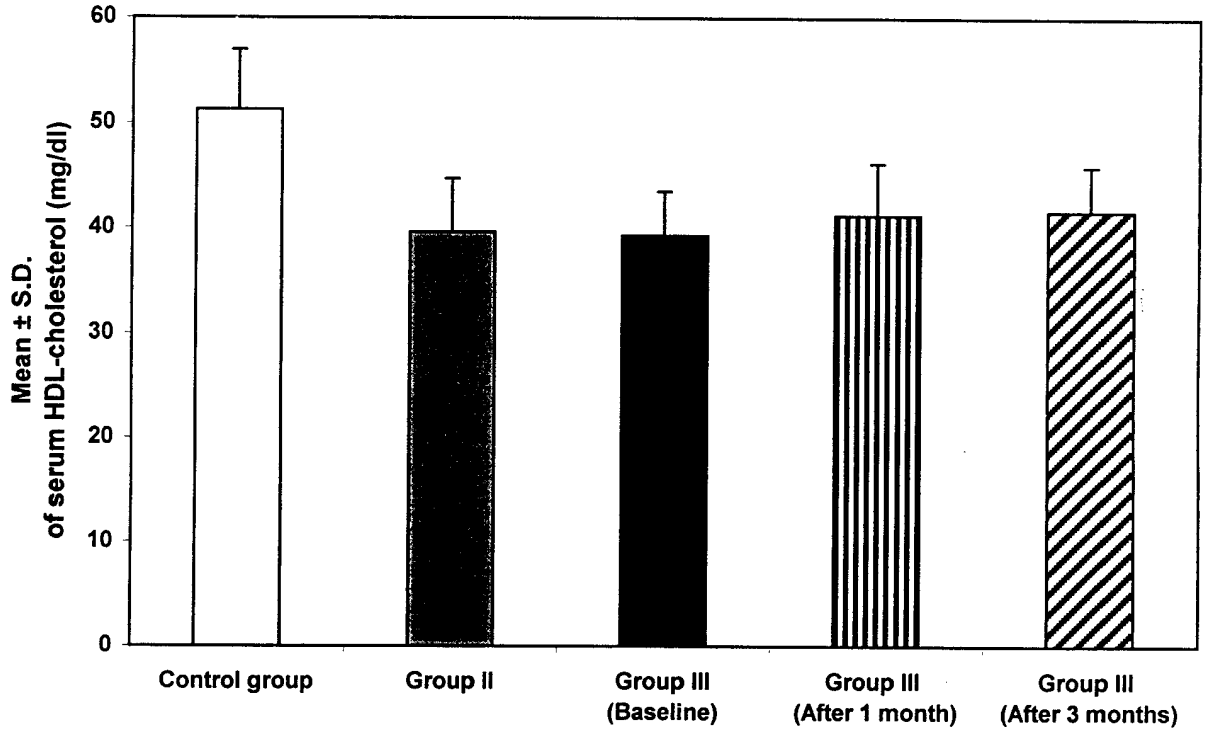

Figure (13) : Mean of serum HDL-cholesterol (mg/dl) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

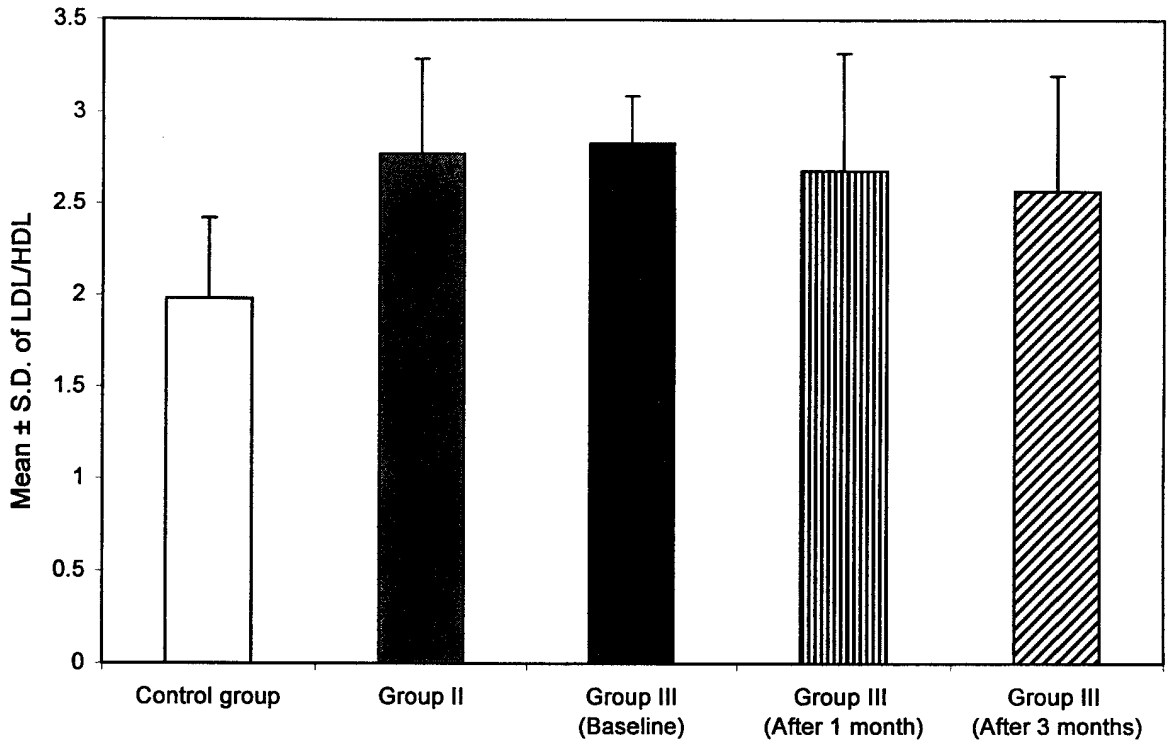

Figure (14) : Mean of LDL/HDL in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

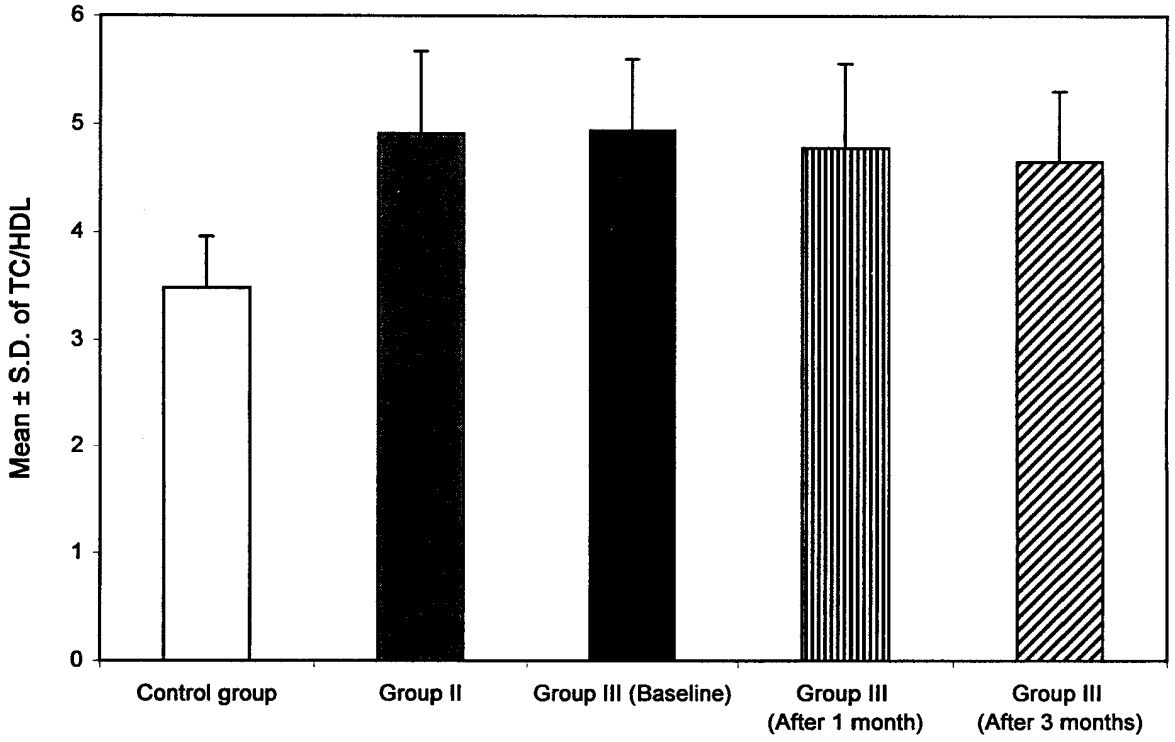

Figure (15) : Mean of TC/HDL in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

The values for the concentration of the Apolipoprotein B (Apo-B) were higher in the plasma of type 2 diabetics than in the plasma of non-diabetic control subjects (Figure 16). The mean concentration of plasma Apo-B in type 2 diabetics without CAD was 27.7% above control, while it was 34.0% above control for type 2 diabetic patients with CAD. Like all other measured lipid parameters, Apo-B was refractory to change following treatment with antioxidants, as its level remained constant during the 3 months follow-up period.

Despite the absence of change in LDL values in type 2 diabetic patients with or without CAD, and refractoriness of LDL to change as a result of antioxidant adjunct therapy, oxidized LDL showed different results (Table R5). A clear and distinct difference in the levels of ox-LDL was obtained in diabetic patients (Figure 17). Type 2 diabetics without CAD had ox-LDL values 26.1 % above non-diabetic controls, which was statistically significant. The increase above normal controls was even higher in diabetics suffering from CAD, as it reached a statistically significant 81.6%. Unlike LDL, the oxidized LDL levels responded favorably to antioxidants therapy. The values of this parameter showed an early and steady decline, which started from the first month. By the end of the 3 months follow-up period, although plasma concentration of ox-LDL declined by a statistically significant 21.4% below the base line value, it was, however, still 42.8% above the non-diabetic control level.

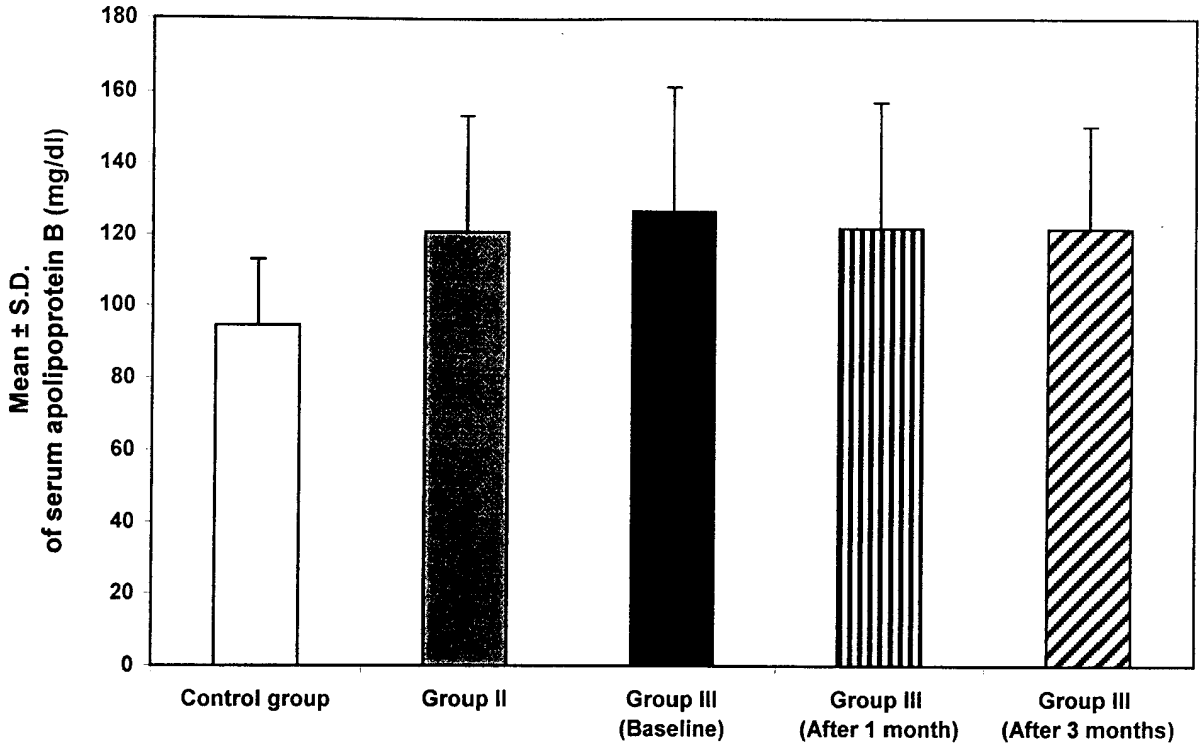

Figure (16) : Mean of serum apolipoprotein B (mg/dl) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

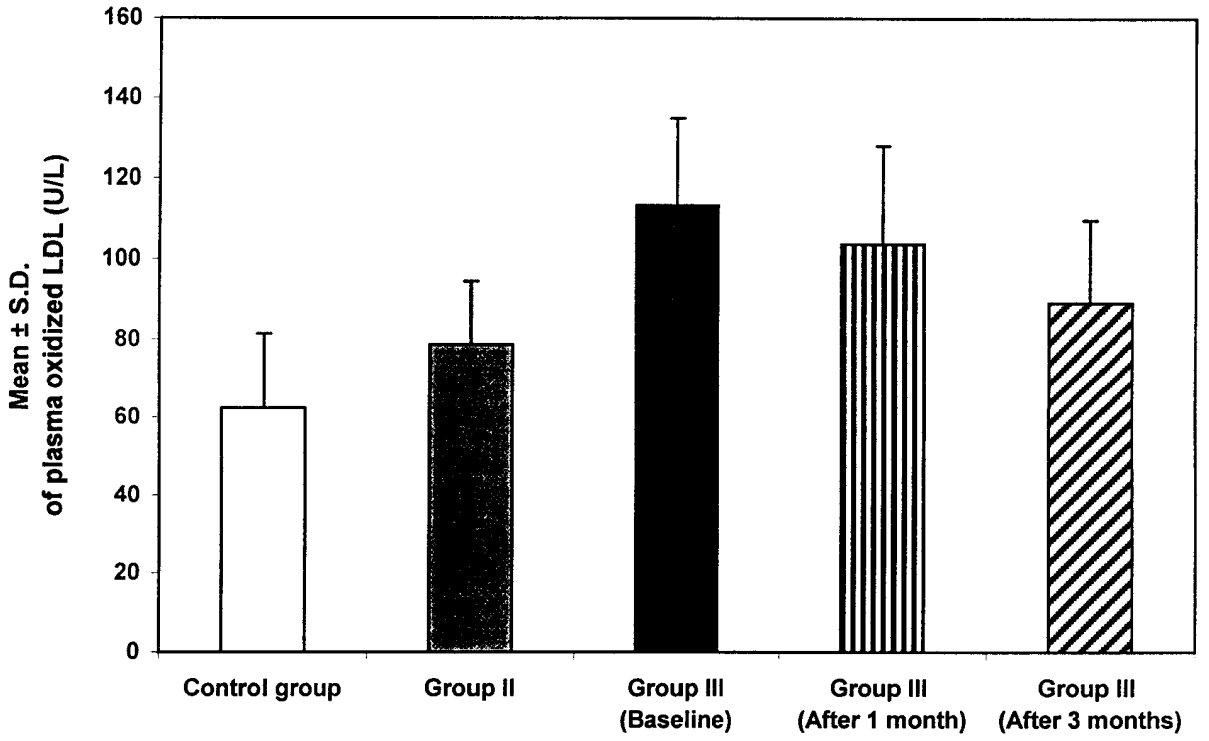

Figure (17) : Mean of oxidized LDL (U/L) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

The change in the mean ox-LDL level in the plasma of type 2 diabetics suffering from CAD and receiving antioxidant adjunct therapy was closely correlated with the average total antioxidant status of that group over the follow-up period (Figure 18). As the total antioxidant status increased, the mean plasma ox-LDL decreased.

Differences were also detected in the oxidized LDL antibodies (OLAb) among the different studied groups (Table R.5 and Figure 19). The OLAb levels were generally higher in diabetic patients than in non-diabetic control. Despite the large difference in mean ox-LDL plasma levels between type 2 diabetics with and without CAD, the mean OLAb values were almost identical reaching 42.2% and 39.7% above control respectively. Adjunct treatment with antioxidants resulted in a very rapid decline in the antibodies to reach a mean value 28.5% above control after one month and to become without statistically significant difference (only 5% increase) from the mean of non-diabetic controls after 3 months. A strong correlation was detected between the change in the mean level of ox-LDL and its antibodies in the plasma of type 2 diabetic patients as a result of antioxidant adjunct therapy. As the mean ox-LDL level in the plasma decreased over the 3-month treatment period, the OLAb also proportionally decreased (Figure 20).

Despite the lack of significant differences among the control and diabetic groups studied in mean plasma LDL values (Table 4), the *in vitro* susceptibility of LDL to oxidation was significantly higher in type 2 diabetics (Table R.5 and Figure 21). Moreover, LDL in diabetics with CAD was more prone to oxidation than in diabetics without CAD. There was also stepwise decrease in the susceptibility of LDL to oxidation with the duration of antioxidant therapy.

Table (R5): Levels of oxidized LDL, oxidized LDL-Ab (OLAb), and susceptibility of LDL to oxidation in vitro (as nmol MDA/mg LDL protein) in type 2 diabetic patients without and with coronary artery disease and the effect of three months adjunct treatment with antioxidants [@].

	Non-diabetic control	Type 2 diabetics without CAD	Type 2 diabetics with CAD		
			Time after start of antioxidant treatment		
			0	1 month	3 months
OxLDL (U/L)	62.4±15.7	78.7±15.7 [a] (+26.1%)	113.3±21.7 [a,b] (81.6%)	103.7±24.4 [a,c] (+66.2%)	89.1±20.5 [a,c] (+42.8%)
OLAb (mU/mL)	323.9±86.4	452.5±142.5 [a] (+39.7%)	460.6±175.6 [a] (+42.2%)	416.3±139.7 [c] (+28.5%)	340.3±97.77 [c] (+5.1%)
MDA-LDL (nmol /mg)	22.8±4.49	34.0±4.58 [a] (+49.1%)	38.0±3.91 [a,b] (+66.6%)	36.6±4.31 [a] (+60.5%)	30.6±4.47 [a,c] (+34.2%)

[@] Data presented as mean ± SD

a Significantly different from control group by ANOVA test .

b Significantly different from group II by ANOVA test .

c Significantly different from baseline of group III by paired t-test.

Number between bracts represents the percent of change from control.

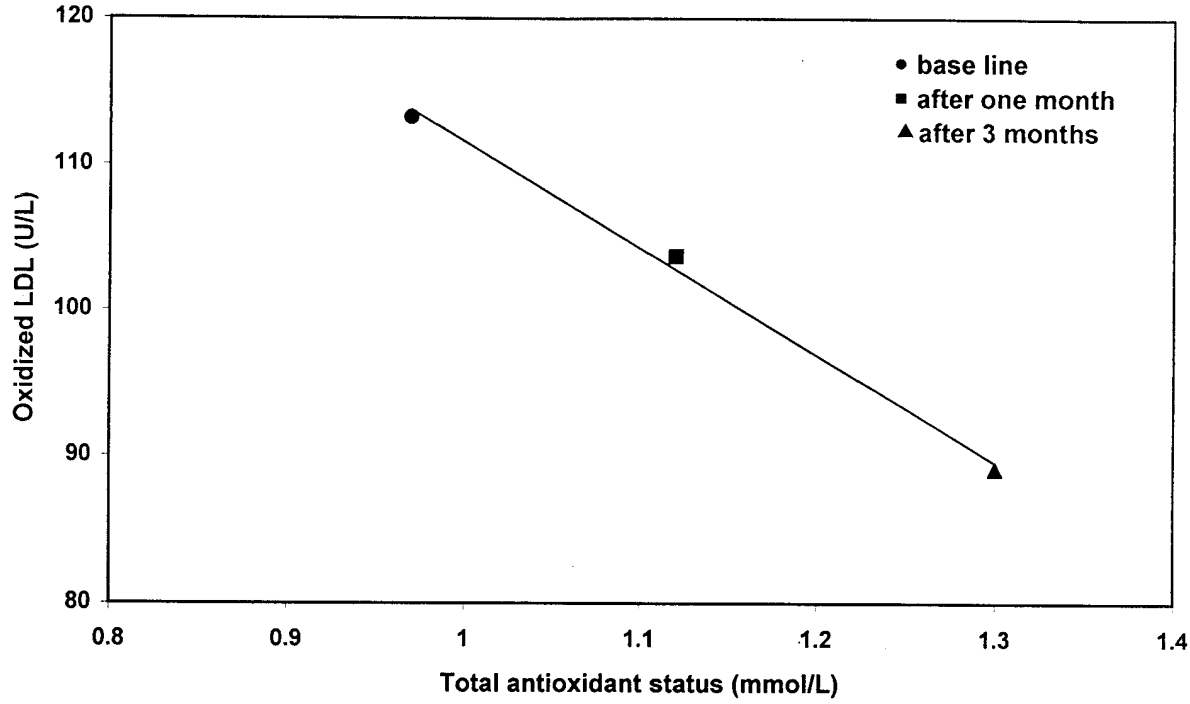

Figure (18) : Relationship between the changes in mean oxidized LDL level with the change in total antioxidant status in the plasma of type 2 diabetic patients with CAD during the 3 month adjunct antioxidant therapy

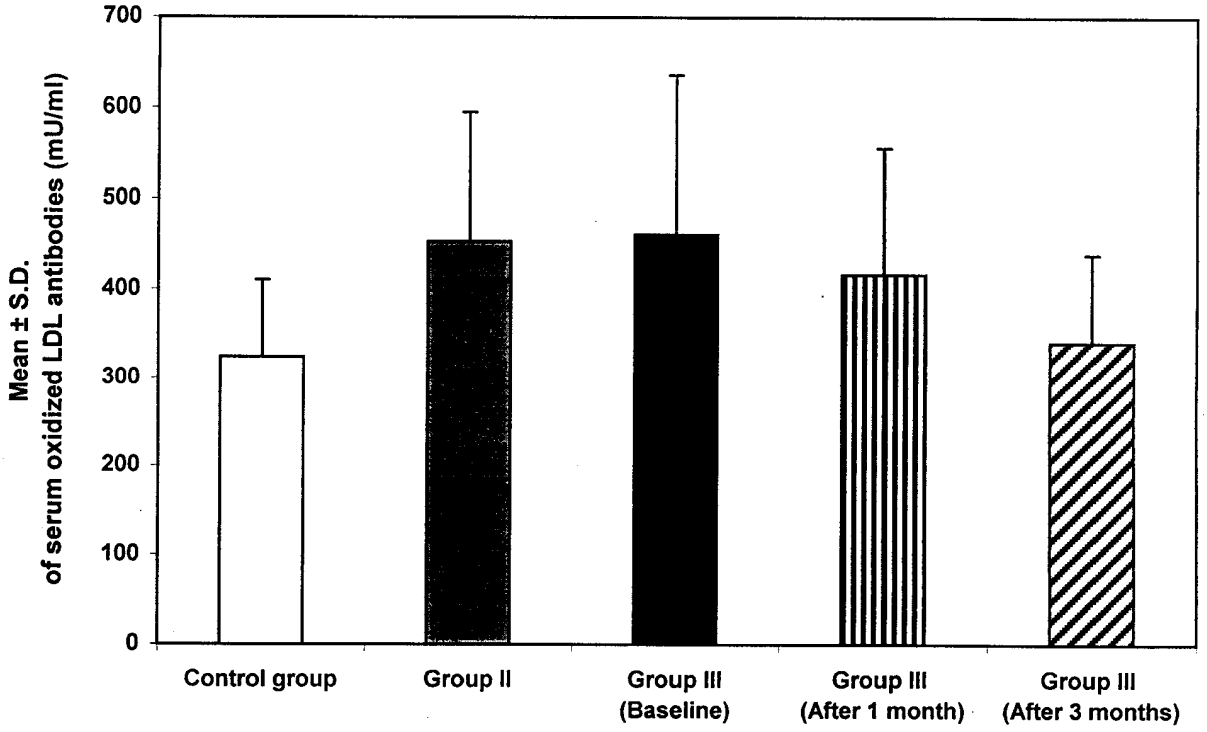

Figure (19) : Mean of serum oxidized LDL antibodies (mU/ml) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

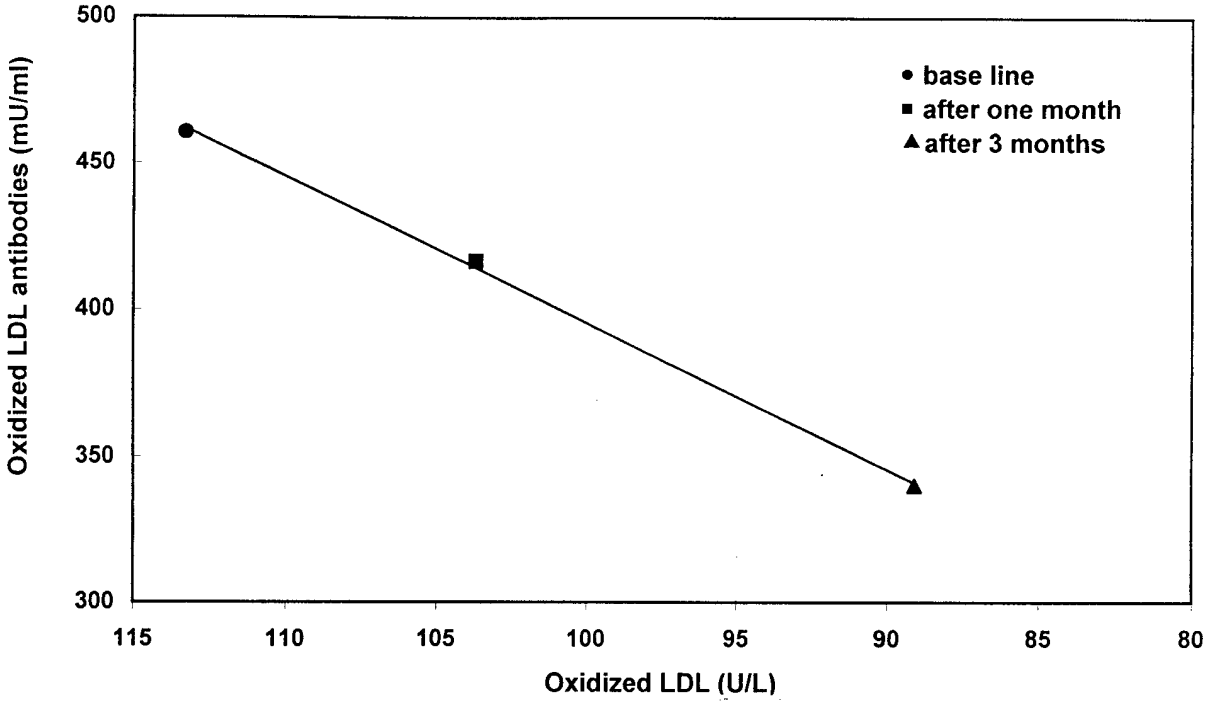

Figure (20) : Relationship between the oxidized LDL levels and oxidized LDL antibodies in the plasma of type 2 diabetic patients with CAD during the 3 month adjunct antioxidant therapy

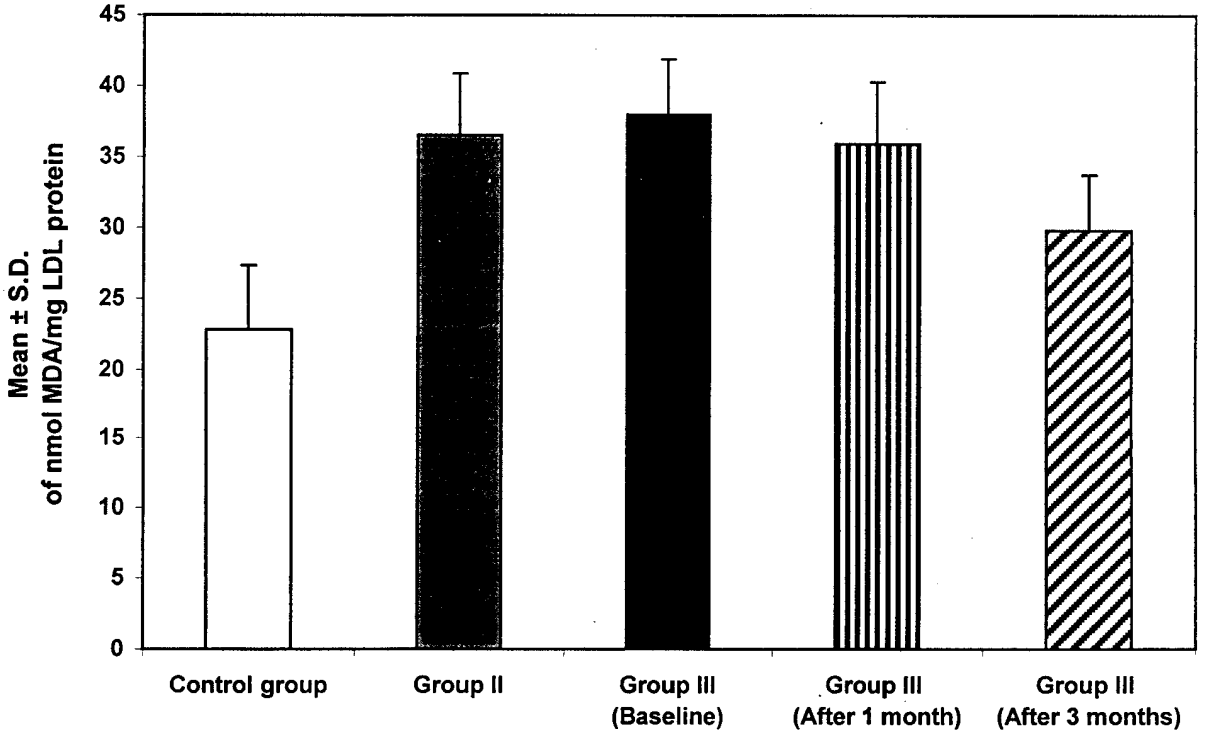

Figure (21) : Mean of susceptibility of LDL to oxidation in vitro (as nmol MDA/mg LDL protein) non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

Thickness of the intima media of the carotid artery:

The thickness of the intima media of the carotid artery was measured as a reflection of the degree of atherosclerosis in type 2 diabetic patients with CAD. The average thickness of the carotid intima media in this group at the beginning of the study was 0.98±0.18 mm. The relationship between the thickness of the intima in individual diabetics with CAD and the plasma levels of ox-LDL is presented in (Figure 22). It could be seen that the thickness of the intima did not exceed the upper normal limit of 0.8 mm until the ox-LDL level exceeded 100-110 U/L. Above this value the thickness of the intima increased sharply with the increase in ox-LDL. As the concentration of ox-LDL increased from 100-110 U/L to 140 U/L the thickness of the intima went up from the clinically acceptable upper limit of 0.8 mm up to 1.4 mm.

Although patients in this group did not receive any hypolipidemic agents, adjunct treatment with antioxidants for 3 months resulted in a decrease in the average thickness of the intima down to 0.90±0.19 mm (Fig 23).

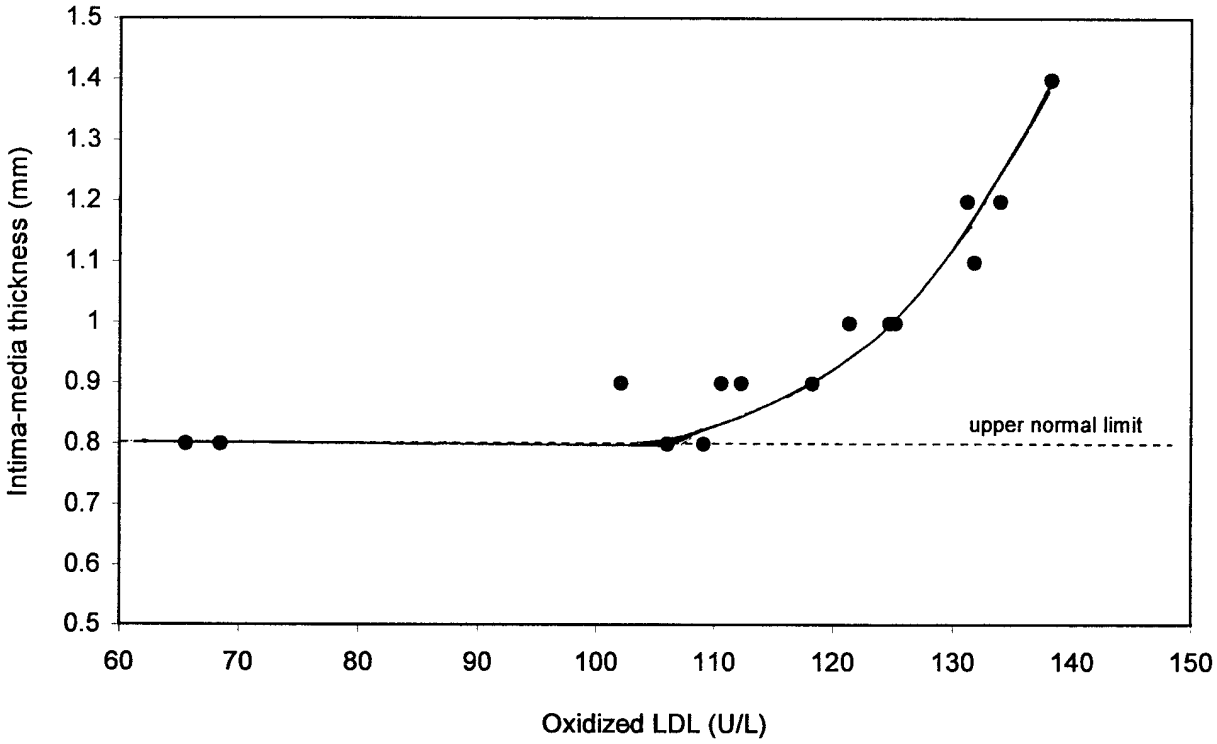

Fig (22) : Correlation curve between OXLDL and IMT in group III patients (r=0.706, P<0.01)

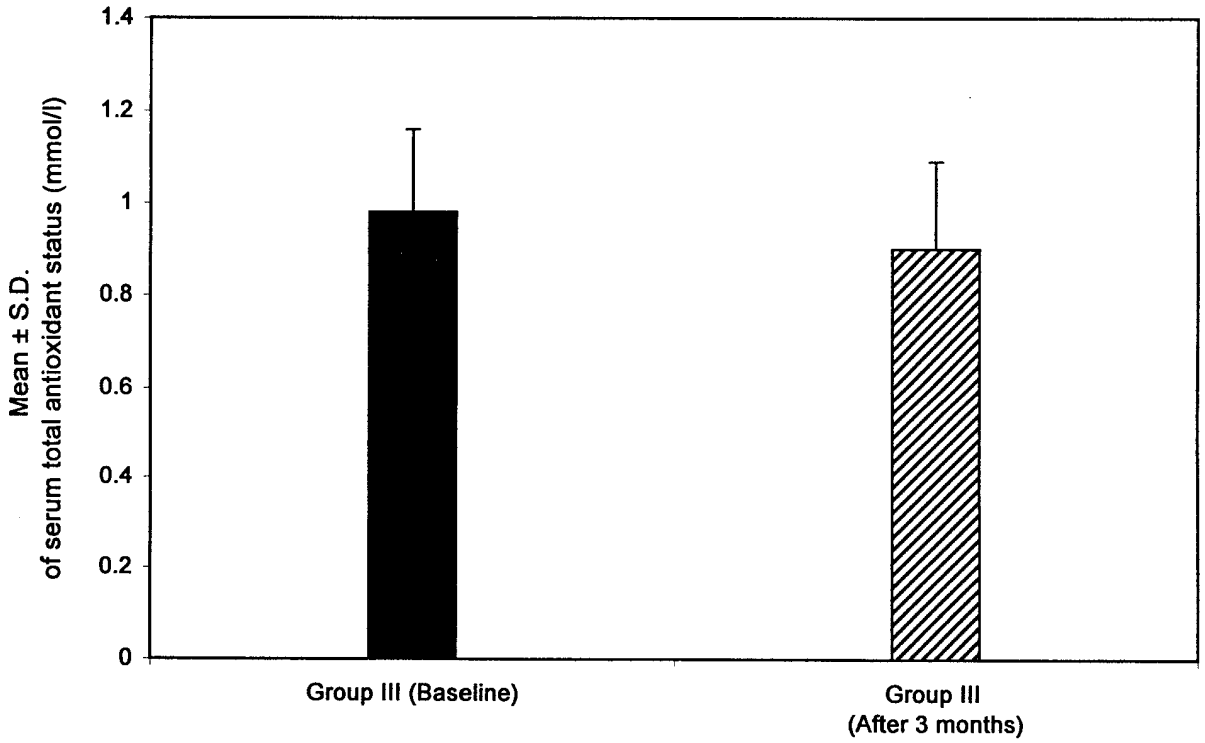

Figure (23) : Mean of serum total antioxidant status levels (mmol/l) in non diabetic control subjects and type 2 diabetic patients without (group II) and with CAD (group III)

DISCUSSION

DISCUSSION

It is well known that diabetes is associated with an increased incidence of macrovascular complications, including coronary artery disease (CAD) and cerebrovascular and peripheral vascular disease[209,210]. These atherosclerotic vascular diseases constitute the principal cause of mortality among diabetic patients [211,212]. Type 2 diabetes is associated with 2- to 4-fold excess risk of coronary heart disease [213].

The data on the glycemic control in the present study were uneventful. As hyperglycemia is the hallmark of diabetes mellitus, it is logical to find that fasting plasma glucose (FPG) levels were higher in the diabetic groups (without complication and with CAD) compared to the control non-diabetics. However, no significant difference was observed in the plasma glucose level between the two groups of diabetic patients.

Also the present study has shown that glycohemoglobin level, which represents patient compliance to proper hypoglycemic treatment and efficacy of the treatment in the 3-months prior to the tested sample, was significantly elevated in the diabetic groups by comparison with the control group. But no significant difference was observed between the two diabetic groups, and the level of glycosylated hemoglobin was equal to or below the 8% recommended cut off value to ensure proper glycemic control and avoid the development of complications. Although the degree of glycemia in diabetic patients is strongly related to the risk of microvascular

complications (retinopathy and nephropathy), the relation of glycemia to macrovascular disease in type 2 diabetes is more modest [213].

The effect of adding antioxidant to the treatment regimen of diabetics with CAD was unexpected. The improvement in the glycemic status, represented by decreases in the mean FPG and HbA_{1C}, although not very prominent but statistically significant, probably reflected a tendency toward better overall improvement in patients general health, better tissue metabolic status and alleviation of oxidative stress.

Various studies have suggested that vitamin E may improve the metabolism of glucose by muscle cells and the circulation to the islets of Langerhans and other tissues [214,215]. Different clinical trials in type 2 diabetic patients have shown that supplementation with vitamin E for duration of 2–4 months results in either a decrease [216,217] or no effect [218,219] on blood HbA_{1C} levels, a decrease [220] or no effect [221] on blood glucose levels.

The results of the present study clearly indicated that there was a definite overproduction of free radicals and excessive exposure of diabetic patients to oxidative stress. The lipid peroxidation index; thiobarbituric acid-reactive substances, was significantly higher in both diabetic groups than in healthy individuals. Moreover, by comparing the two diabetic groups together there was a significant increase in patients with CAD. These findings are compatible with many reports in the literature[222,223].

Hyperglycemia can increase oxidative stress through several pathways. A major mechanism appears to be the hyperglycemia induced intracellular reactive oxygen species (ROS), generated by the mitochondrial electron transport chain and resulting in increased production of superoxide radicals[25]. Two other mechanisms have been proposed that may explain how hyperglycemia causes increased ROS formation. One mechanism involves the transition metal-catalyzed autoxidation of free glucose. Through this mechanism, glucose itself initiates autoxidative reaction and free radical production yielding superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2)[224]. The other mechanism involves the glucose-dependent, nonenzymatic covalent modification of proteins that accompanies the hyperglycemic states (Maillard reaction). It involves the combination of the aldehyde group of glucose in the open chain form with amino groups on proteins to form fructoselysine. The final stage of the Maillard reaction involves the irreversible oxidation, or glycoxidation, of fructoselysine to yield a host of advanced glycation end products (AGEs)[225]. Some of the individual advanced glycation products such as N^{ϵ}-(carboxymethyl) lysine (CML) and pentosidine are formed in reactions of protein with glucose only under oxidative conditions[226,227]. Thus, some AGEs are produced by combined processes of glycation and oxidation and have been termed glycoxidation products[26]. The interaction between AGE epitopes and the cell surface AGE receptors upregulates oxidative stress response genes[228] and releases oxygen radicals[229].

In tissues where glucose uptake is independent of insulin; including retina, lens, kidney, peripheral nerves (all tissue sites of diabetic complications), exposure to elevated glucose levels causes an increase in intracellular sorbitol and fructose levels due to increased activity of aldose reductase (reduces glucose to sorbitol) and sorbitol dehydrogenase (oxidize sorbitol to fructose)[230]. These two enzymes constitute the polyol pathway. Increased substrate flux through the polyol pathway increases not only cellular levels of sorbitol and fructose but also the ratio of NADH-to-NAD^+ [231]. The hyperglycemia-induced increase in the NADH-to-NAD^+ ratio is referred to as hyperglycemic pseudo hypoxia and is thought to play a role in diabetic complications. Part of this idea is based on similarities existing between pseudohypoxia and true hypoxia. Improper blood flow leading to ischemia has been noted in tissues destined for diabetic complications[232]. Both true hypoxia and pseudohypoxia may generate free radicals: the latter via an increased synthesis of prostaglandin H_2 from prostaglandin G_2 as the enzyme hydroperoxidase that uses NADH as cofactor[233], the former via ischemia reperfusion injury[234].

Hyperglycaemia could also enhance endothelial superoxide anion generation via activation of cyclooxygenase pathway which is known to generate ROS with a mechanism involving NAD(P)H oxidase[32]. There is also evidence that acute elevations in glucose levels may depress natural antioxidant defenses[235,236]. It appears that many reactions associated with hyperglycemia may acutely and chronically increase the production of free radicals, resulting in an oxidant/antioxidant imbalance[232].

The increase in free radicals was coupled with disturbance in free radical scavengers, particularly the glutathione system. We found that, GSSG was higher, while the total GSH and reduced GSH were generally lower in diabetic groups than in controls. Many investigations have reported lower concentrations of glutathione in plasma, erythrocytes, aorta, and lenses of diabetic patients compared with healthy subjects [237,238]. In addition to oxidant-antioxidant imbalance, the decreased level of glutathione in diabetic patients could be influenced by the decreased activity of enzymes such as γ-glutamylcysteine synthetase and glutathione reductase, possibly because of their glycation by hyperglycemia[239]. Previous studies have reported that vitamin E *in vitro* [240]and vitamin E supplementation to diabetic patients can lower glycosylation of proteins including enzymes such as γ-glutamylcysteine synthetase and glutathione reductase[216].

As the dominant non-protein thiol in the mammalian cell, GSH is essential for maintaining the intracellular redox balance [241]. It represents the single largest source of reducing equivalent in the cell and accounts for about 90% of all cellular reducing equivalents[242]. Therefore, the depletion of GSH could significantly affect the overall redox potential of the cell. By inspecting the calculated redox potential in the present study, it became clear that redox potential for diabetic groups was shifted to the oxidizing side and the supplementation of group III with antioxidant combination partially corrected the balance of GSH/GSSG couple to restore the reducing potentials specially after three months. These results may

indicated that the calculated level of cell redox potential may provide useful means to quantitatively express the oxidant/antioxidant balance in diabetic condition.

In addition, we found a clear deficiency in total antioxidant status (TAS) in diabetic patients when compared with control non-diabetic subjects. Studies have consistently demonstrated deficiency in individual antioxidants in type 2 diabetic patients [243] with lower concentrations of glutathione, vitamin E, vitamin C and reduced activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase as well as a decrease in total radical trapping antioxidant parameter (TRAP), suggestive of reduced total antioxidant defense[244]. Overall, these studies suggest that oxidative stress and impaired antioxidant defense is a feature of type 2 diabetes that is present early in the disease and may contribute to its progression and even to the development of complications.

There is also evidence that hyperglycemia may compromise natural antioxidant defenses. Under normal circumstances, free radicals are rapidly eliminated by scavengers such as reduced glutathione, vitamin C, and vitamin E. Reduced glutathione content [245], as well as reduced vitamin E [246] have been reported in diabetic patients. Plasma and tissue levels of vitamin C are 40-50% lower in diabetic patients compared with non-diabetic subjects [247]. Supplementation with known free radical scavengers such as vitamins E and C, have a potential role in boosting antioxidant defense[248,249]. In addition, scavenging antioxidants act synergistically [250,251], so supplementation with two or more antioxidant may enhance their

individual effects. Jain et al.[252] found a significant relationship between glutathione and vitamin E concentrations in the erythrocytes of diabetic patients. In addition, they have documented that vitamin E supplementation can increase cellular glutathione concentrations. This relationship could be because of sparing by vitamin E of glutathione utilization for the scavenging of lipid peroxidation reactions. Furthermore, vitamin E supplementation was shown to lower malondialdehyde concentrations. The effect of vitamin E on lowering lipid peroxidation concentrations could be directly on scavenging of lipid peroxides, the stimulation of glutathione peroxidase activity in a fashion similar to the vitamin E stimulation of glutathione peroxidase activity in cultured cardiomyocytes .[253], or the elevated level of its cofactor glutathione.

It is well established that diabetes is one of the major risk factors for atherosclerosis, and diabetic patients have a two- to five fold higher risk of coronary heart disease than non-diabetic individuals [254]. The mechanisms by which diabetes accelerates atherosclerosis are not well understood. The customary clusters of risk factors for CAD, which are more common in patients with diabetes, are not sufficient to explain this phenomenon. It has been proposed that an increased level of modified lipoproteins might be an additional factor contributing to the accelerated development of macrovascular complications in diabetes [255] .

The present study has shown alteration in the lipid profile in diabetic groups as compared to the healthy control subjects. The two diabetic groups had significant increase in the levels of triglycerides, moderately raised cholesterol levels, and lower levels of HDL cholesterol compared to

the control group. On the other hand, LDL-cholesterol concentrations were very similar in control and diabetic groups. The lipid abnormalities found in the diabetic groups are in agreement with the results reported by most workers in the fields [256,257]. These abnormalities (increased triglycerides levels and decreased HDL cholesterol levels) were reported to be highly associated with type 2 diabetes, and to be definite risk factors in atherosclerotic cardiovascular disease [258,259].

Several factors are likely to be responsible for diabetic dyslipidemia: insulin effects on liver apoprotein production, regulation of lipoprotein lipase (LpL), actions of cholesteryl ester transfer protein (CETP), and peripheral actions of insulin on adipose and muscle [260]. Moreover, this dyslipidemia often is found in prediabetic patients with insulin resistance but normal indices of plasma glucose [261]. Therefore, abnormalities in insulin action and not hyperglycemia *per se* are associated with this lipid abnormality [260]. This would explain the lack of correlation observed in the present study between hyperglycemia and the changes in the pattern of different lipid fractions. It has also been established that insulin resistance represents a strong component of the etiology of type 2 diabetes [262]. In patients afflicted with this disease increased lipolysis in adipocytes due to poor insulinization results in increased fatty acid release from fat cells. The ensuing increase in fatty acid transport to the liver, which is a common abnormality seen in insulin-resistant diabetes, may cause an increase in VLDL secretion [260].

Patients with diabetes, especially type 2 diabetes, have increased VLDL production resulting in hypertriglyceridemia[259] . Two factors may

increase VLDL production in the liver : (i), the return of more fatty acids due to increased actions of hormone-sensitive lipase (HSL) in adipose tissue and (ii), insulin actions directly on apoB synthesis. Both of these processes will prevent the degradation of newly synthesized apoB and lead to increased lipoprotein production. VLDL, like chylomicrons, requires lipoprotein lipase (LpL) to begin its plasma catabolism, leading to the production of LDL or the return of partially degraded lipoprotein to the liver, but type 2 diabetic patients have been reported to have reduced LpL activity [263].

Also there are several reasons for the decrease in HDL found in patients with diabetes. Increased rates of secretion of VLDL into plasma appear to drive the exchange of triglyceride from these lipoproteins for the cholesteryl esters found in HDL. This exchange is facilitated by cholesterol ester transfer protein, and generates a TG-enriched HDL which is a substrate for either hepatic lipase or lipoprotein lipase that converts HDL to a smaller particle more rapidly cleared from the plasma [264].

Although LDL is not usually increased in diabetes, qualitative changes in LDL cholesterol may be present. In part this may represent a balance of factors that affect LDL production and catabolism. A necessary step in LDL production is hydrolysis of its precursor VLDL by LpL. A reduction in this step due to LpL deficiency or excess surface apoproteins decreases LDL synthesis [234] . Decrease in size and an increase in density of LDL, like HDL, can be accounted for; CETP-mediated exchange of VLDL triglyceride for cholesteryl esters in the core of LDL [265]

Although patients with type 2 diabetes mellitus do not have higher LDL cholesterol levels than the general population, the resulting dyslipidemia increase the risk of type 2 diabetic individuals to cardiovascular disease. Multiple aspects of their lipid profiles are atherogenic. First, not only are there increased levels of VLDL particles, which can enter the vessel wall and accumulate in atherosclerotic plaques[266,267], but these VLDL, by virtue of receiving CETP-transferred cholesteryl esters, are able to deliver more cholesterol per particle to the vessel wall. Second, reduced HDL cholesterol and apoA-I levels mean that there are fewer HDL particles engaged in cholesterol efflux from peripheral tissues, which is the first step in reverse cholesterol transport. Fewer HDL particles also mean that HDL cannot fulfill several proposed direct antiatherogenic actions at the vessel wall, including the role of HDL as an antioxidant. CETP-mediated transfer of HDL cholesteryl esters to VLDL not only may enrich an atherogenic lipoprotein with cholesterol but also can divert that cholesterol from the specific reverse cholesterol transport pathway. Finally, small dense LDL, may be more atherogenic than an equal number of larger more cholesteryl ester–rich LDL, because small dense LDL may be more liable to oxidation or may more readily penetrate and stick to the extracellular matrix (ECM) of the artery wall.[268]

Apolipoprotein B is the protein moiety of LDL. The clinical interest of this protein lies in the fact that it provides a relatively accurate estimate of circulating LDL particle numbers[269].Our study shown that, ApoB serum concentration was significantly elevated in diabetic groups when compared with healthy control group. That result is also in harmony with number of

studies which have shown that apo B levels are elevated in type 2 diabetes[270,271]. Tissue culture[272], animal experiments[273], and human studies[274] suggest that fatty acids modulate liver apoB secretion. In studies with VLDL, Wu et al.[275] demonstrated that increases in cellular TG but not cholesteryl ester (CE) content were associated with increases in apoB secretion. A second regulatory process may be a direct effect of insulin on liver production of apoB and other proteins involved in degradation of circulating lipoproteins. In some studies insulin directly increased degradation of newly synthesized apoB[276]. Therefore, insulin deficiency or hepatic insulin resistance may increase the secretion of apoB.

LDL may be modified in a way that would make it more atherogenic[277]. LDL oxidation plays a key role in atherogenesis[124,278]. It is now accepted that the first event in the development of fatty streaks is the transport of LDL into the artery wall, a concentration-dependent process that does not require receptor-mediated endocytosis[279]. The oxidation can occur in the microenvironment of the subendothelial space or it can be cell mediated, and the process is known to be dependent on superoxide generated in endothelial cells during mitochondrial respiration[280].

Oxidative modification of LDL has been implicated as a major factor in the pathogenesis of coronary atherosclerosis[281]. To examine whether the presence of type 2 diabetes may affect LDL modification we measured the levels of circulating OxLDL, and OxLDL-Ab and the *in vitro* oxidative susceptibility of LDL subfractions. It was found that circulated OxLDL levels in patients with type 2 diabetes were significantly higher than in control subjects. In addition, diabetic patients with CHD had higher levels

of OxLDL than patients without cardiovascular complication. These results are in agreement with other published data. The work from the laboratory of Holvoet et al,[282-284] who used immunological techniques to measure circulating levels of ox-LDL and MDA-LDL, supports the fact that LDL containing oxidation-specific epitopes seems to differentiate normal patients from patients with CAD, transplant atherosclerosis, and acute coronary syndrome (ACS). They Also showed that circulating oxidized LDL is a sensitive marker of CAD that is correlated with most of the Framingham risk factors[285]. Other studied have demonstrated that OxLDL levels were significantly higher in patients with diabetes mellitus than in control subjects[286]; and that high levels of circulating oxLDL can serve as an independent and significant predictor for future cardiac events in type 2 diabetic patients with CAD[287].

Oxidized LDL exerts several biological effects that may contribute to the progression of the atherosclerotic lesion. During the oxidation of LDL, initially minimally modified LDL (MM-LDL) is formed in the subendothelial space. MM-LDL differs from oxidized LDL in that it has undergone only mild lipid peroxidation, but it is still recognized by the LDL receptor[129,288]. MM-LDL can induce leukocyte-endothelial cell adhesion and promote secretion of monocyte chemotactic protein-1 and macrophage colony-stimulating factor by the endothelium. This produces monocyte binding and migration into the subendothelial space, where macrophage colony-stimulating factor promotes differentiation into macrophages. Macrophages in turn further oxidize MM-LDL to oxidized LDL. Fully oxidized LDL represents the depletion of polyunsaturated fatty

acids and antioxidants within the LDL core and significant derivatization, cross-linking, and fracturing of the apoB of LDL so that the LDL particle is now recognized by scavenger receptors on the monocyte macrophages, which are not regulated by the intracellular cholesterol content. This produces appreciable cholesterol accumulation in macrophages, leading to foam cell formation[108].

Oxidized LDL is cytotoxic and could promote endothelial dysfunction and the evolution of the fatty streak into a more advanced lesion. Oxidized LDL can also promote atherogenesis by stimulating expression of several other genes in the arterial wall such as interleukin-1. Interleukin-1 has been shown to induce smooth muscle cell proliferation, promote a procoagulant state, and stimulate leukocyte-endothelial cell adhesion[289]. Oxidized LDL may also contribute to rapidly progressing coronary atherosclerosis by inducing platelet adhesion, by decreasing the anticoagulant and fibrinolytic capacities of activated endothelium, and by impairing vasodilation and inducing shear stress[22,125,290].

The intima-media thickness (IMT) is at present the best-studied sonographic marker for early atherosclerotic vascular wall lesions[291,292]. A thickening of the intima-media complex not only reflects local alterations, mostly of the common carotid artery (CCA), but also corresponds to generalized atherosclerosis. A direct correlation between IMT and the risk of myocardial infarction and stroke in a population of patients without a prior history of vascular diseases has been shown[293]. By ultrasonography, we have demonstrated that diabetic patients with CAD had increased

intima-media thickness of carotid arteries, suggesting that an increased oxidized LDL level is a marker of atherosclerosis.

The observed relationship between the level of circulating OxLDL and the thickening of the carotid artery intima media deserves further comment. From the results of the present study (Figure 22) it could be seen that the thickness of the intima media of the carotid was within the clinically accepted normal values (equal to or less than 0.8 mm[294]) if the oxidized LDL level was below 100-110 U/L. Once the OxLDL exceeded this range, the thickness of the intima increased sharply with the increase in plasma OxLDL. It seems that the level of OxLDL should be kept below an upper limit of 100-110 U/L in order to avoid the serious atherosclerotic effects of this factor.

Many studies[295,296] strongly indicate that α-tocopherol supplementation decreases susceptibility of LDL to oxidation. Epidemiological studies also support the hypothesis that higher dietary intakes of α-tocopherol are associated with decreased risk for coronary heart disease[297,298] and reduced LDL oxidizability[218,219]. Although adjunct therapy with antioxidant mixture improved the total antioxidant status of the treated diabetic patients, which resulted in significant decrease in OxLDL, there was no immediate reciprocal effect on the thickness of the intima media. It is possible that more time is required for mobilization of the cholesterol precipitated in the atheromatous plaques. The supplementation with exogenous antioxidants is expected to give rapid

improvement in the antioxidant status leading to suppression of free radical production and consequent decrease in OxLDL. The mobilization and disappearance or decrease in size of the atheromatous plaques are expected to proceed at a slower rate, or at least they would not increase in size. Return of the thickness of the intima to values within the clinically normal range, and hence improvement of atherosclerosis in diabetics, is expected to take more time and would probably be expedited by hypolipidimic agents.

The oxidative modification of the apolipoprotein moiety of LDL makes it antigenic and causes it to elicit the production of autoantibodies against OxLDL. These autoantibodies may reflect LDL oxidation *in vivo*[299,300], and oxidatively modified LDL particles do occur in atherosclerotic lesions[301,302]. In the present study, the antibody reactivity level against oxidized LDL was found to be significantly higher in diabetic patients than in control, but no significant difference between the two diabetic groups.

The presence of antibodies against oxidized LDL in patients with diabetes has been reported by several investigators[303-305]. Furthermore, some studies suggest that the presence of a high titer of autoantibodies against oxLDL is associated with the severity of carotid atherosclerosis[300].

However, there is considerable disagreement among different groups about the nature and levels of the abnormalities in modified LDL anti-body levels in diabetic patients. Bellomo et al.[304] reported higher levels of autoantibodies against glycated and oxidized LDL in type 2 diabetes

patients, and Festa et al[306] found a higher level of autoantibodies to oxidized LDL in type 1 diabetes patients. Other investigators[303,307] have found comparable levels of these antibodies in type 1 as well as type 2 diabetic patients.

A very encouraging observation in the present study is the favorable response of OxLDL level to the antioxidant adjunct therapy. The definite and steady decline in OxLDL level in type 2 diabetic patients with CAD was clear and is indicative of improvement in the atherosclerotic condition. The time factor is also important, as the decline in the level below the 100-110 U/L suggested ceiling occurred within only 3 months of therapy. Such lower level indicates cessation of the progress of the atherogenic process, such that the atheromatous plaques in the intima media of the carotid artery would stop growing, before improvement in the level of HDL and HDL-dependent reverse cholesterol transport.

In vivo, free radicals generated by endothelial cells of the arterial wall and activated macrophages are thought to oxidize LDL particles[147]. Vitamin E, mainly α-tocopherol, is the major fat-soluble antioxidant present in the LDL particle. On average, 5-9 vitamin E molecules are carried by each LDL particle and are believed to protect LDL from oxidative damage by acting as a chain-breaking antioxidant and preventing lipid peroxidation of polyunsaturated fatty acids and modification of proteins in LDL by reactive oxygen species (ROS)[147].

Much evidence demonstrated that both cell-mediated and metal-dependent oxidation of LDL can be suppressed by vitamin E supplementation. Enrichment of LDL with vitamin E was reported to protect LDL against *ex vivo* oxidative modification[308]. In survivors of myocardial infarction, vitamin E content in LDL was inversely related to the severity of the coronary stenosis score as determined by angiograms[309,310]. Taken together, these studies clearly demonstrate that as the vitamin E content in LDL or endothelial cells is increased, there is an overall protection against LDL oxidation, with a subsequent reduction in the severity of the coronary stenosis score[311].

Ascorbate, another component of the antioxidant mixture used as adjunct therapy in the present study, can help reduce lipid hydroxyl radicals or recycle the one-electron-oxidized forms of lipid soluble antioxidants. In addition, ascorbate is well known to recycle the α-tocopheroxyl radical[312].

Ceruloplasmin, 15-lipoxygenase, myeloperoxidase (MPO), and inducible (in addition to endothelial) nitric oxide synthase (NOS) have been found in animal and human lesions and can cause or contribute to LDL oxidation *in vitro*[313,314]. MPO and MPO-derived HOCl have been implicated *in vivo* LDL modification and atherogenesis[135]. One likely defense against MPO-mediated LDL oxidation is vitamin C (ascorbate), an important water-soluble antioxidant in biological fluids[315]. Vitamin C scavenges HOCl in a stoichiometric manner[316] and can regenerate amines from HOCl-derived *N*-chloramines[317]. Moreover, as a co-antioxidant,

vitamin C can prevent the pro-oxidant properties of the α-tocopheroxyl radical[318]. Carr et al.[319] found that physiological concentrations of vitamin C can protect LDL lysine and tryptophan residues from oxidation by HOCl and partially protect LDL cysteine residues. Vitamin C also protects against LDL lipid peroxidation initiated by MPO-derived tyrosyl radicals[320,321]. This is likely due to vitamin C's scavenging of these radicals or possibly preventing their formation by MPO[312].

Superoxide anion overproduction in diabetes originates from mitochondria; in these conditions antioxidant enzymes are more relevant to reduce oxygen species than vitamin E. The antioxidant adjunct treatment also contained selenium, which is a major antioxidant trace element and is the co-factor of glutathione peroxidase (Se GSHpx). Low Se GSHpx activity, observed in diabetic patients, is associated with thrombosis and cardiovascular complications[322].

As clinical indices of CVD, ultrasound measurements of intima-media thickness of the carotid artery wall and angiographic scores of coronary artery stenosis have been reported to be inversely related to vitamin E status[323,324]. High levels of vitamin E in RBC were associated with less thickening of the arterial wall in French patients[325].

In contrast, two other studies[326,327] reported that vitamin E treatment of CVD patients had no effect on reducing the primary end points, which included death, nonfatal myocardial infarction or stroke. It was suggested that the genetic background, type and dose of vitamin E and dietary habit

and lifestyle of study subjects might have contributed to the differential results in these studies[328,329].

Although these two studies have raised some doubts on the efficacy of vitamin E in the prevention of progression of atherosclerotic lesions, the overwhelming observational and experimental studies strongly support its positive effect on the reductions of risk of atherogenesis. Investigators of the Heart Outcomes Prevention Evaluation (HOPE) study suggested that a longer treatment time and follow-up might be necessary to suppress early events and observe a positive effect of vitamin E. In many observational studies in which the beneficial effects of vitamin E have been noted, intake of other vitamins and micronutrients and their interaction with vitamin E might have contributed to the observed positive effects[330].

SUMMARY AND CONCLUSION

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Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. It is estimated that the worldwide burden of this disease in adults is around 173 million in the year 2002.

It is well known that diabetes is associated with an increased incidence of macrovascular complications, including coronary artery disease (CAD) and cerebrovascular and peripheral vascular disease. These atherosclerotic vascular diseases constitute the principal cause of mortality among diabetic patients. Type 2 diabetes is associated with 2- to 4-fold excess risk of coronary heart disease. The atherosclerotic process is indistinguishable from that affecting the non-diabetic population but begins earlier and may be severe. The mechanisms by which diabetes accelerates atherosclerosis are not well understood. The customary clusters of risk factors for coronary artery disease (CAD), which are more common in patients with diabetes, are not sufficient to explain this phenomenon.

The present study was under taken to evaluated the possible relationship between the circulating levels of the modified derivatives of LDL and the development of angiopathy in type 2 diabetic patients with coronary artery disease. The status of the antioxidant defenses and the role of supplementation with antioxidant combination, as a free radical scavenger was also studied in these patients.

This study was conducted on 3 groups:

-Group I (control), Group II: type 2 diabetic patients without complications and, Group III: type 2 diabetic patients with stable coronary artery disease. After establishing the baseline values for different studied parameters, patients in this group received adjunct treatment of antioxidant tablets for three months and the assessed parameters were re-evaluated after three months of supplementation. The supplements consisted of one daily tablet of antioxidant combination containing: Vitamin E, Ascorbic acid, Vitamin A acetate, and Selenium.

The assessed parameters were included:

-Glycemic Control: fasting plasma glucose (FPG), and glycated hemoglobin (HbA1c).

-Lipid pattern: triglycerides, Total cholesterol, HDL–cholesterol, LDL–cholesterol, and Apolipoprotein B (Apo B). Modified LDL: Circulating oxidized LDL, Circulating oxidized LDL– antibody, and susceptibility of LDL to Oxidation in vitro.

-Oxidative stress and antioxidant defense: thiobarbituric acid reactive substance (TBARS), free thiol group, and total antioxidant status.

-Carotid artery intima-media thickness (IMT) was measured by carotid ultrasound scanner for patients of group III at baseline and after 3 months of supplementation.

As hyperglycemia is the hallmark of diabetes mellitus, it is logical to find that fasting plasma glucose (FPG) levels were higher in the diabetic

groups. However, no significant difference was observed in the plasma glucose level between the two groups of diabetic patients.

The results of present study clearly indicated that there was a definite overproduction of free radicals and excessive exposure of diabetic patients to oxidative stress. The lipid peroxidation index; thiobarbituric acid-reactive substances, was significantly higher in both diabetic groups than in healthy individuals. Moreover, by comparing the two diabetic groups together there was a significant increase in patients with CAD. The increase in free radicals was coupled with disturbance in free radical scavengers, particularly the glutathione system. We found that, GSSG was higher, while the total GSH and reduced GSH were generally lower in diabetic groups than in controls.

By inspecting the calculated redox potential in the present study, it became clear that redox potential for diabetic groups was shifted to the oxidizing side and the supplementation of group III with antioxidant combination partially corrected the balance of GSH/GSSG couple to restore the reducing potentials especially after three months. These results may indicate that the calculated level of cell redox potential may provide useful means to quantitatively express the oxidant/antioxidant balance in diabetic condition. In addition, we found a clear deficiency in total antioxidant status (TAS) in diabetic patients when compared with control non-diabetic subjects.

The present study has shown alteration in the lipid profile in diabetic groups as compared to the healthy control subjects. The two diabetic

groups had significant increase in the levels of triglycerides, moderately raised cholesterol levels, and lower levels of HDL cholesterol compared to the control group. On the other hand, LDL-cholesterol concentrations were very similar in control and diabetic groups. Apolipoprotein B is the protein moiety of LDL. The clinical interest of this protein lies in the fact that it provides a relatively accurate estimate of circulating LDL particle numbers. Our study shown that, ApoB serum concentration was significantly elevated in diabetic groups when compared with healthy control group.

Oxidative modification of LDL has been implicated as a major factor in the pathogenesis of coronary atherosclerosis. To examine whether the presence of type 2 diabetes may affect LDL modification we measured the levels of circulating OxLDL, and OxLDL-Ab and the *in vitro* oxidative susceptibility of LDL subfractions. The levels of circulated OxLDL in patients with type 2 diabetes were significantly higher than in control subjects. In addition, diabetic patients with CHD had higher levels of OxLDL than patients without cardiovascular complication.

The intima-media thickness (IMT) is at present the best-studied sonographic marker for early atherosclerotic vascular wall lesions. By ultrasonography, we have demonstrated that diabetic patients with CAD had increased intima-media thickness of carotid arteries, suggesting that an increased oxidized LDL level is a marker of atherosclerosis .

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Although adjunct therapy with antioxidant mixture improved the total antioxidant status of the treated diabetic patients, which resulted in significant decrease in OxLDL, there was no immediate reciprocal effect on the thickness of the intima media. It is possible that more time is required for mobilization of the cholesterol precipitated in the atheromatous plaques. The supplementation with exogenous antioxidants is expected to give rapid improvement in the antioxidant status leading to suppression of free radical production and consequent decrease in OxLDL. The mobilization and disappearance or decrease in size of the atheromatous plaques are expected to proceed at a slower rate, or at least they would not increase in size. Return of the thickness of the intima to values within the clinically normal range, and hence improvement of atherosclerosis in diabetics, is expected to take more time and would probably be expedited by hypolipidimic agents.

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A very encouraging observation in the present study is the favorable response of OxLDL level to the antioxidant adjunct therapy. The definite and steady decline in OxLDL level in type 2 diabetic patients with CAD was clear and is indicative of improvement in the atherosclerotic condition. The time factor is also important, as the decline in the level below the 100-110 U/L suggested ceiling occurred within only 3 months of therapy. Such lower level indicates cessation of the progress of the atherogenic process, such that the atheromatous plaques in the intima media of the carotid artery would stop growing, before improvement in the level of HDL and HDL-dependent reverse cholesterol transport.

From the data obtained in the present study and accompanying discussion it is clear that the plasma levels of oxidized LDL correlate with the extent of coronary artery disease in type 2 diabetic patients and suggest that elevated levels of oxidized LDL, rather than oxidized LDL antibodies, can serve as an independent and significant predictor for future cardiac events in type 2 diabetic patients with CAD.

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ARABIC SUMMARY

الملخص العربى

الملخص العربى

يتصف داء السكرى (البول السكرى) بارتفاع مزمن فى نسبة سكر الدم وخلل فى أيض كلا من الكربوهيدرات والدهون والبروتينات، وذلك نتيجة لنقص فى إفراز أو تأثير هرمون الأنسولين أو كلاهما. ويقدر الذين يعانون من هذا المرض من البالغين فى العالم بحوالى ١٧٣ مليون شخص حسب إحصائيات عام ٢٠٠٢.

ومن المعروف أن داء السكرى يصاحبه زيادة فى معدلات الإصابة بالمضاعفات القلبية الوعائية بما فيها مرض الشريان التاجى وأمراض الأوعية الدماغية والأوعية الطرفية. وتعتبر هذه الأمراض ذات الصلة بتصلب الشرايين السبب الرئيسى فى حالات الوفيات فى مرضى النوع الثانى من الداء السكرى. وقد وجد أن معدلات الإصابة بمرض الشريان التاجى تزيد فى مرضى النوع الثانى من داء السكرى بنسبة تتراوح من ٢ إلى ٤ أضعاف.

ولا تختلف طبيعة مرض تصلب الشرايين فى مرضى الداء السكرى عن غيرهم من المرضى إلا فى كونه يظهر مبكراً ويكون أكثر حدة فى مرضى الداء السكرى. ولكن الآلية التى يتسبب بها داء السكرى فى تسريع الإصابة بتصلب الشرايين لم تفهم بعد بشكل واضح، حيث لا يكفى كون العوامل المسببة لمرض الشريان التاجى أكثر انتشاراً فى مرضى الداء السكرى بتفسير هذه الظاهرة.

وكان هدف هذا البحث هو تقدير إمكانية وجود علاقة بين مستويات البروتين الدهنى المعدل منخفض الكثافة فى الدم وتطور مرض الشريان التاجى فى مرضى النوع الثانى من داء السكرى. وكذلك دراسة حالة مضادات الأكسدة والدور الذى يمكن أن يلعبه إضافة خليط من مضادات الأكسدة- والتى تمثل مصائد للشقوق الحرة - إلى علاجات هؤلاء المرضى.

وقد أجريت هذه الدراسة على ثلاثة مجاميع:

- **المجموعة الأولى (القياسية)**: وقد اشتملت على ١٥ شخص من الأصحاء.
- **المجموعة الثانية:** وقد ضمت ١٥ مريض بالنوع الثانى من داء السكرى غير المصحوب بمضاعفات.
- **المجموعة الثالثة**: وقد ضمت ١٥ مريض من مرضى النوع الثانى من داء السكرى المصحوب بمرض الشريان التاجى المستقر. وبعد استقراء قيم الدلالات المختلفة لهذه المجموعة عند بداية

التجربة تم تزويد أفرادها بأقراص من خليط من مضادات الأكسدة لمدة ثلاثة شهور وقد أعيدت لهم نفس التحاليل بعد شهر وثلاثة شهور من بداية العلاج.

وقد احتوت هذه الأقراص على فيتامين هـ وفيتامين أ وفيتامين س وعنصر السيلينيوم.

- **وقد تم قياس ما يلى فى دم كل أفراد المجموعات الثلاثة:**

أ- **<u>على مستوى قياس التحكم فى سكر الدم تم قياس:</u>**

- مستوى سكر الدم الصائم.
- مستوى سكر الدم المرتبط بالهيموجلوبين فى الدم.

ب- **<u>على مستوى الدهون ثم قياس:</u>**

- الدهون الثلاثية • الكوليسترول الكلى
- كوليسترول البروتين الدهن عالى ومنخفض الكثافة
- الأبوبروتين ب
- مستوى البروتين الدهن منخفض الكثافة المؤكسد وكذلك أجسامه المضادة فى الدم.
- قابلية البروتين الدهن منخفض الكثافة للأكسدة فى أنبوب الاختبار.

ج- **<u>على مستوى الإجهاد التأكسدى ودفاعات مضادات الأكسدة تم قياس:</u>**

- المالون داى ألدهيد كمؤشر على الأكسدة الفائقة للدهون.
- مجموعة الثيول الحرة.
- حالة مضادات الأكسدة الكلية.

- بالإضافة إلى ذلك تم قياس سمك الطبقة الباطنة الوسطى للشريان السباتى بواسطة تقنية الفحص الدقيق بالموجات فوق الصوتية لمرضى المجموعة الثالثة عند بداية الدراسة وبعد ٣ شهور من الإمدادات التكملية بمضادات الأكسدة.

وجاءت نتائج الدراسة كما يلى:

- ارتفاع نسبة سكر الدم فى مرضى مجموعتى الداء السكرى بالنسبة إلى المجموعة القياسية بينما لا يوجد فرق ذو دلالة بين مجموعتى مرض الداء السكرى.

- دلت هذه الدراسة بشكل واضح عن فرط فى إنتاج الشقوق الحرة عند مرضى الداء السكرى وتعرضهم للإجهاد التاكسدى بشكل كبير وقد دل على ذلك دليل الأكسدة الفائقة للدهون. المالون داى ألدهيد. حيث وجد عالى بشكل ذو دلالة فى كلا مجموعتى مرضى الداء السكرى بالمقارنة بالمجموعة القياسية والأهم أنه بمقارنة مجموعتى مرضى الداء السكرى وجدنا زيادة ذات دلالة فى المجموعة الثالثة والمقترنة بمرض الشريان التاجى عن الغير مقترنة.
- إضافة إلى ذلك وجد أن زيادة الشقوق الحرة مترافقة مع اضطراب فى كاسحات هذه الشقوق وبخاصة نظام الجلوتاثيون من خلال ارتفاع نسبة الجلوتاثيون المؤكسد فى مقابل انخفاض واضح فى الجلوتاثيون الكلى والمختزل فى مجموعتى داء السكرى بمقارنتها بالمجموعة الضابطة.

وبحساب جهد التأكسد فى المجموعات وجد أنه يميل ناحية الأكسدة فى مجموعتى مرضى داء السكرى وأن العلاج التكميلى بمضادات الأكسدة فى المجموعة الثالثة يعمل على إصلاح التوازن بين الجلوتاثيون المختزل والمؤكسد ويعيد الجهد الاختزالى خاصة بعد ٣ شهور من العلاج التكميلى بمضادات الأكسدة. وتدل تلك النتائج على أن حساب مستوى الجهد التاكسيدى للخلية ربما يزودنا بطرق قياس كمية لتوازن المؤكسدات ومضادات الأكسدة فى حالة داء السكرى.

بالإضافة إلى ذلك، وجد نقص واضح فى حالة مضادات الأكسدة الكلية فى مرضى داء السكرى بالمقارنة مع المجموعة القياسية.

وبالمقارنة أيضا بالمجموعة القياسية وجد اختلاف فى صورة الدهون فى مجموعتى مرضى داء السكرى. حيث وجدت زيادة ذات دلالة واضحة فى مستوى الدهون الثلاثية وزيادة بسيطة فى مستويات الكوليسترول ونقص ذو دلالة فى مستوى تركيز البروتين الدهنى عالى الكثافة بينما كان تركيزات البروتين الدهنى منخفض الكثافة متساوية بشكل كبير بين مجموعتى مرضى الداء السكرى والمجموعة القياسية.

أما بالنسبة للشق البروتينى الأبوبروتين "ب" والذى يمثل قياس دقيق نسبيا لعدد جزئيات البروتين الدهنى منخفض الكثافة، فقد وجدنا أنه مرتفع بشكل ذو دلالة فى مجموعة مرضى داء السكرى عندما تقارن مع المجموعة القياسية.

ويعتبر التعديل التأكسدى للبروتين الدهنى منخفض الكثافة عامل أساسى فى عملية نشوء مرض تصلب الشريان التاجى ولاختبار تأثير النوع الثانى من

داء السكرى فى تعديل البروتين الدهنى منخفض الكثافة تم قياس مستويات البروتين الدهنى منخفض الكثافة المؤكسد ومضاداته وقابلية هذا المركب للأكسدة فى أنبوب الأختبار. وقد وجد أن مستويات هذا البروتين الدهنى المؤكسد فى الدم فى مرضى النوع الثانى من داء السكرى عالى بقدر ذو دلالة أحصائية فى هؤلاء المرضى مقارنة بالمجموعة الضابطة. وبالإضافة إلى ذلك وجد هذا البروتين الدهنى المؤكسد أعلى بمقدار ذو دلالة فى مجموعة مرضى داء السكرى المقترن بمرض الشريان التاجى عن الذين هم بدون هذه المضاعفات.

ويعتبر قياس سمك الطبقة الباطنة الوسطى حالياً دليل جيد لبؤر تصلب جدار الأوعية مبكراً. وعن طريق جهاز القياس الدقيق بالموجات فوق الصوتية وجدت أن زيادة فى سمك الطبقة الباطنة الوسطى فى الشريان السباتى فى المجموعة الثالثة من مرضى الداء السكرى مما يؤكد أن زيادة مستوى البروتين الدهن المنخفض الكثافة المؤكسد مؤشر على مرض تصلب الشرايين.

وهذه العلاقة بين منسوب هذا البروتين الدهنى المؤكسد وسمك الطبقة الباطنة الوسطى للشريان السباتى تستحق الملاحظة. حيث أن نتائج هذه الدراسة تشير إلى أن سمك الطبقى الباطنة الوسطى للشريان السباتى تكون فى حدود القيم الطبيعية المقبولة طالما أن مستوى البروتين الدهنى منخفض الكثافة المؤكسد تحت القيم من ١٠٠ إلى ١١٠ وحدة/لتر. وعندما يرتفع هذا المستوى أعلى من هذا المدى يظهر زيادة حادة فى سمك الطبقة الباطنة الوسطى وعلى ذلك يجب أن يظل مستوى هذا البروتين الدهنى المؤكسد أقل من مدى ١٠٠-١١٠ وحدة/لتر لتفادى تأثير هذا العامل.

وبالرغم أن العلاج المكمل بخليط مضادات الأكسدة يحسن حالات مضادات الأكسدة الكلية للمجموعة المعالجة بما يؤدى إلى نقص ذو دلالة فى مستوى البروتين الدهنى المنخفض الكثافة المؤكسد إلا أنه ليس هناك تأثير تبادلى على سمك الطبقة المبطنة الوسطى. وربما لابد من وقت أطول لتحريك الكوليسترول المرسب على جدار الشريان المتصلب.

ولعودة سمك الطبقة الباطنة إلى القيم الداخلة فى المدى الطبيعى وتحسن حالة تصلب الشريان فى الداء السكرى يجب أن تتوقع أن يأخذ هذا وقت أطول وربما يحتاج إلى تسهيل بواسطة عوامل خافضات الدهون.

وأيضا وجدنا مستوى نشاط الأجسام المضادة للبروتين الدهنى منخفض الكثافة المؤكسد أعلى بشكل ذو دلالة فى مرض داء السكرى عن المجموعة الضابطة ولكن لا يوجد هذا الفرق بين مجموعتى مرض الداء السكرى.

والملاحظة المشجعة جداً فى هذه الدراسة هى الاستجابة المؤاتية فى مستوى البروتين الدهنى منخفض الكثافة المؤكسد للعلاج التكميلى بمضادات الأكسدة. ومثل هذا الانخفاض فى مستوى البروتين الدهنى منخفض الكثافة يدل على انقطاع عملية التصلب فى الشرايين حيث وقف نمو الدقائق التصلبية فى الطبقة الباطنة الوسطى للشريان السباتى قبل تحسن مستوى نقل الكوليسترول العكسى المعتمد على البروتين الدهنى المنخفض والمرتفع الكثافة.

هكذا تظهر هذه الدراسة بوضوح أن مستويات البروتين المنخفض الكثافة والمؤكسد فى البلازما تتلازم مع مدى مرض الشريان التاجى فى مرضى النوع الثانى من الداء السكرى وأن زيادة مستويات البروتين الدهنى منخفض الكثافة المؤكسد ـ مفصلا على الأجسام المضادة له ـ يمكن أن يصلح كنظام تنبؤ مستقل وذو دلالة وقيمة عالية فى التوقعات المستقبلية المتصلة بحالة القلب فى مرضى النوع الثانى من داء السكرى المقترن بمرض الشريان التاجى.

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الدراسات العليا

تم موافقة أ.د./ عميد المعهد على تشكيل لجنة الحكم بالتفويض فى ٢٠٠٥/٥/١٠ .

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موافقة مجلس المعهد على منح درجة الدكتوراه

يعتمـــد

وكيل المعهد للدراسات العليا والبحوث

أ.د. إبراهيم محمد العكارى

العلاقة بين مستويات البروتين الدهنى المعدل منخفض الكثافة ومرض الشريان التاجى فى مرضى النوع الثانى من داء السكرى

رســـــــــــــالة
مقدمة إلى معهد البحوث الطبية – جامعة الإسكندرية
إيفاءاً جزئياً لشروط الحصول على درجة
الدكتـــــــــوراه
في
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تم تحمل التعديلات المطلوبة

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٢٠٠٥