ANTIOXIDANT ENZYME ACTIVITIES IN RAT LIVER TISSUES OF DIABETIC RATS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

GÖKHAN SADİ

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

JULY 2004

Approval of the Graduate School of Natural and Applied Sciences.

_____

Prof. Dr. Canan ÖZGEN
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____

Prof. Dr. Orhan ADALI
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____

Prof. Dr. Tülin GÜRAY
Supervisor

Examining Committee Members

Prof. Dr. Zeliha BÜYÜKBİNGÖL (Ank. Uni., Pharmacy) _____

Prof. Dr. Orhan ADALI (METU, Biology) _____

Prof. Dr. Hüseyin Avni ÖKTEM (METU, Biology) _____

Assoc. Prof. Dr. Nursen ÇORUH (METU, Chemistry) _____

Prof. Dr. Tülin GÜRAY (METU, Biology) _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: **Gökhan SADİ**

Signature:

ABSTRACT

ANTIOXIDANT ENZYME ACTIVITIES IN RAT LIVER TISSUES OF DIABETIC RATS

SADİ, Gökhan

M.Sc., Department of Biochemistry

Supervisor: Prof. Dr. Tülin GÜRAY

July 2004, 115 pages

Free radicals are the compounds having one or more unpaired electrons in their outer orbital and this unpaired electron make these compounds very reactive. Especially as their concentration increases, they initiate a chain oxidation reaction of lipids, proteins and nucleic acids. The condition, in which the production of free radicals exceeds their elimination or tissue defense mechanism decrease against them or both occur together, is called oxidative stress. In diabetes mellitus which is a glucose metabolism disorder, there occurs excessive non-enzymatic protein oxidation, glucose autoxidation and enhanced activity of polyol pathway enzymes, which are the possible sources of the oxidative stress in this disease.

In this study, the conditions of the activity measurements of major antioxidant enzymes, namely superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, 1.11.1.9) and glutathione S-transferase (GST, EC 2.5.1.18) were studied and the optimum conditions (pH, temperature and substrate concentrations) for each assay were determined.

Further objectives of the study were to characterize the enzymatic antioxidant systems (catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferase), tissue oxidation status (concentrations of TBARS, protein carbonylation, and lipid/protein ratios) and nonenzymatic antioxidant (reduced glutathione) levels of the diabetic rat liver tissues.

According to our results, the hepatic SOD and GPx activities significantly increased whereas CAT activity markedly decreased in diabetic rats compared to control group. Also, GST activities did not change in diabetes. As a result of oxidative stress, TBARS concentration, lipid/protein ratios and protein carbonylation increased and GSH levels decreased in diabetic rats compared to control rats. This increase in tissue damage, in spite of the increase in antioxidant enzyme activities, could have been due to the overproduction of reactive oxygen species that exceeded the capacity of the antioxidant enzymes during the eight week of diabetes.

Key words: Antioxidants, Oxidative stress, Diabetes mellitus, Lipid Peroxidation, Protein carbonylation.

ÖZ

DİYABETİK SIÇAN KARACİĞER DOKULARINDA ANTİOKSİDAN ENZİM AKTİVİTELERİ

SADİ, Gökhan

Yüksek Lisans, Biyokimya Bölümü

Tez Danışmanı: Prof. Dr. Tülin GÜRAY

Temmuz 2004, 115 sayfa

Serbest radikaller dış orbitallerinde eşlenmemiş bir yada birden fazla elektron sahibi moleküllerdir ve bu eşlenmemiş elektronlar bu molekülleri çok reaktif hale getirirler. Özellikle konsantrasyonları arttığında, lipitler, proteinler ve nükleik asitler ile zincirleme reaksiyonları başlatırlar. Serbest radikallerin üretiminin artması, yada doku savunma mekanizmaları tarafından kaldırılmasının azalması yada bu iki durumun aynı anda meydana gelmesi durumuna oksidatif stress adı verilir. Bir glukoz metabolizması bozukluğu olan diyabette meydana gelen yüksek miktardaki enzimatik olmayan protein şekerlenmesi, glukoz otoksidasyonu ve polyol zincirindeki enzimlerin aktivitelerinin artması, bu hastalıktaki muhtemel oksidatif stres kaynağıdır.

Bu çalışmada, en önemli antioksidan enzimler, yani süperoksit dismutaz (SOD, EC 1.15.1.1), katalaz (CAT, EC 1.11.1.6), glutatyon peroxidaz (GPx, EC 1.11.1.9) ve glutatyon S-transferaz (GST, EC 2.5.1.18), aktivite ölçüm şartları çalışıldı ve en uygun şartlar (pH, sıcaklık ve substrat konsantrasyonu) her bir enzim için belirlendi.

Çalışmanın sonraki amaçları ise enzimatik antioksidan sistetemlerinin (katalaz, süperoksit dismutaz, glutatyon peroksidaz ve glutatyon S-transferaz), doku oksidasyon durumunun (TBARS konsantrasyonu, protein karbonilasyonu, ve lipit/protein oranları) ve enzimatik olmayan antioksidan (GSH) seviyelerini diyabetik sıçan karaciğer dokularında tanımlamaktır.

Sonuçlarımıza göre, diyabetik sıçan karaciğer SOD ve GPx aktiviteleri anlamlı derecede arttı, fakat CAT aktivitesi kontrol grubuna göre önemli ölçüde azaldı. Ayrıca, GST aktiviteleri diyabette değişmedi. Oksidatif stresin sonucu olarak diyabette TBARS kosantrasyonu, lipit/protein oranları ve protein karbonilasyonu yükseldi ve GSH seviyesi kontrol grubuna göre azaldı. Buna göre, sekiz haftalık diyabette, antioksidan enzim aktivitelerinin artmasına karşı, doku hasarının artması, reaktif oksijen moleküllerinin üretiminin, antioksidan enzimlerinin kapsitesini aşmasının bir sonucu olabileceğini düşünmekteyiz.

Anahtar kelimeler: Antioksidanlar, Oksidatif stres, Diyabet, Lipid peroksidasyonu, Protein karbonilasyonu

To my mother…

ACKNOWLEDGEMENTS

I would like to thank to my supervisor Prof. Dr. Tülin Güray for her encouraging guidance, incredible patience, and supervision throughout this thesis study. Her lovely attitude was not restricted to this thesis study.

I would like to thank to my lab mates, Y. Senem Su, Güvenç Görgülü, Belgin İşgör, Ebru Saatçi and Metin Konuş for their sincere friendship and supports.

I wish to thank my friends Aytaç Kocabaş, Tahir Bayraç, Sefa Denizoğlu, and Murat Yatağan for their friendship, encouragement and support.

I would like to send my ultimate appreciation to my fiancée Özlem Türk for her endless patience, encouragement, support and love.

I am very grateful to family for their eternal love, encouragement and trust.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER I

INTRODUCTION

The recent growth in knowledge of free radicals and reactive oxygen species (ROS) in biological systems is producing a medical revolution that promises a new age of health. They play an important role in living systems through their beneficial and detrimental effects (Halliwell, 1999, Gutteridge and Halliwell, 2000).

Atoms contain a nucleus, and electrons move around it usually in pairs. A free radical is any atom or molecule that contains one or more unpaired electrons. The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical. The hydrogen radical (same as hydrogen atom) contains 1 proton and 1 electron (therefore unpaired), is the simplest free radical by definition. By interacting with cellular components, free radicals may cause cellular and genetic damage. Their involvement has been implicated in several diseases such as atherosclerosis, HIV/AIDS, cancer, chronic fatigue syndrome, asthma, arthritis, cystic fibrosis, multiple sclerosis, fibromyalgia, and other immune disorders (McCord, 1985, Loft and Poulsen, 1997).

Radicals can be produced in the cells and tissues of our bodies by various processes and reactions. For instance; the impact of radiation such as high energy radiation, ionizing radiation, visible light with photosensitizes and thermal degradation of organic materials can produce hemolytic fission, by the loss of a single electron from a non-radical, or by the gain of a single electron, where the electron pair of a covalent bond is shared between two atoms resulting in each having an odd electron (Halliwell and Gutteridge, 1989).

The body makes another oxygen radical (i.e. the unpaired electron is located on oxygen), superoxide ($O_2^{\cdot -}$) anion. Superoxide is made by adding one electron to the oxygen molecule. It is generally poorly reactive. Some superoxide is made by "accidents of chemistry", in that many molecules in the body react directly with oxygen to make superoxide. Examples include the catecholamines, tetrahydrofolates, and some constituents of mitochondrial and other electron transport chains and such superoxide generation is unavoidable. During the production of ATP in the electron transport chain, where the terminal electron acceptor is oxygen, approximately 1-5% of all oxygen escapes as free radical intermediates. The various Fe-S proteins and NADH dehydrogenase have also been implicated as possible sites of superoxide and hydrogen peroxide formation (Turrens *et al*., 1982). Probably, the most important sources of superoxide anion in vivo in most aerobic cells are electron transport chains of mitochondria and endoplasmic reticulum.

Various oxidative processes, including oxidation, hydroxylation, dealkylations, deaminations, dehalogenation and desaturation, occur on the smooth

endoplasmic reticulum. Mixed function oxygenases that contain a heme moiety add an oxygen atom into an organic substrate using NAD(P)H as the electron donor (McKersie, 1996). The generalized reaction catalyzed by cytochrome P450 is:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_3O^+$$

Superoxide is produced by microsomal NAD(P)H dependent electron transport involving cytochrome P450 (Winston and Cederbaum, 1983). One possible site at which this may occur is shown in Figure 1.1. After the univalent reduction of the substrate (RH) and the addition of triplet oxygen to form the complex P450 - RHOO the complex may decompose to P450-RH and release superoxide.

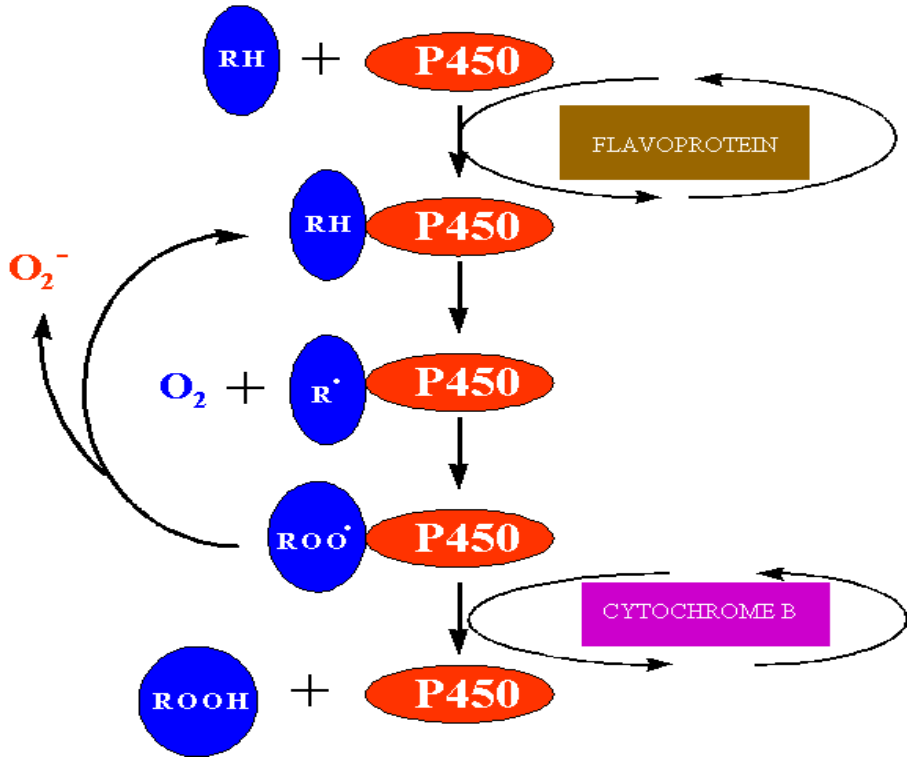


Figure 1.1: Schematic representation of the cytochrome P450 electron transport system on the endoplasmic reticulum showing one possible site of superoxide production.

Another well known active source of superoxide anion is xanthine oxidase. Radicals are formed during the oxidation of xanthine to uric acid (Ghe *et al*., 1985). If an aerobic cell is subjected to insufficient oxygen, tissue energy stores are depleted of high energetic phosphorus compound, such as ATP, leaving hypoxanthine. Upon reoxidation, hypoxanthine is utilized to restore ATP. If, however, tissue hypoxia is prolonged, xanthine oxidase is activated and it catabolizes hypoxanthine to xanthine and subsequently to uric acid to form superoxide radicals. Xanthine oxidase, urate oxidase, and NAD(P)H oxidase generate superoxide as a consequence of oxidation of their substrates (McKersie, 1996).

Cells of the immune system have a special role in production of superoxide anion. Neutrophiles contain a plasma membrane bound enzyme complex, the NADPH oxidase, which reduces oxygen with NADPH to produce copious amounts of superoxide anion which is essential for effective bacterial killing. Absence of this enzyme activity is responsible for an inherited human condition called chronic granulomatous disease that is characterized by recurrent infections.

Superoxide anion is not particularly reactive molecule by itself but, it can diffuse considerable distances from its site of production. It may combine with other reactive species such as nitric oxide or hydrogen peroxide. Under normal cellular conditions, superoxide anions are readily converted into hydrogen peroxide by enzymatic reactions mostly catalyzed by superoxide dismutases. Even though hydrogen peroxide is not a free radical, it is the major sources of the most reactive free radical namely hydroxyl radical ($OH^{\cdot}$). In the presence of a transition cation such

as iron or copper, superoxide anion in combination with hydrogen peroxide, can give rise to the highly reactive **hydroxyl radical** species ($HO\cdot$) by the Haber-Weiss Reaction.

$$Fe^{III} + O_2\bullet^- \rightarrow Fe^{II} + O_2$$

$$+ \underline{Fe^{II} + H_2O_2 \rightarrow Fe^{III} + HO\bullet + HO^-}\ \underline{\textit{(fenton reaction)}}$$

Haber-Weiss Reaction: $O_2\bullet^- + H_2O_2 \rightarrow O_2 + HO\cdot + HO\cdot$

Hydroxyl radical is considered to be a principal actor in the toxicity of partially reduced oxygen species, since it is very reactive with all kinds of biological macromolecules, producing products that cannot be regenerated by cell metabolism.

Low-wavelength electromagnetic radiation (e.g., gamma rays) cans split water in the body to generate hydroxyl radical, OH^-. This fearsomely reactive radical, once generated, attacks whatever it is next to. Its lifetime in vivo is vanishingly small because hydroxyl radical reacts at its site of formation, usually leaving behind a legacy in the form of propagating free-radical chain reactions.

Another physiological free radical is nitric oxide (NO^-), which is made by vascular endothelium as a relaxing factor, and also by phagocytes. Nitric oxide has many useful physiological functions, but excess nitric oxide can be toxic. Neither superoxide nor nitric oxide is highly reactive chemically, but under certain circumstances they can generate more toxic products.

Actions of some toxins and drugs may also induce the production of free radicals and their metabolites. For instance, metabolism of drugs such as

phenylbutazone, penicillamine and nitrofurantoin (İşcan *et al.,* 1995) generate oxygen free radicals in some in vitro experiments.

Factors such as the site of production, the availability of transition metals, and the action of enzymes determine the fate of each radical species and its availability for reaction with cellular molecules. The noxious consequences of free radicals in biological systems are manifold and include damage to all cell constituents which are prone to oxidative attack. Mainly uncontrolled production of free radicals often leads to damage of cellular macromolecules and other small antioxidant molecules (Figure 1.2).

The reactions of free radicals with organic substrates are complex in biological systems due to the properties of macromolecules such as electrical charges, binding properties, and compartmentalization of enzymes, substrates, and catalysts. Thus, various sites even within a single cell differ in the nature and extent of the reactions with free radicals.

The nature of oxidative injury is not always obvious but mechanism by which oxygen radicals' damage to the membrane lipids are well accepted and consequently oxidative damage is often exclusively associated with peroxidation reactions in membrane lipids. Peroxidation of membrane phospholipids may result in altered fluidity of membranes and altered permeability characteristics (e.g. leading to abnormal influx of Ca^{++} with the resulting disregulation of cell functions). Oxygen free radicals also damage DNA by oxidative scission, leading to mutations and

carcinogenesis. Oxidative destruction of polysaccharides may result in altered functioning and finally oxidation of proteins may result in loss of the functions.

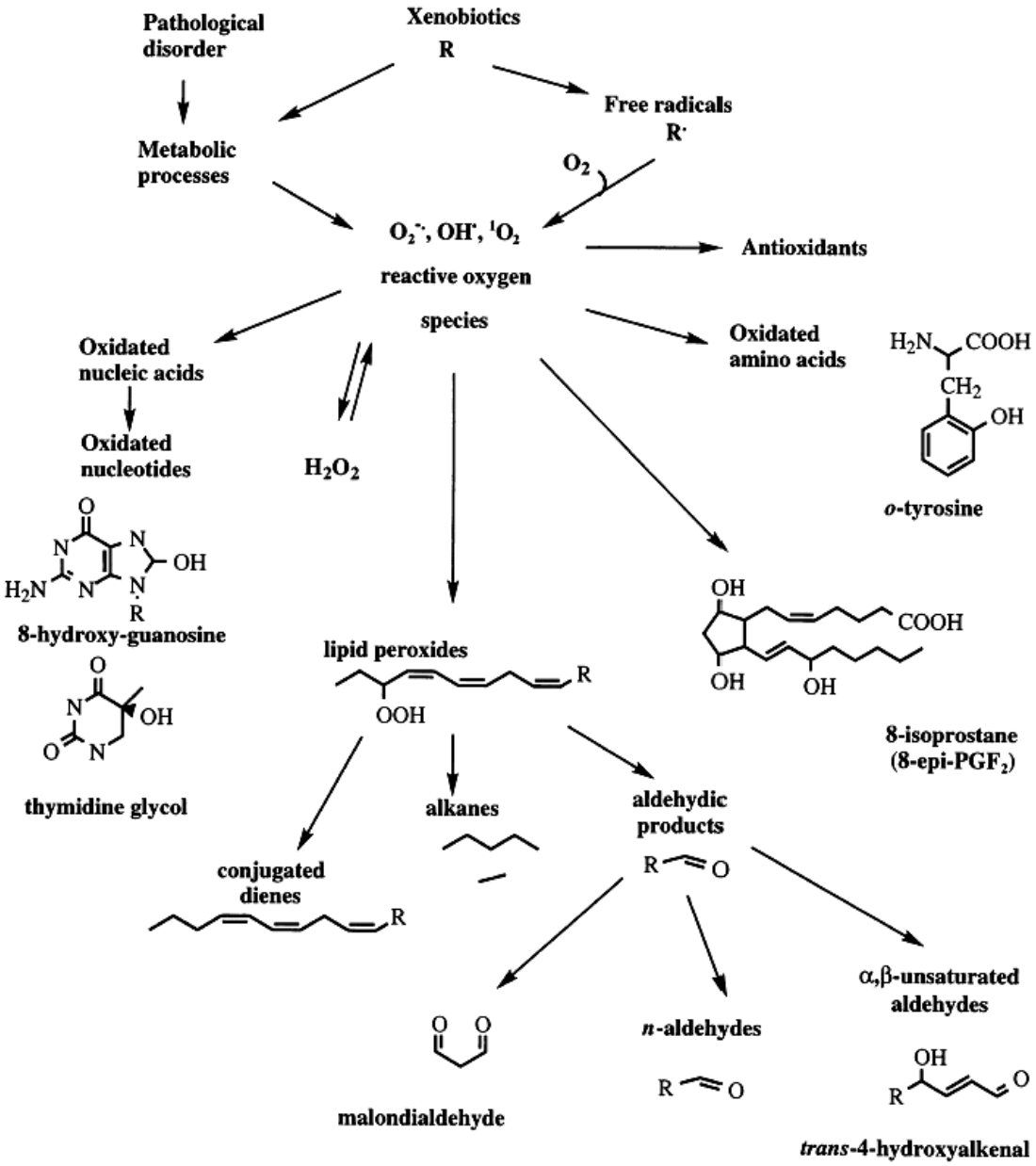


Figure 1.2: Products of free radical damage. Free radicals may react with different cellular macromolecules, such as DNA, components of cell membranes or proteins, which eventually may lead to many different products.

A number of major cellular antioxidant defense mechanisms exist to neutralize the damaging effects of free radicals. Antioxidant defenses have been evolved to protect the cells against free radicals. Superoxide dismutases which are present in cytosol and mitochondria convert superoxide to hydrogen peroxide. Catalases are found in peroxisomes in most tissues and they remove hydrogen peroxide. Glutathione peroxidases are major enzymes that remove hydrogen peroxide generated by SOD in cytosol and mitochondria by oxidizing the tripeptide glutathione (GSH) into its oxidized (GSSH) form (Chance *et al*., 1979). Also, xenobiotic metabolizing phase 2 detoxification enzymes (glutathione S-transferase) are working in the detoxification reactions of oxidatively modified molecules and peroxides in cells and they are mainly found in cytosol.

Iron and Cupper ions are powerful promoters of free radical damage, and therefore, a complex system of transport and storage proteins ensure that these essential metals are rarely allowed to be "free". Ferritin is the usual storage form of iron and iron within ferritin will not stimulate free radical reactions. Other antioxidant defenses are the macromolecules such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), reduced glutathione (GSH), melatonin, and β-carotene (precursor of vitamin A). The lipophilic ones, mainly vitamin E, located inside the membrane and in the structure of lipoproteins. They block the chain reaction of lipid peroxidation and produce much less reactive tocopherol radical, which are further reduced by ascorbate (Figure 1.3). The final oxidized ascorbate is reduced enzymatically via

ascorbate reductase. Urate is present in body fluids and is the end product of purine metabolism. It also scavenges several free radicals (Kaur and Halliwel, 1990).

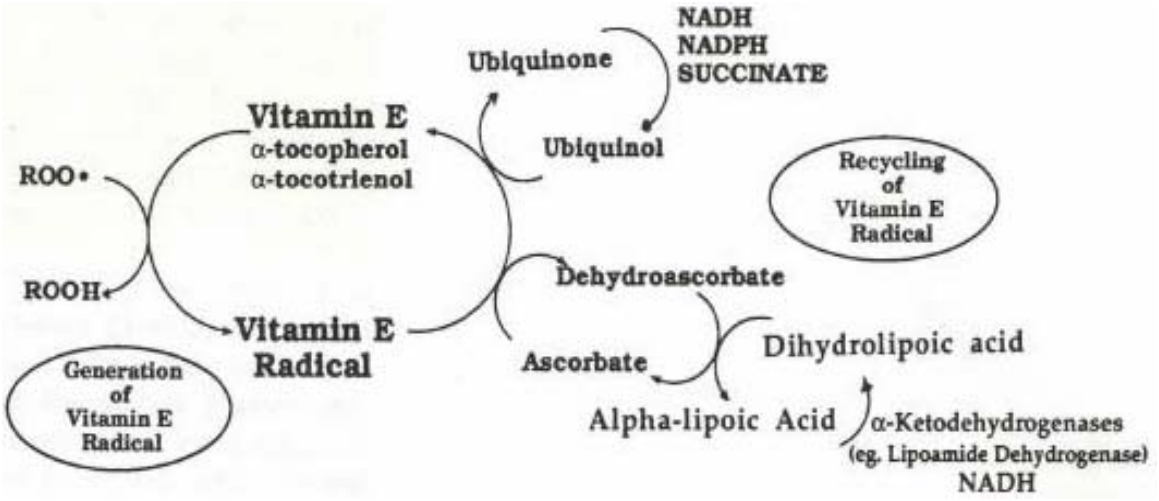


Figure 1.3: Vitamin E and vitamin C cycle as an antioxidant mechanism.

Despite all these antioxidants, some free radicals still escape to do damage, and repair enzymes exist to remove free-radical damaged proteins, oxidized fatty acids and damaged DNA molecules (Braimer, 1991)

Oxidative stress occurs when there is an imbalance between free radical reactions and the scavenging capacity of antioxidative defense mechanism of the organism (Sies, 1991). In principle, oxidative stress can be caused by increased production of free radicals or diminished antioxidants (e.g. mutations affecting antioxidant defense systems and depletions of dietary antioxidants). As a result; consequences of oxidative stress will be adaptation or cell injury, disruption in cellular homeostasis and accumulation of damaged molecules.

The tissue level of antioxidants critically influences the susceptibility of various tissues to oxidative stress. Enhanced oxidative stress and oxidative damage to

tissues are general features of some chronic diseases such as Alzheimer's disease, cancer, atherosclerosis and diabetes mellitus (Berlett and Stadtman, 1997, Beal, 2002). If mild oxidative stress occurs, tissues often respond by making extra antioxidant defenses. However, severe oxidative stress can cause cell injury and death. Free radical induced cell death can proceed as necrosis or apoptosis, and "anti-apoptosis genes" in certain cells appear to encode free radical scavengers (Sarafian and Bredesen, 1994)

1.1 DIABETES MELLITUS

Diabetes mellitus, from the Greek words meaning "siphon" and "sweet," is a common disease of altered glucose utilization that is usually characterized by three symptoms when not adequately controlled by therapy: polyphagia, polydipsia and polyuria. In the 19th century, prior to the availability of standard chemical tests for the measurement of glucose, physicians or their technicians would actually taste the urine of suspected diabetics to diagnose this disease. A sweet taste indicated the likelihood of diabetes. In diabetes mellitus the body's cells experience a relative starvation for glucose because of the lack of insulin action. Insulin is the pancreatic hormone that functions to assist in the uptake and utilization of glucose, the most important fuel source of the body. The uptake of glucose into cells, where it functions as a metabolic fuel, is dependent upon insulin in many different organs and tissues. Insulin also aids in enhancing a number of important enzymes involved in the subsequent metabolism of glucose within the cell. Without adequate insulin secretion or activity of insulin (e.g., decreased insulin sensitivity), the blood glucose levels become elevated, while

the cells are starving for this metabolic fuel. Often, cells will then convert to metabolizing fats and proteins, which are nonpreferred fuels in the cell. This can lead to the accumulation of metabolic by-products that can be toxic to the cells when present in higher than normal levels (e.g., ketone bodies and ammonia from fat and protein metabolism, respectively). The starvation sensed by the cells leads to increased food intake (polyphagia). The increased blood glucose leads to incomplete glucose reabsorption in the kidneys' tubules, with the resultant spilling of glucose in the urine. Increased urine volume (polyuria) is needed to keep this excreted glucose in solution. Finally, as the patient urinates larger quantities, the body will compensate by increasing its fluid intake (polydipsia).

Two types of diabetes mellitus have been identified according to dependence of disease to insulin. Type 1 diabetes or insulin-dependent diabetes mellitus (IDDM) is a disease involving severe destruction of the insulin-producing pancreatic β-cells and it is generally associated with a young or juvenile onset (Lernmark, 1999). In Type 1 diabetes mellitus, there is almost a complete lack of pancreatic beta cell function such that essentially no, or very little, insulin is released. This type of diabetes, previously called juvenile-onset diabetes, and sometimes called insulin-dependent diabetes mellitus, requires insulin injections, in addition to careful attention to diet and exercise.

Type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) typically occurs with older age and obesity (Lebowitz, 1999). This type of diabetes is observed when the affinity of insulin receptors decreased toward its ligand or it

develops as there is insufficient insulin receptor on cellular membranes and the latter case is usually developed during obesity. Type 2, is called maturity-onset or insulin-independent diabetes mellitus. This non insulin dependent diabetes mellitus is a multifactorial disease, which is characterized by hyperglycemia and lipoprotein abnormalities (Scoppola *et al.,* 2001). These traits are hypothesized to damage cellular macromolecules which results in elevated production of reactive oxygen species (ROS). This generation of oxygen-free radicals during cellular metabolism, and by certain environmental factors, including lifestyle, appears to play a critical role in the pathogenesis of diabetes mellitus (Hartnett *et al.,* 2000).

Type 2 diabetes may also be characterized by decreased release of insulin from the pancreas. However, some patients, especially those that are obese, actually secrete above normal amounts of insulin, but because the body's cells are insensitive to the secreted insulin, blood glucose still increases since the cells are incapable of properly accumulating and utilizing the circulating glucose. While many of these patients can be treated with oral hypoglycemic agents that enhance insulin's actions, some will still require insulin injections daily. Exercise and diet are also two very important aspects of the therapy of this type of diabetes.

Several theories have emerged to explain the adverse effects of hyperglycemia on tissues. Hyperglycemia may initiate and promote progression of diabetic disease include insulin resistance and hyperinsulinemia (Grill and Bjorklund, 2000), altered fatty acid metabolism-dyslipidaemia (Best and O'Neal*.,* 2000, Betteridge, 1999),

hypertension, ketoacidosis, osmotic effects, vasoactive hormones and dysfunction in sympathetic regulation of glucose and fat metabolism (Nonogaki, 2000).

1.1.1 Oxidative stress in diabetes mellitus

Hyperglycemia, the primary clinical manifestation of diabetes, has been accepted as being essential for the development of diabetic complications most probably due to the oxidative stress. Much evidence has indicated that some biochemical pathways, strictly associated with hyperglycemia, can increase the production of free radicals (Figure 1.4) (Giugliano *et al.,* 1996). It has been found that non-enzymatic glycosylation, glucose autoxidation, and polyol pathway are the major contributor for the fabrication of free radicals and oxidative stress in diabetes mellitus.

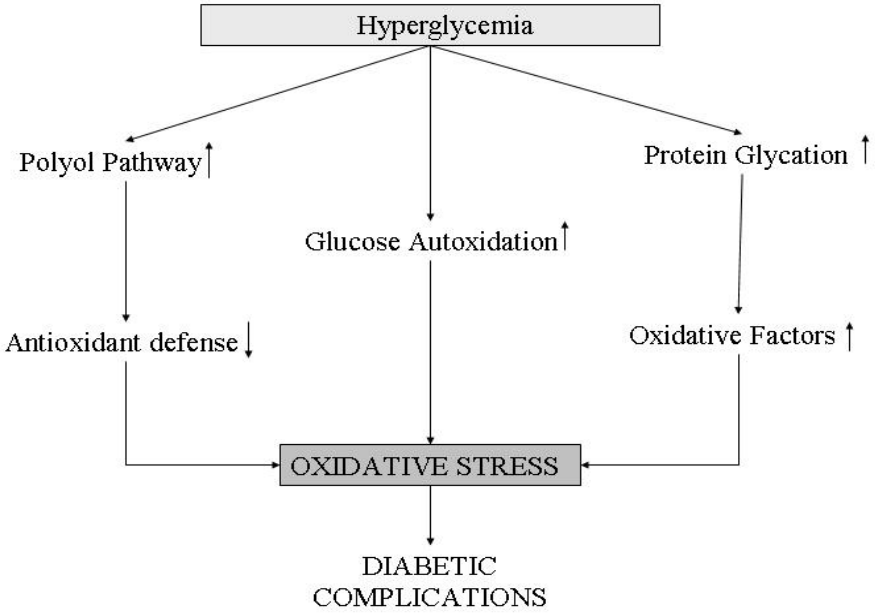


Figure 1.4: Possible links between hyperglycemia-induced oxidative stress and diabetic complications.

1.1.1.1 Free radicals generated by non-enzymatic glycosylation

It has been proved that presence of hyperglycemia, which is considered to be the main factor contributing the complications of diabetes mellitus, may initiate the non-enzymatic glycation of proteins. This glycosylation increase the formation of free radicals through a multistep mechanism. Initially, glucose can slowly condense nonenzymatically with proteins' amino groups forming a Schiff base which may rearrange to form the Amadori product (Figure 1.5). This early stage of the reaction is called nonenzymatic glycosylation or, more properly, "glycation". The Amadori product subsequently degrades into alpha-ketoaldehyde compounds such as 1- and 3-deoxygiucosones (Figure 1.6) (Wolff *et al.*, 1991)

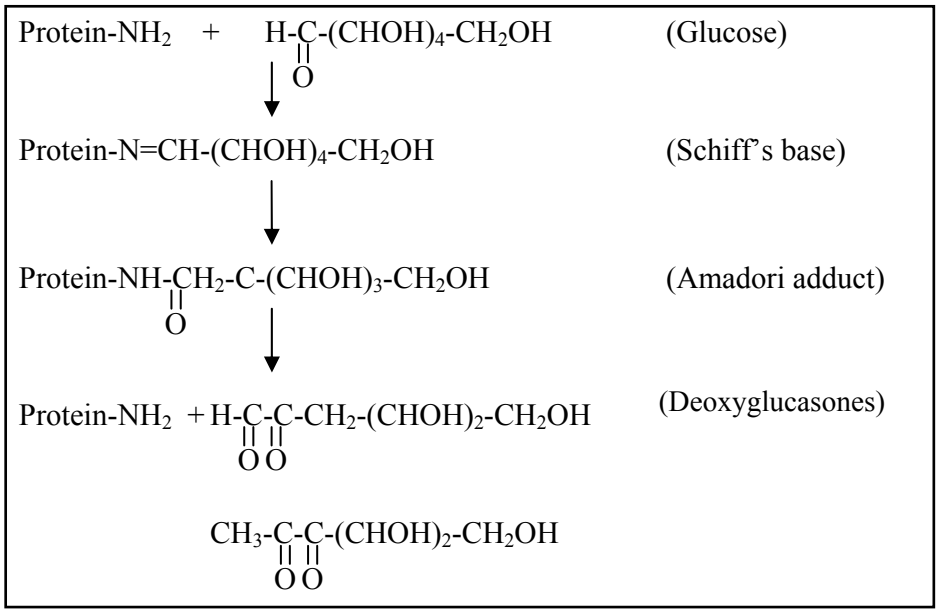


Figure 1.5: Non-enzymatic glycosylation. The addition of glucose to protein is followed by rearrangements and dehydrations (Wolff *et al.,* 1991).

These secondary compounds are more protein-reactive than the parent monosaccharide and can react with proteins to form cross-links, as well as chromo/fluorophoric adducts called Maillard products or Advanced Glycation Endproducts (AGE) (Wolff *et al.,* 1991).

The Amadori products have been also implicated in the formation of H_2O_2. They may form H_2O_2 via two pathways (Figure 1.6). One pathway is the 1,2-enolization pathways, which lead to 3-deoxyglucosone formation under anaerobic conditions. In the presence of a suitable electron acceptor, however, enolization would occur to form H_2O_2 and glucosone (Elgawish, 1996). The other pathway is 2,3-enolization pathway, which leads to 1-deoxyglucosone and the putative 1,4-deoxyglucosone. Under oxidative conditions, the 2,3-enediol is thought to generate H_2O_2 and carboxymethyllysine (Elgawish, 1996).

3-deoxyglucosones has been known to be a major and highly reactive intermediate in the non-enzymatic glycosylation and a potent crosslinker responsible for the polymerization of proteins to AGEs. AGEs tend to accumulate on long-lived macromolecules in tissues and crosslinking between advanced glycation end products and other macromolecules in tissues results in abnormalities of cell and tissue functions.

Recent studies have shown that formation of free radicals is associated with Maillard reactions (Ortwerth *et al.,* 1998, Mossine *et al.,* 1999, Lee Ch *et al.,* 1998, Yim, 1995). Maillard reaction is amplifier of oxidative stress and glycated proteins produce nearly 50 fold more free radicals than non glycated proteins. (Baynes, 2000)

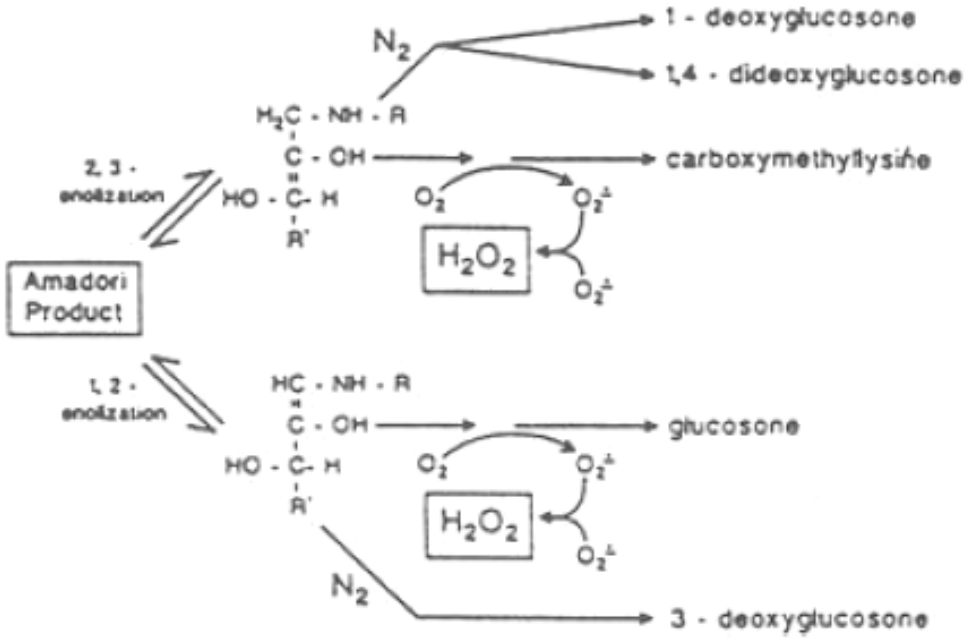


Figure 1.6: Degradation of Amadori product and H_2O_2 formation. H_2O_2 formation can be generated via 1,2- and 2,3-enolization and oxidation of the enolate anion. (Elgawish, 1996)

1.1.1.2 Free radicals generated by glucose autoxidation

Glucose, like other alpha-hydroxyl aldehydes, can enolize and thereby reduce molecular oxygen under physiological conditions, catalyzed by transition metals, yielding alpha-ketoaldehydes and oxidizing intermediates (Wolff and Crabbe, 1985, Wolff and Dean, 1988) (Figure 1.7). This process entails the reduction of oxygen, producing oxidizing intermediates, such as O_2^-, OH^- and H_2O_2 (Sakurai and Tsuchiya, 1988, Jiang *et al.,* 1990) Evidence suggests that free radicals and hydrogen peroxide slowly produced by glucose "autoxidation" are the substantial cause of the structural damage in tissues. Free radicals which are formed through autoxidation process also

accelerate the formation of advanced glycosylation end-products, which in turn generate more free radicals.

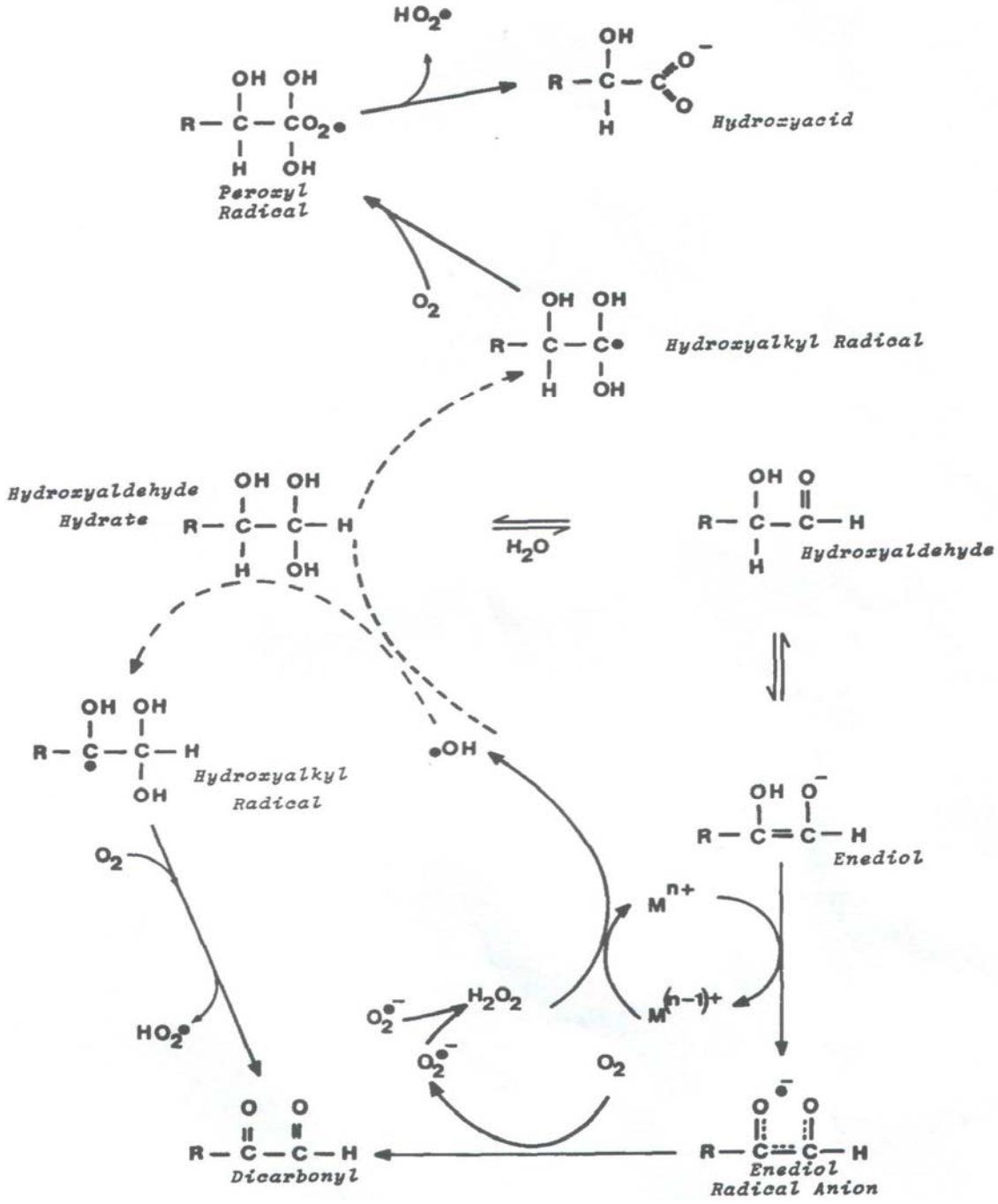


Figure 1.7: The process of autoxidation of a monosaccharide, showing how free radicals are produced when excess glucose is present (Wolff and Dean, 1988).

1.1.1.3 Free radicals generated by polyol pathway

An increase in the concentration of glucose (hyperglycemia) contributes to an enhanced activity of the two enzymes used in the polyol pathway, aldose reductase and sorbitol dehydrogenase (Figure 1.8) (Baynes, 1991). With the increased activity of these two enzymes, the concentration of both sorbitol and fructose increase. This increased activity also causes the NADPH/$NADP^+$ ratio to decrease and the NADH/NAD^+ ratio to increase (Tesfamariam, 1993). The change in these ratios can cause changes throughout various systems in the cell. An increase in NAD^+/NADH ratio is linked to $O_2^{\cdot -}$ formation via the reduction of prostaglandinG_2 (PGG_2) to prostaglandin H2 (PGH_2) by prostaglandin hydroperoxidase that use NADH or NADPH as a reducing cosubstrate (Baynes, 1991).

The reduction in the amount of NADPH may also cause an inhibition in enzymes which are NADPH-dependent and lead to a shortage of the NADPH available for the many pathways it is involved in. Furthermore, such an altered redox state may influence the availability of tetrahydrobiopterin (BH_4), which is an essential cofactor for nitric oxide synthatse (NOS). During BH_4 depletion NOS is "uncoupled", (Figure 1.9) leading to increased superoxide, rather than NO production. In diabetic animal models BH_4 supplementation has been shown to improve impaired endothelium-dependent vasodilatation (Schmidt *et al*, 1992).

In diabetes, process of production of free radicals, mainly by non-enzymatic glycation, transition metal catalyzed autoxidation, and polyol pathway result in a site

specific attack on proteins, with consequent protein damage, lipids with lipid peroxidation and DNA to form DNA adducts (Figure 1.10).

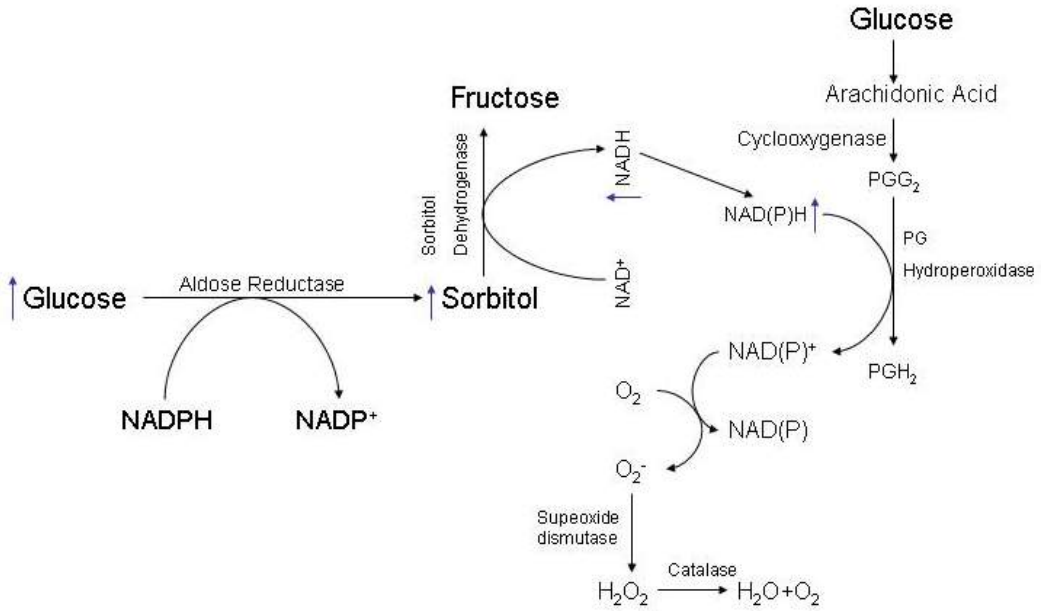


Figure 1.8: The polyol pathway in relation with the redox imbalance (NAD^+/NADH ratio) and free radical production (Lee and Chung, 1999)

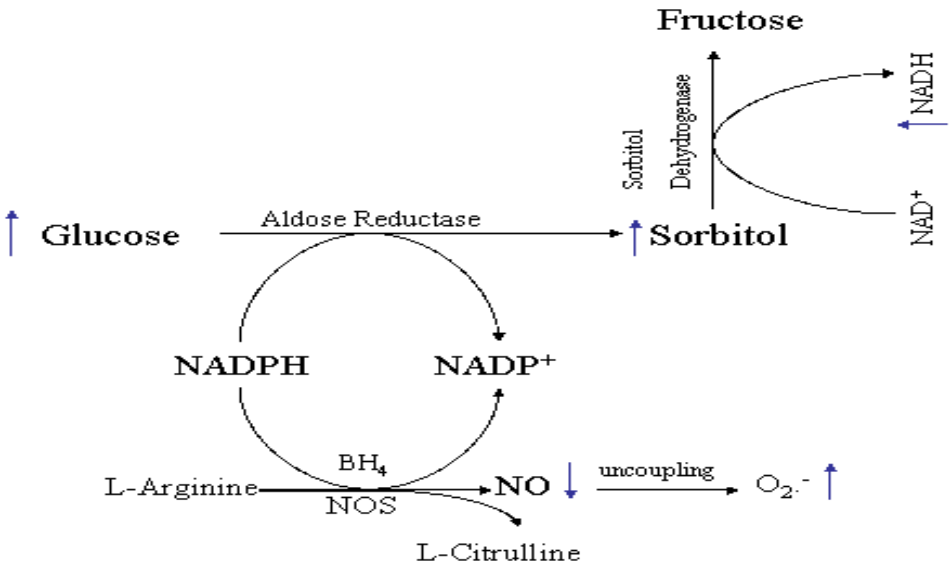


Figure 1.9: The polyol pathway in relation with the NOS uncoupling leading to reduced NO production but increased superoxide formation (Tesfamariam, 1993).

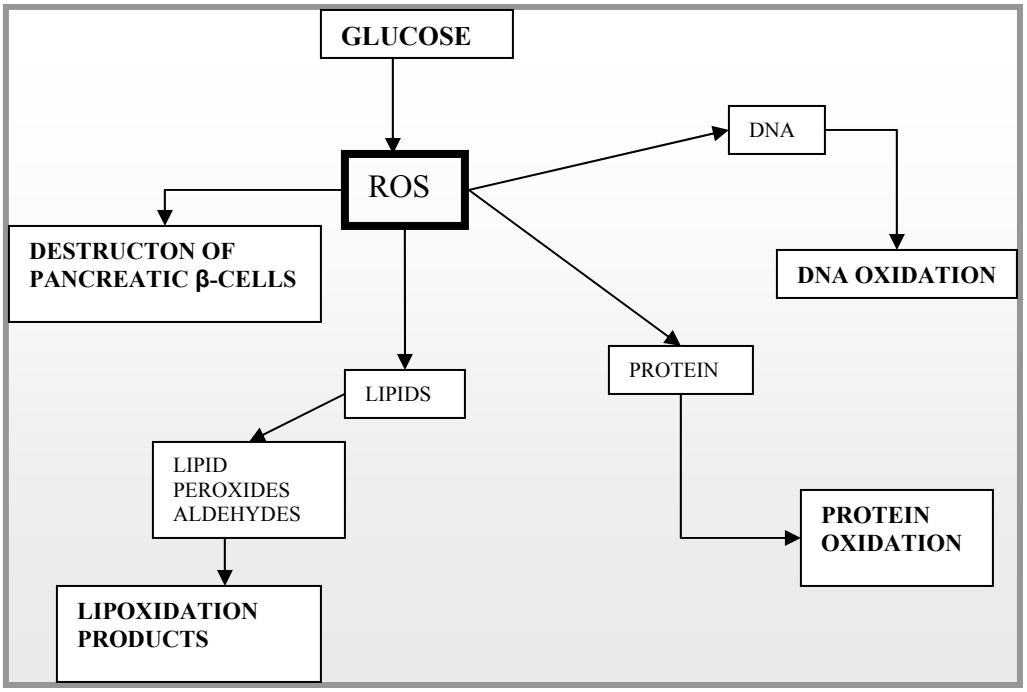


Figure 1.10: Fates of free radicals, mainly ROS which can be produced in hyperglycemia

1.1.2 Hyperglycemia, insulin, insulin resistance and oxidative stress

Several studies show that acute hyperglycemia can impair the physiological homeostasis of many systems in living organisms. Excessive hyperglycemia may impair insulin activity and sensitivity by the mechanism of glucose toxicity (Mooradian and Thurman, 1999). Insulin stimulates the uptake and utilization of glucose in muscle and adipose tissue, inhibits glycogenolysis and gluconeogenesis in the liver and inhibits lipolysis in adipose tissue. Deficient action of insulin reverses the metabolism in the opposite direction. Thus, increased lipolysis enhances the level of free fatty acids and their oxidation in liver. In animal models, it was found that hyperglycemia increases fatty acid availability in muscle. Both hyperglycemia and

insulin resistance are accompanied by reduced insulin action (De Fronzo *et al.,* 1992, Zierath *et al.,* 2000) and they can also be accompanied by oxidative stress (Ceriello, 1995 -1997).

1.1.3 Experimental Models of Diabetes Mellitus

To investigate the effect of diabetes mellitus on tissues and to see the molecular changes, working with lab animals provide several advantages. Therefore, to induce diabetes mellitus in animal models, there would be various experimental procedures such as;

Surgical Diabetes: This method includes surgical removal of total or subtotal pancreas of animals but it is seldomly used for studying diabetes and its complications. ***Spontaneous Diabetes***: diabetes may occur spontaneously in the population of various animal species like Chinese Hamsters and BB Wister Rats.

Viral Diabetes: In both human and animal, several viruses like Coxsaclike viruses, foot and mouth disease viruses, Rubella, and some retroviruses may be a cause of diabetic incidences.

Chemical Diabetes: Various drugs and chemicals elicit somehow situations similar with diabetes mellitus. These are diabetes inducing substances like alloxan (ALL) and streptozotocin (STZ) and the latter is more specific and convenient one (Öztürk *et al.*., 1997). These chemicals induce diabetes by increasing the level of free radicals in pancreas and cause destruction of β-cells. STZ is an antibiotic extracted from *Streptomyces Achromogenes* and cause diabetes by direct action on the pancreatic β-cells by massive degranulation and necrosis associated with an increased serum

insulin level previously and later, it cause prolonged hyperglycemia (Junod *et al*., 1967).

1.2 BIOMARKERS OF OXIDATIVE STRESS

Many direct and indirect methods have been proposed and are generally used to assess in vivo oxidative stress. These methods evaluate not only levels of damaged biological products, but also antioxidant status (Oberley, 1988). Several biomarkers of DNA oxidation, lipid peroxidation, amino acids oxidation, glycoxidation and lipoxidation reactions have been identified and can be measured in oxidatively modified tissues by some chemical and physical methods. Some of these methods require intensive care and equipments such as sensitive high-performance liquid chromatography or gas chromatography-mass spectrometry, requiring both complex analytical instrumentation and derivatization procedures. Also sensitive immunohistochemical and ELISA assays are now available for many of these biomarkers (Onorato *et al.,* 1998). Immunochemical techniques are becoming an increasingly important part of the methodology for detection and measurement of oxidation products in tissues. The only technique that can detect free radicals directly is the spectroscopic technique of electron spin resonance (ESR). However, this method often is too insensitive to directly detect superoxide and hydroxyl radicals in living systems. Another method is trapping (for example spin trapping), in which a radical is allowed to react with a trap molecule to give one or more stable products, which are then measured. Although, such methods are quite complex, they give more reliable information about the oxidative status of a tissue.

Biochemists usually prefer to use simpler, less time consuming, but reliable enough methods to measure the antioxidant status. For example, one of the general method to measure the total antioxidant capacity are described by various authors and they used synonyms of same terms as Total antioxidant capacity (TAC) (Miller, 1993) total antioxidant activity (TAA) (Koracevic *et al.,* 2001), total antioxidant power (TAOP) (Benzie and Strain, 1996), total antioxidant status (TAS) (Rice and Miller, 1994), total antioxidant response (Erel, 2004) or other synonyms. All these methods give general information about the total capacity for the removal of total free radicals and their basis is same. Other methods that biochemists usually prefer are more specific and give detailed information about site of the oxidative stress and oxidative modifications.

1.2.1 Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) parameter summarizes overall activity of antioxidants and antioxidant enzymes. The depletion of TAC induced by oxidative stress is eliminated by release of stock organ antioxidants, mainly from liver and adipose tissue and the induction or activation of antioxidant enzymes. At a later phase of oxidative stress, the TAC falls due to depletion of antioxidants. Low molecular weight antioxidants penetrate specific locations in the cell where oxidative stress may occur and protect against reactive oxygen species (ROS). The clinical importance of determining TAC consists in identifying patients with increased risk of the diseases and, deficient nutrition (Berry and Kohen, 1995). At present, assessment of the antioxidant status of the body, as a clinical marker of oxidative stress, is established

using three approaches: (i) determination of the concentration of total or individual low-molecular antioxidants in serum or plasma (ii) determination of activity of selected enzymes (iii) monitoring markers of oxidative stress, e.g. 8-hydroxyguanosine or products of LDL lipoperoxidation damage. Determination of TAC is based on the evaluation of a total reduction effect of individual low molecular weight antioxidants, of either a hydrophilic or a hydrophobic character. It provides information about antioxidant types and their concentration without exact qualitative differentiation.

1.2.2 Modified Lipid Structures as Biomarkers of Oxidative Stress (Lipid Peroxidation)

A free radical (R*) that has sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid LH, can initiate a chain reaction in bulk lipid. The resulting carbon-centered radical (L*) reacts rapidly with a molecular oxygen ($K=10^{9}$-10^{10} $M^{-1}.s^{-1}$) to form a peroxy radical, which itself can abstract a hydrogen atom from an unsaturated fatty acid, leaving a carbon centered radical and a lipid hydroperoxides (LOOH) (Figure 1.11) (Halliwell and Gutteridge, 1999). Peroxyl radicals can combine with each other or they can attack membrane proteins, but they are also capable of abstracting hydrogen from adjacent fatty acid side chains in a membrane and so propagating the chain reaction of lipid peroxidation (Gutteridge and Halliwell, 1990)

A free radical chain reaction propagates until two free radicals destroy each other to terminate the chain. The length of the propagation chain depends upon many

factors, including the lipid/protein ratio on a membrane. The chance of a radical reacting with a membrane protein increases as the protein content of the membrane raises. Lipid peroxidation is probably the most extensively investigated process which is induced by free radicals. The abundant presence of membrane phospholipids at sites where radicals in general and, more specifically, reactive oxygen species are formed render them easily accessible endogenous targets, rapidly affected by free radicals. Especially the group of polyunsaturated fatty acids (PUFAs) is highly susceptible to reactions with free radicals. Peroxidation of lipids in fatty acids may lead to a radical chain reaction (Figure 1.10).

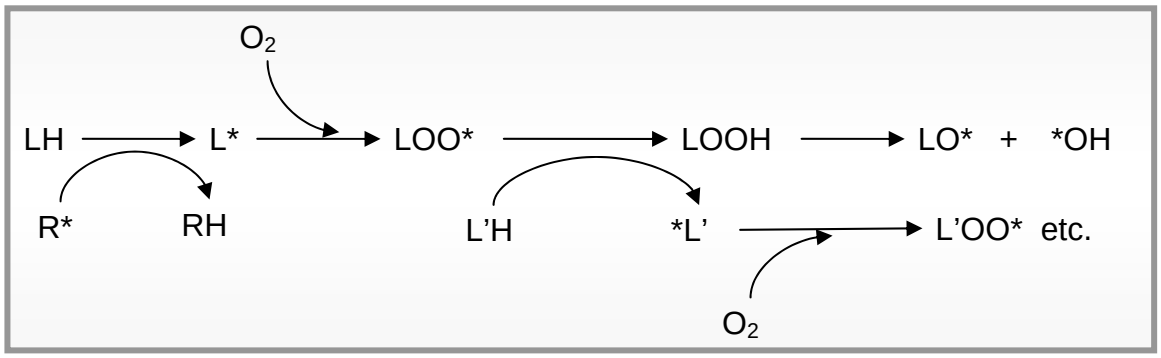


Figure 1.11: Schematic proceed of lipid peroxidation, chain reactions resulting in the formation of many lipid peroxide radicals.

Because of these chain reactions, one substrate radical (R•) may result in the formation of many equivalents of lipid peroxides (LOOH). These degenerative propagation reactions in lipid membranes are usually accompanied by the formation of a wide variety of products, including alkanes and carbonyl compounds. Because some of these products, especially hydroxyalkenals, are toxic by themselves, they may serve as second messengers for radical damage (Kneepkens *et al*., 1994).

Products resulting from lipid peroxidation are thus attractive parameters to monitor radical damage.

Figure 1.12 shows an example for the mechanism of lipid peroxidation chain reaction. The mechanism of polyunsaturated fatty acid oxidation is exemplified by the autoxidation of linoleic acid. The initiating radical, $ROO^{\cdot}$, is usually of unknown origin or composition. Step 1 generates the first polyunsaturated fatty acid radical that then, after electron redistribution, adds dioxygen in step 2. The hydroperoxyl radical from step2 can attack linoleic acid, step 3, to generate the first linoleyl radical and a linoleyl hydroperoxide. This is a free radical chain reaction. Two linoleyl hydroperoxyl radicals can react to give non-radical products, step 4, and terminating free radical chain oxidation. Antioxidation by vitamin E takes place at step 5, giving linoleyl hydroperoxide and a tocopheroxyl radical. A second linoleyl hydroperoxyl radical can react with a tocopheroxyl radical to give a non-radical product, step 6. If the concentration of linoleyl hydroperoxyl radical is too low the α-tocopheroxyl radical will attack linoleic acid giving the linoleic acid radical and regenerating vitamin E, step 7. In vivo the oxidation of ascorbic acid may regenerate vitamin E without oxidizing linoleic acid, step 8.

Measuring the loss of unsaturated fatty acids, amounts of primary and secondary peroxidation products can quantitate the amount of lipid damage by the process of chain reaction of peroxidation (Halliwell and Chirico, 1993). Analysis with HPLC, GLC, Light emission, Fluorescence, Antibody techniques can be useful for the detection of lipid peroxidation products. On the other hand; biochemical TBA

test is the simplest and oldest one which measures the product of lipid peroxidation, namely malonedialdehyde (MDA)

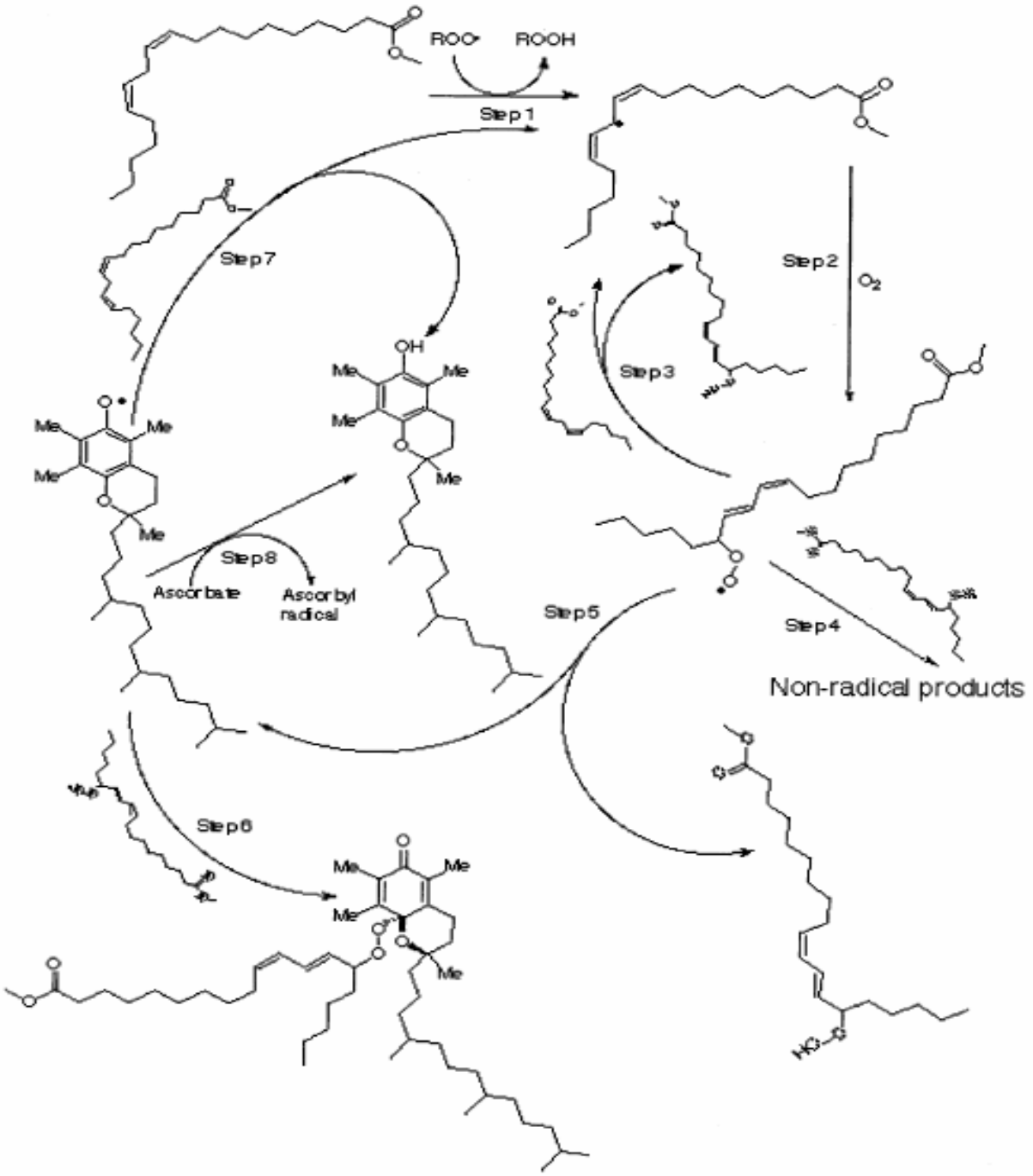


Figure 1.12: An example for the mechanism of lipid peroxidation chain reaction.

1.2.3 Protein Carbonyl Groups As Biomarkers of Oxidative Stress (Protein Oxidation)

Carbonyl (CO) groups (aldehydes and ketones) are produced on proteins' side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized (Figure 1.13). These moieties are chemically stable, and so their detection and storage can be possible.

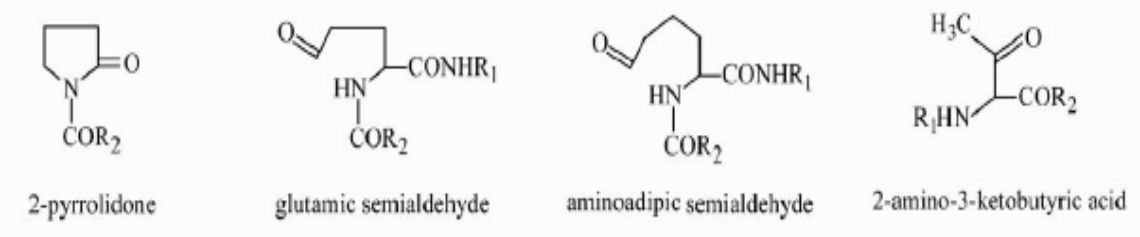


Figure 1.13: Products of Protein Carbonylation which are produced by direct oxidation amino acids side chains.

Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation (Chevion *et al.,* 2000, Shacter, 2000, Beal, 2002) and accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease (AD), diabetes, inflammatory bowel disease (IBD), and arthritis, just for citing a few (Halliwell and Gutteridge, 1999, Chevion e*t al.,* 2000)

The classical approach to the detection of protein carbonyl groups involves their reaction with DNPH followed by the spectrophotometric quantification of the acid hydrazones at 370 nm (Levine *et al.,* 1990). Spectrophotometric DNPH method

is useful to quantify carbonyl content in mixture of proteins, such as plasma, tissue homogenates, cellular extracts, or in isolated proteins.

1.2.4 Modified DNA Structures as Biomarkers of Oxidative Stress (DNA Oxidation)

Under conditions of oxidative stress damage to cellular biomolecules such as lipids, proteins and DNA occurs. Oxygen free radicals induce a variety of lesions in DNA, including oxidized bases, abasic sites, DNA strand breaks and formation of cross-links between DNA and proteins (Shigenaga and Ames, 1991)

It has been shown that hydroxyl radical which is produced by the Fenton reaction in the presence of transition metal ions is responsible for DNA damage. A major site of radical attack is at the 8-position of guanine to produce 8-hydroxydeoxyguanosine (8-oxo-*d*Guo) (Cheng *et al*., 1992). The 8-oxo-*d*Guo is strongly mutagenic, having the propensity to mispair with adenine residues, leading to an increased frequency of spontaneous G:C → T:A transversion mutations unless repaired prior to DNA replication. The 8-oxo-*d*Guo can be repaired by base excision repair system. Oxidative damage to DNA has been demonstrated by measuring levels of 8-oxo-*d*Guo as a biomarker of oxidant-induced DNA damage in lymphocytes from diabetic subjects by using high pressure liquid chromatography (Dandona *et al.,* 1996). An alternative method for detecting DNA damage is single cell gel electrophoresis, the comet assay. Comet assay can be used for determination of oxidized purines on DNA by using damage specific-repair endonucleases such as endonucleaseIII and formamidopyrimidine DNA glycosylase (Fpg). There are a few

studies showing enhanced DNA damage as strand breaks by the comet assay or DNA unwinding technique in diabetic patients (Lorenzi *et al.,* 1987 and Öztok *et al.*¸1999) and one of them reported that Vitamin E supplementation decreases DNA damage in those patients (Sardas *et al.*, 2001).

1.3 PROTECTION AGAINST FREE RADICAL INDUCED CELLULAR DAMAGE

In conjugation with the omnipresent free radical generation, the organism is well equipped with a complex armory of effective defense systems in order to prevent radical damage to cell constituents. The structural characteristic of the cell membrane forms a defense in itself. Penetration of free radicals into the hydrophobic layer, where the initiation reaction of lipid peroxidation takes place, will not occur readily. Moreover, the dispersion of free radical chain reaction in membranes is hampered by proteins. Other protective mechanisms vary from low molecular weight free radical scavengers to complex enzyme systems.

1.3.1 Non-Enzymatic Antioxidants

Antioxidants are the compounds that are functioning for the neutralization of the oxidizing effects of the free radicals. As they accept the free electrons of the radicals, they don't behave like a radical. Since, they have the ability to accommodate free electrons without reacting with other molecules. A number of compounds act as non-enzymatic antioxidants. However; the major macromolecular antioxidants in a cell can be described as α-tocopherol (vitamin-E) which is a lipophilic molecule, and

its major role is to block the chain reaction of lipid peroxidation (Halliwell and Gutteridge, 1999). The second most important antioxidant in a cell is a hydrophobic molecule which is ascorbic acid (vitamin C). A-tocopherol (vitamin E) and ascorbic acid (vitamin C) cooperate in cellular defense against ROS. Ascorbic acid reduces the oxidized α-tocopheryl radical back to α-tocopherol (Freeman and Crapo, 1982). In addition, vitamin C, a hydrophilic scavenger of ROS, is considered the most important antioxidant in extracellular fluids (Sies *et al.*, 1992). Since lipid peroxidation occurs on unsaturated fatty acid chains that reside within the lipid bilayer, and vitamin E is dipped through the membranes, it terminates the free radical induced lipid damage. Oxidized vitamin E, is then neutralized by vitamin C which is further neutralized by glutathione pool or with specific vitamin C reductase enzyme. In addition to being essential in the glutathione redox cycle, glutathione can scavenge ROS non-enzymatically (Freeman and Crapo, 1982), as well as detoxify xenobiotics via the glutathione S-transferase reaction. Glutathione protects SH-groups of proteins from oxidation and maintains intracellular vitamin-C levels (Meister, 1994). On the other hand, other then being an antioxidant, ascorbic acid can promote ROS injury in the presence of free transition metals (Heffner and Repine, 1989).

1.3.2 Antioxidant Enzymes

Non-enzymatic scavengers are essential in the protection of cellular components from undiscriminate damage by the most reactive oxidizing species, but this protection does not cope with reducing radicals such as superoxide, or with metastable hydro-peroxides. Such primary peroxides are continuously produced

especially by aerobic cells (Chaudie, 1994) and they provide a major source of much more oxidizing species such as hypervalent iron or copper complexes, peroxynitrite, and hydroxyl or alkoxyl radicals, which all behave as initiators of chain reactions.

Specific antioxidant enzymes have been designed by nature to destroy superoxide and hydroperoxides. The advantage of using enzymes is that the steady-state concentration of peroxides can be adapted to cellular requirements: several of the antioxidant enzymes can be induced, inhibited or activated by endogenous effectors, and they play an important role in the regulation of metabolic pathways and specific functions of aerobic cells.

Enzymatic degradation of superoxide is catalyzed by superoxide dismutases (SOD), while that of hydroperoxides is ensured by catalase, glutathione peroxidases (GPx) or ascorbate peroxidases. SOD and catalase are dismutases, and therefore they do not consume cofactors. Thus, the primary, reactions catalyzed by such enzymes are not associated with any energetic cost. Conversely, glutathione peroxidases and ascorbate peroxidases are reductases whose reducing coenzymes are regenerated by NAD(P)H equivalents produced in metabolic pathways.

1.3.2.1 Superoxide Dismutases (E.C:1.15.1.1)

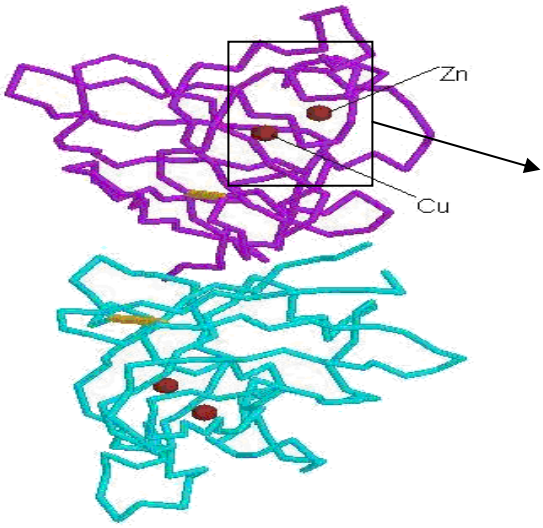
Superoxide dismutases are metalloenzymes, which catalyze the dismutation of superoxide anion ($O_2^{\cdot}$) into H_2O_2 and O_2 at a rate 104 times faster than spontaneous dismutation at neutral pH (Halliwell and Gutteridge, 1989). SOD has been shown to play a central role in protecting cells and tissues against oxidant stress (Fridovich and Freeman 1986, and Tsan, 1997). Four different kinds of SODs have been

characterized in eukaryotes; a copper- and zinc-containing form (CuZnSOD) localized in the cytosol, a manganese-containing form (MnSOD) in the mitochondria, iron containing FeSOD in some prokaryotes and plants' outer mitochondrial membrane, and a copper- and zinc-containing form in the extracellular matrix (ECSOD) (Marklund, 1982).

Copper-zinc superoxide dismutase, a homodimer with a molecular weight of 32 kD (Figure 1.14), was found by McCord and Fridovich (1969). It is mainly localized in the cytosol, but is also found in the nucleus and peroxisomes, at least in human cells. CuZnSOD contains both Cu (II) and Zn (II) at its active sites (Figure 1.15).

Manganese superoxide dismutase, which constitutes approximately 10 to15% of total cellular SOD activity, is a homotetramer with a molecular weight of 88 kD (Figure 1.16) (Fridovich and Freeman, 1986). MnSOD, having manganese (III) at its active site, is localized in the matrix of the mitochondria, this location is important for removal of $O_2^{\cdot}$ produced by the respiratory chain. MnSOD activity can be distinguished from that of CuZnSOD by its resistance to 1 mM potassium cyanide.

Extracellular superoxide dismutase is a secretory tetrameric Cu/Zn containing glycoprotein, with a molecular weight of around 135 kD (Marklund, 1982). ECSOD is the least abundant of the SODs in tissues, but it is the major SOD in extracellular fluids such as plasma and extracellular matrix (Marklund 1984, Sandstrom *et al.*, 1993, and Oury *et al.*, 1994).



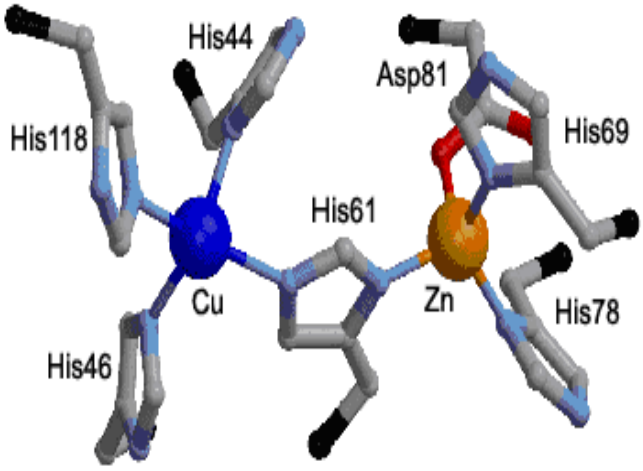


Figure 1.14: Protein Structure of CuZnSOD

Figure 1.15: Active site

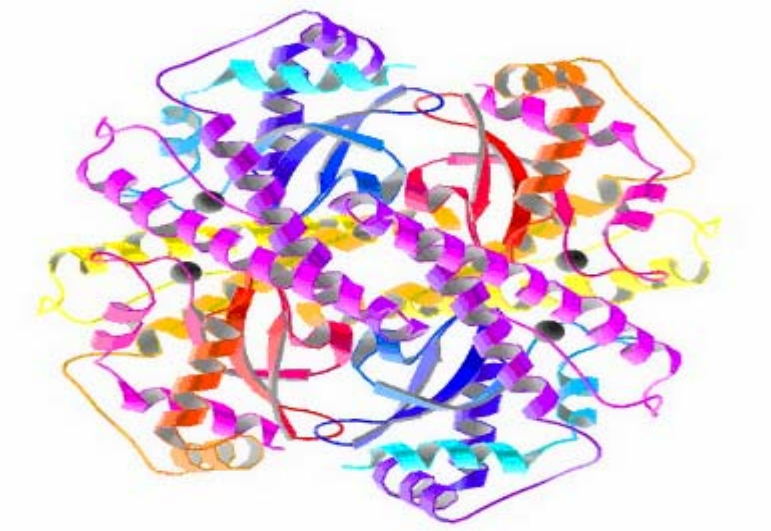


Figure 1.16: Structure of MnSOD

1.3.2.2 Catalase (E.C: 1.11.1.6)

Catalase is a tetrameric hemoprotein (porphyrin-containing) with a molecular weight of 240 kD (Figure 1.17). A typical catalase has 4 identical subunits, each with a heme in active site and they decompose H_2O_2 into water and oxygen, in addition to

that, they have peroxidative functions, so as to convert peroxides (ROOH) into alcohol (ROH) and water. Therefore, they protect cells from adverse effects of lipid peroxidation. There are many forms of catalase and most of them contain Fe-heme, but some contains Mn. Although most of anaerobic bacteria don't contain catalase, most aerobic ones contain it.

Catalases are mainly localized in the peroxisomes (Davies *et al.*, 1979), but are also found in the cytosol of human neutrophiles (Ballinger *et al.*, 1994) and in rat-heart mitochondria (Radi *et al*., 1991). They are found also in periplasmic membrane of some bacteria such as E.Coli. Although a significant portion of the catalase activity, detected in homogenates of animal and plant tissues, is found not to be bound to organelles; this could be, in part or in whole, due to the rupture of fragile peroxisomes during homogenization.In mammalian cells, NADPH is bound to catalase and may protect the enzyme from inactivation by H_2O_2 (Kirkman and Gaetani, 1984). In erythrocytes, which have a high content of non-peroxisomal catalase, hydrogen peroxide is essentially degraded by glutathione peroxidases (Nicholls, 1972).

1.3.2.3 Enzymes Working In Glutathione Redox Cycle

The low molecular weight reduced glutathione (GSH) (Figure 1.18) is a tripeptide which contains Gly-Cys-Glu residues, and it is a participant of intracellular antioxidant system. Oxidation reactions may produce oxidized form (GSSG) by combining two GSH units via -S-S- bonds of cystein residues.

Figure 1.17: Structure of Catalase

Glutathione is abundant (3 to 10 mM) in cytoplasm, nuclei, and mitochondria and is a soluble antioxidant in these cell compartments. Reactive protein sulfhydryls are abundant in both soluble proteins and in membrane-bound proteins.

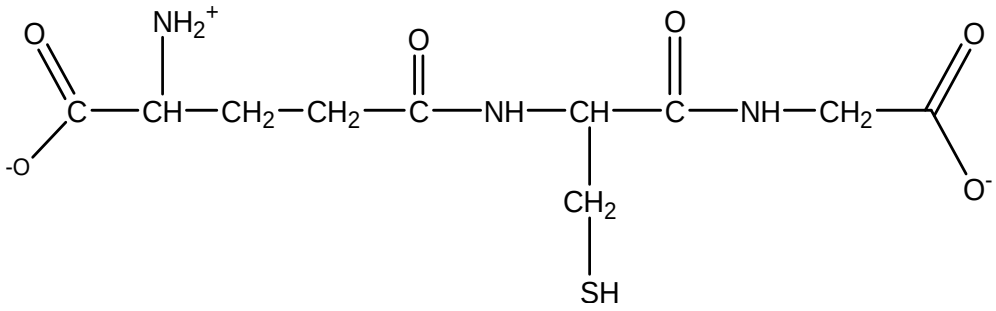


Figure 1.18: Structure of reduced glutathione

The sulfur atom in sulfhydryl groups easily accommodates the loss of a single electron and the lifetime of radical species of sulfur, i.e., a thiyl radical, may be significantly longer than many other radicals generated during stress. The glutathione

redox cycle (Figure 1.19) is primarily mediated by enzyme-catalyzed reactions. In this cycle, Glutathione is oxidized by hydrogen peroxide to glutathione disulfide by the selenium-containing enzyme, glutathione peroxidase (GPx), and also by other enzymes that may use lipid peroxides rather than hydrogen peroxide as the oxidant. Thus, glutathione can detoxify both soluble and lipid peroxides. Glutathione disulfide is subsequently reduced by glutathione reductase (GR), using NADPH as the reductant. Cellular NADPH, produced by the pentose-P pathway and other cytoplasmic sources, provides the major source of reducing power for detoxifying many peroxides.

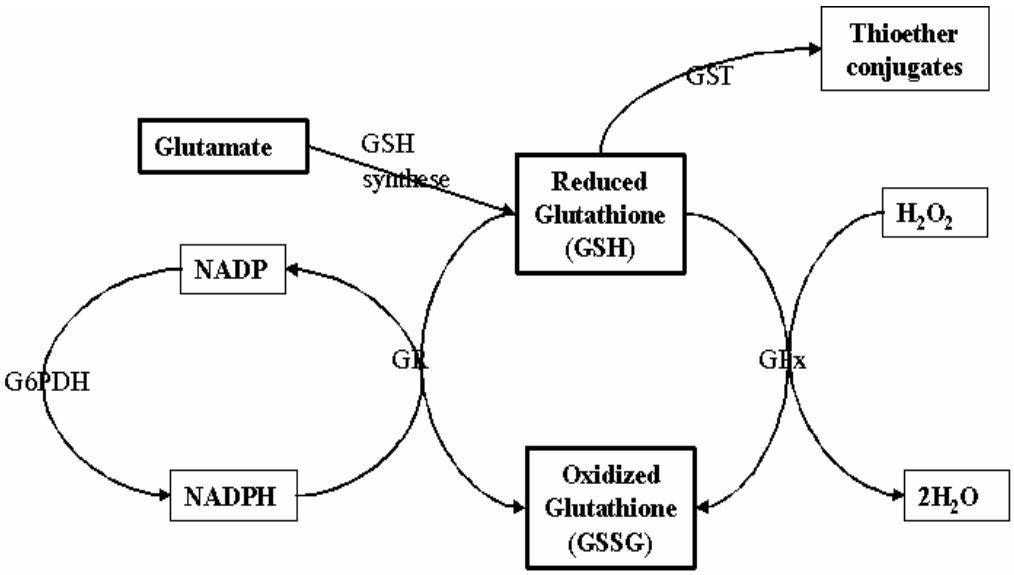


Figure 1.19: Glutathione redox cycle; GST: glutathione S-transferase, G6PDH: glucose-6-phosphate dehydrogenase, GR: glutathione reductase, GPx: glutathione peroxidase.

The concentration of cellular glutathione has a major effect on its antioxidant function and it varies considerably as a result of nutrient limitation, exercise, and

oxidative stress. Under oxidative conditions, the concentration of glutathione can be considerably diminished through conjugation to xenobiotics, and by secretion of both the glutathione conjugates and glutathione disulfide from the affected cells. A considerable amount of glutathione may also become protein-bound during severe oxidative stress. During oxidative stress, large families of proteins that contain reactive sulfhydryls are modified by oxidation to mixed-disulfides with attached glutathione (S-thiolation). Three antioxidant enzymes are important and working in the glutathione redox cycle, these are; Glutathione Peroxidase (GPx), Glutathione S-Transferase (GST) and Glutathione Reductase (GR).

1.3.2.3.1 Glutathione Peroxidase (E.C: 1.11.1.9)

In animal cells, glutathione peroxidases (GPx) are homotetrameric water soluble selenoenzymes (Figure 1.20) which catalyze the reduction of hydroperoxides at the expense of GSH (Flohe, 1989 and Ursini *et al.,* 1995). In this process, hydrogen peroxide is reduced to water, whereas organic hydroperoxides are reduced to alcohols. As in other selenoenzymes of animal cells, such as deiodinases and thioredoxin reductase, GPx active sites contain selenium in the form of a selenocysteine residue, which is incorporated into the polypeptide backbone (Flohe *et al.,* 1973, Rotruck *et al.,* 1973 and Stadtman, 1991). Se-GPx is generally abundant in cytosolic and mitochondrial compartments (Epp *et al.,* 1983). There is also monomeric Se-GPx, which is partially bound to membranes and which is not strictly GSH-specific, can directly reduce phospholipid hydroperoxides of membrane bilayers (Flohe *et al.,* 1994 and Roveri *et al.,* 1994). However, the regulation of its

biosynthesis, enzyme activity, intracellular compartmentalization and tissue distribution is complex, and it is not clearly established that its antioxidant properties are the most important ones.

The advantage of using selenium rather than sulfur to reduce hydroperoxides at the active site of GPx has been demonstrated by site directed mutagenesis (Rocher *et al.,* 1992) the sulfur S-GPx mutant of murine Se-GPx exhibits extremely weak GPx activity, and it is rapidly inactivated during catalysis.

In most animal cells, hydrogen peroxide is reduced by selenium-dependent GPx, but there are isoforms of glutathione S-transferases (GSTs) which exhibit non-selenium dependent GPx activity on organic hydroperoxides, such as those derived from membrane lipids or from DNA bases. The kinetics of such enzymes is much slower than those of selenium-dependent GPx, but they are probably of biological significance in cells or cellular compartments where Se-GPx is normally not expressed, or in situation of selenium deficiency (Lawrence *et al.,* 1978).

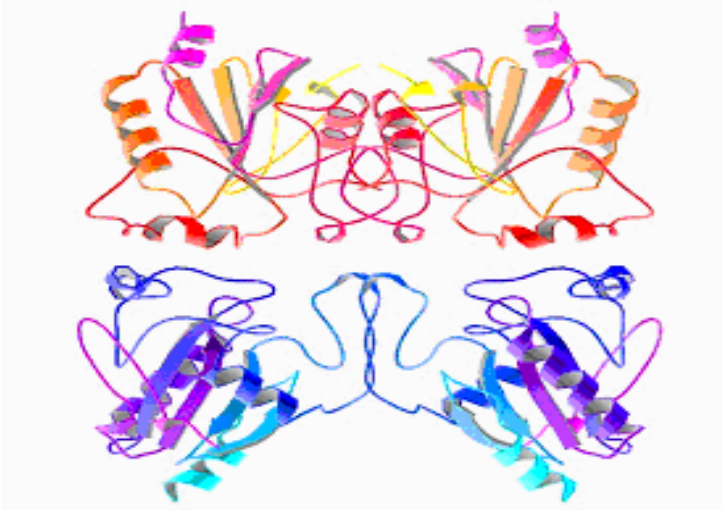


Figure 1.20: Structure of GPx

1.3.2.3.2 Glutathione-S-Transferases (E.C:2.5.1.18)

Glutathione S-transferases (GSTs) are dimeric, ubiquitous multifunctional enzymes composed of two polypeptide subunits. They play a key role in cellular detoxification (Dixon *et al.,* 2002). The GST proteins have evolved by gene duplication to perform a range of functional roles using the tripeptide glutathione (GSH) as a cosubstrate or coenzyme.

GSTs are predominantly expressed in the cytosol, where their GSH dependent catalytic functions include the conjugation and resulting detoxification of herbicides, the reduction of organic hydroperoxides formed during oxidative stress and the isomerization of maleylacetoacetate to fumarylacetoacetate, a key step in the catabolism of tyrosine (Edwards *et al.,* 2000 and Marrs, 1996). GSTs also have non-catalytic roles, binding flavonoid natural products in the cytosol prior to their deposition in the vacuole (Edwards *et al.,* 2000). Recent studies have also implicated GSTs as components of ultraviolet-inducible cell signaling pathways and as potential regulators of apoptosis (Loyall *et al.,* 2000). Initially five independent classes of GST subunits, alpha, mu, pi, sigma and theta were classified (Figure 1.21). (Buetler and Eaton 1992; Drogg *et al*., 1995; Ketterer *et al*., 1993 and Neuefiend 1997), but later studies have shown that there also exist beta, delta, tau, zeta form of these familiy (Dixon, 2002). GSTs alpha, mu and pi form are active in drug metabolism and sigma form is functioning in the prostoglandin synthesis (Rowsey *et al.,* 2001). Zeta and theta GSTs are found in both animals and plants but tau and phi is plant specific (Dixon *et al.,* 2002). Theta, phi and tau have been shown to have glutathione

peroxides activity to reduce organic hydroperoxides of fatty acids to the corresponding monohydroxy alcahols (Roxas *et al.,* 1997 and Cummins *et al.,* 1999) and zeta form functions in tyrosine metabolism in the isomerization of maleylacetoacetate to fumarylacetoacetate which is the ultimate step in the tyrosine degradation (Dixon *et al.,* 2000).

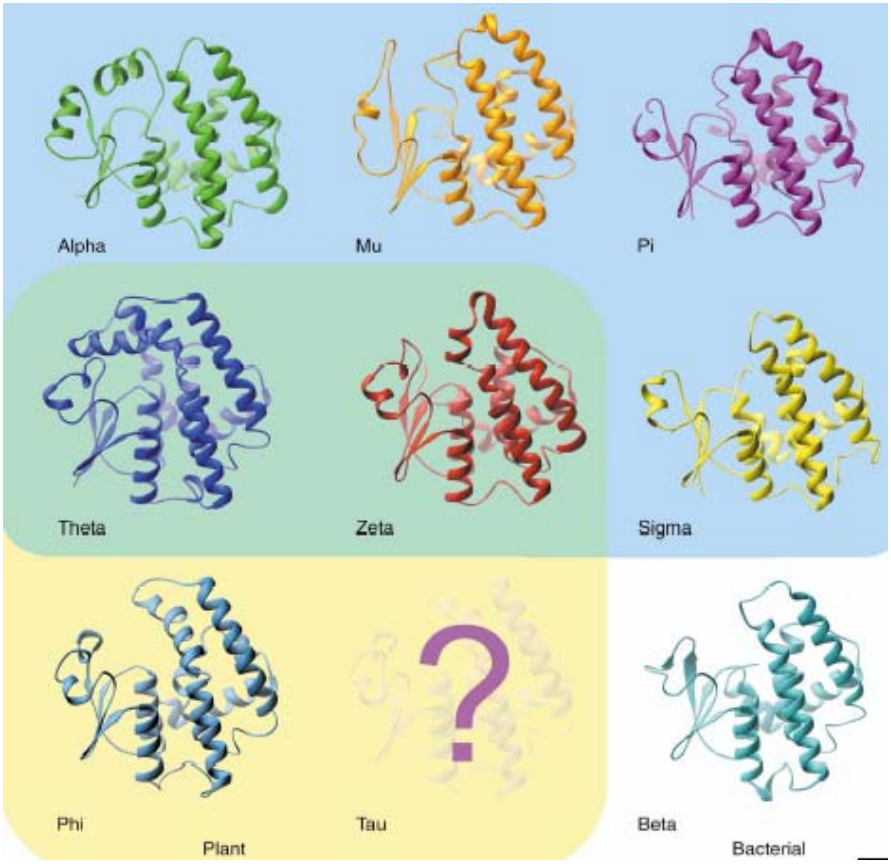


Figure 1.21: Ribbon representations of the structures of GST subunits. The GSTs specific to mammals (alpha, mu, pi and sigma) have a blue background; the plant specific (phi) and bacteria-specific (beta) GSTs have yellow and white backgrounds, respectively; GSTs (theta and zeta) that have counterparts in both animals and plants have green backgrounds. The structure of a tau GST has yet to be reported. Although there is little sequence similarity between enzymes of different classes, there is significant conservation in overall structure (Dixon *at al.,* 2002).

1.3.2.3.3 Glutathione Reductase (E.C:1.6.4.2)

Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH). It has molecular weight of 104.8 kD and two identical subunits (Figure 1.22). This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress. They are present in cytoplasm and mitochondria.

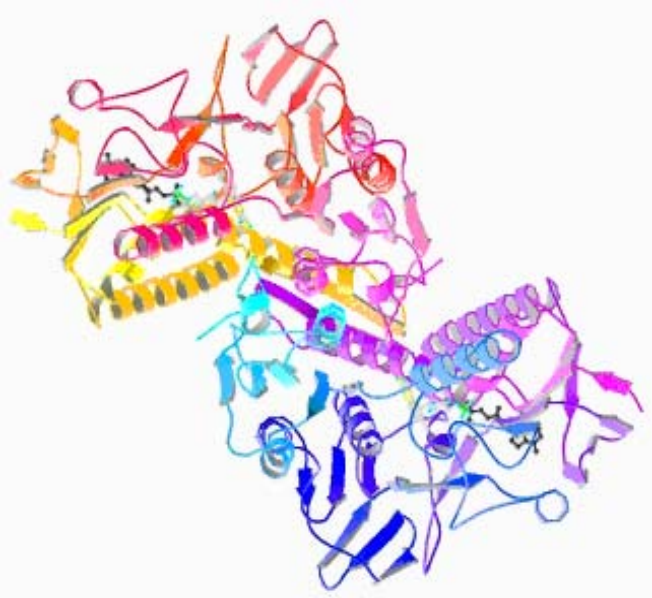


Figure 1.22: Structure of GR

1.4 Aim of the Study

In this study, our aim is to optimize the conditions of the activity measurements of the major antioxidant enzymes, which are MnSOD and CuZnSOD, CAT, GPx and GST for the diabetic and control rat liver tissues. Furthermore, the determination methods of total TBARS content (indicating degree of lipid peroxidation), reduced glutathione (indicating tissue level of this antioxidant), microsomal suspensions' lipid to protein ratios (showing the degree of change in membrane fluidity), total proteins' carbonyl content (screening the oxidative protein damage) are aimed to be optimized. Finally, optimized assay conditions are utilized to compare these biomarkers of oxidative stress between diabetic (n=7) and control (n=6) rat liver tissues to evaluate the presence of oxidative stress and oxidative changes which may occur during the diabetes mellitus.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Bovine serum albumin (BSA), ethanol (95%), o-phosphoric acid (85%), sodium-potassium tartarate, cupper sulfate, sodium hydroxide, Folin-Ciocalteu Phenol Reagent, hydrogen peroxide (30%), streptozotocin, potassium dihydrogen phosphate, Triton X-100, sodium carbonate, ethylenediaminetetraaceticacid, ammonium sulphate, 1-chloro-2,4-dinitrobenzene, reduced glutathione, glutathione reductase, pyrogallol, tris(hydroxymethyl)aminomethane, hydrochloric acid, nicotinomideadeninedinucleotide, potassium cyanide, 5-5'-dithiobis-2-nitrobenzoic acid, methanol, 2,4-dinitrophenylhydrazine, trichloroacetic acid, guanidinehydro-chloride, ethanol, ethyl acetate, streptomycin sulfate.

All chemicals were analytical grade and were obtained from commercial sources at the highest purity available.

2.2 Animals and Induction of Diabetes

13 male 3 months old Wistar rats (250-300g), which were divided into 2 groups: Control (n=6) and Diabetic (n=7) were obtained from Dicle University, Elazığ. All the animals were fed with standard diet and water. Diabetes was induced by a single intraperitoneal dose of streptozotocin (STZ) (50 mg/kg) dissolved in 0.05M citrate buffer (pH 4.5). Control group received 50 mg/kg physiological saline solution. After two weeks, blood glucose levels were weekly checked and the rats who have blood glucose levels above 200 mg/dL were considered as diabetic. At the end of the eight week, rats were weighed, decapitated and livers were removed and kept at -80 ^{o}C until use.

2.3 Methods

2.3.1 Preparation of Rat Liver Cytosolic Fractions and Microsomes

Principle:

Basis of the preparation of cytosolic fractions for the determination of enzyme activities and microsomal suspensions for the TBA test is to make a homogenate of rat liver tissues with the teflon glass homogenizer and destroy cellular boundaries. Then, by the help of differential centrifugation, different cellular fractions and microsomes were isolated.

Reagents:

1. Homogenization Buffer: 1.15% (w/v) KCl, 25mM K_2HPO_4, 5mM EDTA, 0.2mM PMSF, 2mM DTT, pH 7.4

2. Washing buffer: 0.5M Tris, 1mM EDTA, pH 7.4
3. Suspension buffer: 25% (v/v) glycerol and mM EDTA, pH 7.4

Preparation:

Livers were washed twice or more with cold washing buffer to remove blood, then dried on a filter paper, and fatty tissues were removed. Then, livers were cut into small pieces and weighed. 4 mL homogenization buffer is added to each gram wet weight of tissue. All these steps were carried at 0-4^{o}C. The tissues were homogenized using Potter-Elvehjem glass homogenizer packed in crush ice, coupled motor – driven (Black & Decker, V850, multispeed drill) teflon pestle at 2400 rpm for 3x20 sec.

Homogenate was first centrifuged at 1000*g* (Sorvall RC5Plus, Newtown St., USA) using SS-34 rotor for 10 min and the resulting nuclear pellet was discarded. From the supernatant small aliquot was removed for the determination of activity of catalase and SOD isozymes. Then, the rest of supernatant was centrifuged at 16000*g* (Sorvall RC5Plus, Newtown St., USA) using SS-34 rotor for 20 min and the mitochondrial pellet was discarded. Afterwards, the supernatant was further centrifuged at 125000*g* (Sorvall Combi Plus Ultracentrifuge, Newtown St., USA) using A-841 rotor for 60 min. The resulting cytosolic fractions were stored as small aliquots at -80^{o}C, for determination of other cytosolic antioxidant enzyme activities. The resulting pellets were suspended in washing buffer and recentrifuged at 125000*g* for further one hour. After discarding the supernatant, the microsomal pellet was suspended in suspension buffer at a volume of 0.25 mL for each gram of initial wet

liver tissues. The resuspended microsomes were homogenized manually using the teflon-glass homogenizer and stored at -80^{o}C as small aliquots.

2.3.2 Protein Determinations

(Lowry *et al.*, 1951)

Principle:

The principle of protein determination was based on the reduction of Folin reagent (phosphomolybdate and phosphotungstate) by Cu^{++} treated proteins' tryptophan and tyrosine residues. The color due to reduction of molybdate in Folin reagent is directly proportional to protein content and measured at 660 nm.

Reagents:

1. Reagent I: 2% w/v $CuSO_4.5H_2O$
2. Reagent II: 2% w/v Na-K Tartarate
3. Reagent A: 2% w/v Na_2CO_3 in 0.1 N NaOH
4. Lowry ACR Reagent (alkaline cupper reagent): Reagent I, II and A were mixed respectively with a ratio of 1:1:100 (v/v/v)
5. Folin Phenol Reagent: 2N stock reagent were diluted to 1N with dH_2O and kept in dark.

Assay:

Before preparation of reaction tubes, samples from differential centrifugation were diluted initially 200 fold with distilled water and further 2.5 and 5 fold dilutions were done in test tubes. Standard bovine serum albumin solutions (0.02, 0.05, 0.10, 0.15, and 0.20 mg/mL) were also prepared. Then in separate duplicate tubes, 0.5 mL diluted samples and standard BSA solutions were added.

Then to each tube, 2.5 mL Lowry ACR was added, mixed and incubated for 10 minutes at room temperature for copper reaction in alkaline medium. After that, 0.25 mL Folin Reagent was added to each tube and mixed within 8 seconds. The tubes were then incubated at room temperature for further 30 min. The intensity of color developed was measured at 660nm (Shimadzu 1601 UV/Vis Double-beam Spectrophotometer, Kyoto, Japan) against blank cuvette containing 0.5 mL dH_2O instead of standard BSA solutions. Finally, a standard BSA calibration curve was constructed from the absorbance readings of standards and used for the calculation of protein amounts of samples.

2.3.3 Total Lipid Determination of Microsomal Suspensions (Frings *et al.*, 1972)

Principle:

Total lipid determination is based on reaction of lipids' double bonds with phosphovanillin reagent in acidic medium to form a colored complex whose

absorbance can be measured at 540nm. As standard 700 mg/dL olive oil or torein in ethanol are used.

Reagents:

1. Concentrated sulfuric acid
2. Standard Olive oil: 700 mg in 100 mL absolute ethanol
3. Phosphovanillin reagent: 1.2 g of vanillin was dissolved in 200mL water and 800mL concentrated phosphoric acid was added and then stored in dark bottle.

Assay:

Hundred microliters of 2 fold diluted microsomal suspension (or standard olive oil: 700mg/dL) and 2mL of concentrated sulfuric acid were mixed in a test tube and heated in a boiling water bath for 10 min, then tubes were cooled to room temperature. From these solutions, 100µL were transferred to another test tube and 6mL phosphovanillin reagent was then added (blank tube was contain 100µL H_2SO_4 instead of sample). After incubating at room temperature for 10min, absorbances were measured at 540nm (Shimadzu 1601 UV/Vis Double-beam Spectrophotometer, Kyoto, Japan). Total lipid content was calculated from the following equation:

Total lipid (mg/dL) = (OD_{sample}/OD_{std}) x 700mg/dL

2.3.4 Determination of Catalase Activities

(Aebi, 1974)

Principle:

In the UV range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240nm (ε_{240}=0.00364 L/mmol.mm), and the difference in absorbance per unit time is the measure of catalase activity.

Reagents:

1. Phosphate Buffer: 50mM, pH=7.0
2. Hydrogen peroxide: 30mM
3. Triton X-100: 1% (v/v)

Assay:

Before mixing the contents of the assay medium, 1000g cytoplasmic fractions, containing catalase in peroxisomes were diluted with 1% (v/v) Triton X-100 detergent to burst the peroxisomes and release the catalases to the medium. Then, further 200 or 300 fold dilutions were done with 50 mM phosphate buffer according to protein contents of the aliquots. After that, in a quartz cuvette, 2mL of enzyme solution was added. Enzymatic reaction was started with the addition of 1mL 30mM hydrogen peroxide and the rate of H_2O_2 decomposition was followed at 240nm for 1 minute with Shimadzu 1601 UV/Vis double-beam spectrophotometer.

Effect of pH on CAT Activity

- *Acidic region* activities were measured by 50 mM acetate buffers with pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0
- *Neutral region* activities were measured by 50 mM Potassium phosphate buffers with pH 6.0, 6.5, 7.0, 7.5, 8.0
- *Basic region* activities were measured by 50 mM Tris buffers with pH 7.5, 8.0, 8.5, 9.0, 9.5

Effect of Temperature on CAT Activity

After the addition of 2 mL enzyme suspension into quartz cuvettes, they were incubated in circulatory water bath, at varied temperatures (0, 10, 20, 25, 30, 35, 40, 45, 50, 55, and 60) for 5 min. With the addition of H_2O_2 (same temperature), reaction was started and the rate of reaction was monitored in order to calculate the activities.

Effect of Substrate (H_2O_2) Concentration and K_m&V_{max} Determination

10, 20, 30, 40, 50, 60, 70, 80 mM stock H_2O_2 solutions were used to determine K_m&V_{max} values for catalase.

2.2.5 Determination of Total, MnSOD and CuZnSOD Activities (Marklund and Marklund, 1974)

Principle:

The superoxide radical, which is the substrate for SOD, is generated by the autoxidation of pyrogallol under illumination at alkaline pH with the action of

atmospheric oxygen. As superoxide builds in the solution, the formation of yellow chromophore of oxidized pyrogallol accelerates because superoxide also reacts with pyrogallol. SOD neutralizes this radical and therefore slows down the pyrogallol oxidation. Because of this slowing process, SOD is said to inhibit the oxidation of pyrogallol.

Reagents:

1. Tris-EDTA buffer: 50mM-Tris, 10mM-EDTA, pH 8.2

Note 1: *In MnSOD activity measurements, this buffer were contained 1mM KCN which inhibits only CuZnSOD but not MnSOD.*

Note 2: *pH of TE buffer is very important since autoxidation of pyrogallol follows linear form at that pH. Above this value, curvature was observed and below this value (< pH: 8.0) there were no pyrogallol autoxidation.*

2. Pyrogallol solution: 15mM (*light sensitive)*

Assay:

In 3mL plastic spectrophotometer cuvettes, 2.80mL Tris-EDTA buffer and varied volume (5, 10, 15, 20, 25, 30, 40, 50μL) of cytosolic fractions (1000g) were mixed. Then, final volume was completed to 2.90mL with TE buffer. Pyrogallol autoxidation was started with the addition of 100μL pyrogallol and illumination, and the rate of autoxidation was followed at 440nm for 3 minutes. Enzyme blank readings were measured (no enzyme solution) to determine uninhibited autoxidation. In all readings, reference cuvette contained only TE buffer.

Same procedure for the same samples was repeated but this time, TE buffer also contain 1mM KCN, so that CuZnSOD activities were inhibited and only MnSOD activity could be determined. Percent inhibition of pyrogallol autoxidation was calculated according to equation 1 given below for each protein amount that was added to the reaction medium.

$$\% \text{ Inhibition} = 100x\ [(\Delta OD/min)_{blank} - (\Delta OD/min)_{sample}] / (\Delta OD/min)_{blank} \quad \text{(eq. 1)}$$

Percent inhibition versus mg protein graphs were constructed and converted into percent inhibition versus log protein graphs. Then, one unit total SOD activity is calculated as the amount of protein causing 50% inhibition of pyrogallol autoxidation. Next, in order to find out the amount of total SOD activity in 1 mg protein containing homogenate, equation 2 was applied.

$$1 / (\text{mg prot.}_{\text{50\% inhibition of autoxidation of pyrogallol}}) \quad \text{(eq. 2)}$$

After calculating total SOD activities by using TE buffer only, and calculating the MnSOD activities by using KCN containing TE buffer, CuZnSOD activities were calculated from equation 3.

$$\text{Total SOD activity} = \text{CuZnSOD activity} + \text{MnSOD activity} \quad \text{(eq. 3)}$$

Effect of pH on total SOD Activity

Autoxidation process of pyrogallol was dependent highly on pH, and it was very difficult even impossible to determine pH for SOD activities. Since above pH 8.5 autoxidation process occurred not linearly and leveled of immediately, reading an activity did not give reliable results. Furthermore; below pH 8.0, there was no autoxidation of pyrogallol.

Effect of Temperature on total SOD Activity

Autoxidation process was also highly dependent on temperature and below 15^{o}C and above 35 ^{o}C linearity of autoxidation process was demolished.

Effect of Substrate (Superoxide radical) Concentration and K_m&V_{max} Determination

Since the half life of superoxide radical is very small to be detected, and since it is not supplied directly to the assay medium, it was not possible to see the effect of substrate concentration on SOD activities and to determine K_m&V_{max}

2.2.6 Determination of Glutathione Peroxidase (GPx) Activities (Paglia and Valentine, 1967)

Principle:

GPx activity measurement was based on the measurement of degree of NADPH oxidation at 340nm with glutathione reductase which use oxidized glutathione and NADPH as a substrate. Since oxidized glutathione is produced by GPx, the degree of NADPH reduction is directly proportional to GPx activity (Figure 2.1).

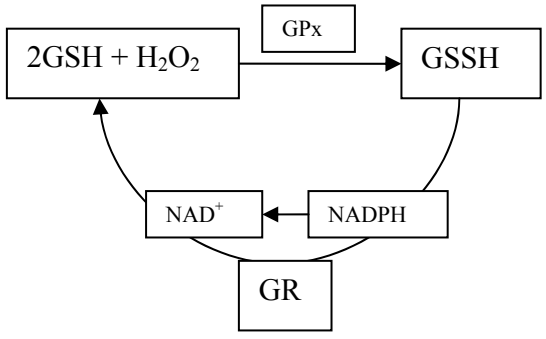


Figure 2.1: Principle of GPx assay

Reagents:

1. Tris-HCl buffer: 0.1M, pH 8.0
2. Reduced Glutathione: 80 mM
3. NADPH: 2mM
4. Glutathione Reductase: 0.24 U

Note: *Appropriate dilution must be done with freshly prepared 3.6M Ammonium Sulphate.*

5. Hydrogen Peroxide: 1.5mM
6. Sodium Azide (NaN_3): 30mM

Assay:

In 3 mL quartz cuvettes, 2.425 mL Tris buffer, 75 µL GSH, 100 µL glutathione reductase, 100 µL 40 fold diluted cytosolic fraction (≈0.075mg protein), 100 µL NaN_3 (inhibit catalase), were mixed and incubated for 5 min at room temperature. Then, enzymatic reaction was initiated with the addition of 100 µL hydrogen peroxide, and the rate of disappearance of NADPH at 340nm (ε_{340}=6220 $M^{-1}.cm^{-1}$) was followed for 3 minutes. Glutathione peroxidase activity was then described as the amount of NADPH consumed in one minute by 1 mg protein containing cytosolic fraction.

Effect of pH on GPx Activity

- *Acidic region* activities were measured by using 0.1 M acetate buffers with pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0

- *Neutral region* activities were measured by using 0.1 M Potassium phosphate buffers with pH 6.0, 6.5, 7.0, 7.5, 8.0
- *Basic region* activities were measured by using 0.1 M Tris buffers with pH 8.0, 8.5, 9.0, 9.5

Effect of Temperature on GPx Activity

After mixing all ingredients of the assay medium before starting the enzymatic reaction, 5 min incubation period was carried out in a circulatory water bath at varied temperatures (0, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60 ^{o}C). Then, normal procedure for the GPx assay was performed and effect of temperature on GPx activity was determined.

Effect of Substrate Concentration and K_m&V_{max} Determination

K_m and V_{max} values for GPx were determined independently by fixing one substrate at saturating concentration and changing the concentration of second substrate. That is, K_m of GPx against GSH was determined by using 2.5, 5, 10, 20, 30, 40, 50, 75, 100, 150, and 200mM stock solutions of GSH and 3 mM stock H_2O_2 at which GPx was saturated. Similarly, 0.1, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5mM H_2O_2, and 100mM stock reduced GSH were used for the determination of K_m of GPx to the H_2O_2. At that stock concentration of GSH, it was found that GPx was saturated.

2.2.7 Determination of Total Glutathione S-Transferase (GST) Activities (Habig *et al.*, 1974)

Principle:

1-chloro-2,4-dinitrobenzene (CDNB) is a common substrate for all isozymes of GST. It was used as substrate for total GST catalyzed GSH oxidation which was monitored by the increase in the absorbance at 340nm due to the colored adducts formation (Figure 2.2).

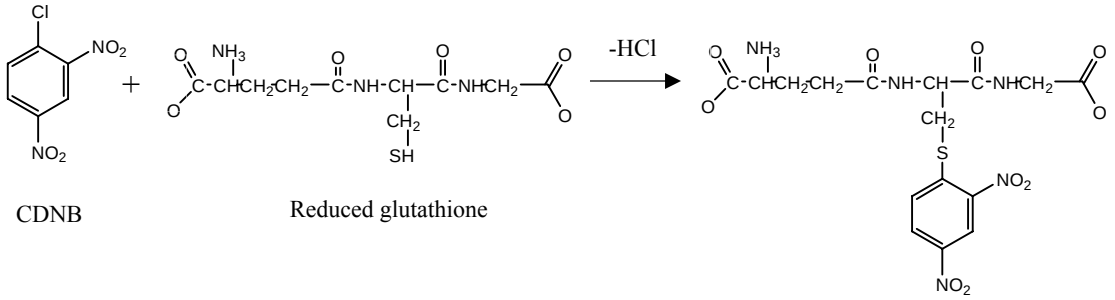


Figure 2.2: Mechanism of GST catalyzed reaction

Reagents:

1. CDNB: 25 mM (in ethanol/dH_2O (3/2))

   Note: It should be first dissolved in ethanol and then appropriate amount of dH_2O (2/3 of ethanol) is added before storing in dark.

2. Potassium Phosphate Buffer: 50mM, pH 7.0
3. GSH: 20mM

Assay:

Into the 3mL quartz cuvette, 2500µL potassium phosphate buffer, 200µL GSH, and 150µL enzyme (1/50 diluted cytosol) were added and the reaction was started with the addition of 150µL CDNB solution. Then, thioether formation was followed at 340 nm for 2 minutes. Each time, there were blank readings (reaction with no enzyme) for subtracting non-enzymatic product formation from the GST assay. Then, the enzyme activity was calculated as the amount of thioether (nmol) formed by 1mg total protein containing cytosol in one minute by using 0.0096 $\mu M.cm^{-1}$ as an extinction coefficient of thioether formed by GST.

Effect of pH on total GST Activity

- *Acidic region* activities were measured with 50mM Acetate buffer; pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0
- *Neutral region* activities were measured with 50mM Potassium phosphate buffers; pH 6.0, 6.5, 7.0, 7.5, 8.0
- *Basic region* activities were measured with 50mM Tris buffer; pH 7.5, 8.0, 8.5, 9.0, 9.5

Effect of Temperature on GST Activity

After mixing all ingredients of the assay medium, except CDNB, 5 min incubation period was carried out in circulatory water bath at varied temperatures namely 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 ^{o}C, then the normal procedure

for the GST assay was performed and effect of temperature on GST activity was determined.

Effect of Substrate Concentrations and K_m&V_{max} Determination

The effect of both substrates, GSH and CDNB, were studied and the individual K_m and V_{max} values were calculated independently by fixing one substrate concentration at saturating level and changing the concentration of second substrate. That is, K_m of GST against GSH was determined by using 0.1, 0.2, 0.5, 1, 2.5, 5, 10, 15, 20, 25, 40 and 50mM reduced glutathione stock solutions and 30mM CDNB at which GST was saturated. Similarly, 1, 2.5, 5, 10, 15, 20, 25, 30, 50mM CDNB and 30mM stock GSH were used as a substrate for the determination of K_m of GST to the CDNB. At that stock concentration of GSH, it was found that GST was saturated. While using different concentrations of substrates, blank readings were carried out for each of above concentrations.

2.3.8 Determination of Reduced Glutathione (GSH) Concentration (Sedlak and Lindsey, 1968)

Principle:

GSH determination is based on the oxidation of reduced GSH by 5,5'-dithiobis-(2-nitrobenzoic acid), [DTNB] to produce pale yellow color that gives its maximum absorbance at 412 nm. DTNB method measures the ability of SH groups to reduce DTNB to form 1 mole of 2-nitro-5-mercaptobenzoic acid (yellow) per mole of SH group.

Reagents:

1. Tris-EDTA (TE) Buffer: 0.2M Tris, 20mM EDTA, pH 8.2
2. DTNB: 10mM *(freshly prepared!)*
3. Methanol
4. Standard GSH solutions: 0.1 to 1mM

Assay:

To the 0.25mL appropriately diluted cytosolic fractions (1/5 and 1/10), or GSH standard solutions ranging between 0.1 to 1mM, 0.75mL Tris buffer, 0.05mL DTNB and 3.95mL methanol were added and mixed. Then, they were incubated at room temperature for 30 min with occasional shaking. After incubation, the samples were centrifuged at 3000g for 15 min to remove proteins having SH group and reacting with DTNB. GSH standard calibration curve was drawn and the slope was used to determine GSH concentration. The results were expressed as μmol GSH per mg protein.

2.3.9 Determination of TBARS Concentration (Jain and Levine, 1995)

Principle:

Malonedialdehyde which is an end product of lipid peroxidation gives reaction with thiobarbituric acid (TBA) to form a colored complex whose absorbance can be read at 532 nm. Since, not only MDA, but also other aldehydes in sample give

reaction with TBA, this method determines the total thiobarbituric acid reactive substances and called TBARS method.

Reagents:

1. Trichloro Acetic Acid (TCA): 30% (w/v)
2. Butylated Hydroxy Toluene (BHT): 0.04M in ethanol
3. Thiobarbituric Acid (TBA): 1% (w/v) in 0.05N NaOH
4. EDTA: 0.1M
5. Phosphate Buffered Saline (PBS): 18mM NaCl, 18mM Na_2HPO_4, pH 7.4
6. NaOH: 0.05N

Assay:

Two hundred microliters of microsomal suspension was mixed with 0.8mL PBS and 0.025mL of BHT to prevent artificial increase of MDA during the experiment. Then, 0.5mL TCA was added and tubes were allowed to stand in ice for at least two hours with occational shaking. After incubation, tubes were centrifuged at 2000*g* for 15 min and 1mL of each supernatant was transfered to another tube containing 0.075mL EDTA and 0.25mL TBA. Then, tubes were mixed and incubated in boiling water bath for 45 min. The samples were cooled to room temperature and the absorbances were measured at 532nm and 600nm in Shimadzu 1601 UV/Vis Double-beam Spectrophotometer (Kyoto, Japan). The absorbances of the samples at

600 nm were subtracted from 532 nm. This process was applied for the deduction of free MDA in the sample. Finally, TBARS concentrations were determined by using extinction coefficient of colored complex as 1.56 x 10^5 L/mol.cm and expressed for one mg of protein. TBARS test determines any compound that reacts with thiobarbituric acid, but the most abundant product is malondialdehyde.

2.2.10 Determination of Carbonyl Content of Protein (Levine *et al.*, 1990)

Principle:

A spectrophotometric assay was used for the quantification of protein carbonyl groups, as a biomarker of oxidative damage in diabetic liver tissues. This highly sensitive assay for detection of protein carbonyls, involve derivatization of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), which leads to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product, which could be measured specrophotometrically at 360nm.

Reagents:

1. Hydrochloric acid (HCl): 2 M
2. 2,4-Dinitrophenylhydrazine (DNPH) : 10 mM in 2M HCl
3. Trichloroacetic acid (TCA): 30% (w/v)
4. Ethanol/ethyl acetate: 1/1 (v/v)
5. Guanidine hydrochloride (GndHCl): 6 M in 20mM phosphate buffer, pH 2.5
6. Streptomycin sulfate: 10% (w/v)

Assay:

Before measuring the carbonyl contents, the impurities of homogenate from nucleic acids were checked, since nucleic acids also contain carbonyl groups and would also react with carbonyl reagents and interfere with the results. In general, interference is minimized when the ratio of absorbance at 280nm to that at 260nm is greater than one. Whenever the ratio of 280/260nm was less than one, samples were incubated with 10% streptomycin sulfate until a final concentration of 1% streptomycin sulfate were reached. The test tubes were left at room temperature for 15 minutes and then centrifuged at 6000g for 10 minute to remove insoluble nucleic acid precipitates.

After removing nucleic acid contaminants, 200µL protein solution (≈5mg) was added into 2.0mL eppendorf tubes and 800µL DNPH solution was added, (for each sample, a blank was prepared by adding 800µL of 2M HCl instead of DNPH). Then tubes were allowed to stand at room temperature for 1 hour with mixing every 10-15 minutes. After incubation, 500µL of 30% TCA was added and samples were incubated in ice for further 10 minutes, and then tubes were centrifuged in a tabletop microcentrifuge at 11000g for 5 minutes and supernatant was discarded. Resulting pallets were washed 3 times with 1.5mL ethanol-ethyl acetate (1:1) in order to remove unbound reagent (by mixing and centrifugating at 11000g for 5 minutes and discarding the supernatant each time). Then, the precipitated proteins were redissolved in 1.5mL guanidine hydrochloride solution. Proteins usually redissolved within 45 minutes at room temperature. Any insoluble material was removed by

centrifugation in the microcentrifuge for 5 min at 11000g and absorbances at 360nm were read against blanks and the carbonyl contents of the proteins were calculated by using a molar absorption coefficient of $2.2x10^4$ lt/mol.cm.

2.3.11 Statistical Analysis

Differences in measured parameters between normal and diabetic subjects were assessed by the Student T-test with the help of MINITAB 13.0 statistics software. Data were expressed as mean ± S.E.M. The relationships between oxidative parameters characterizing diabetic and control rat liver status were analyzed and a probability of 0.05 was set as the level of statistical significance. Throughout this thesis, (*) indicates significance at 95% confidence (i.e. $p<0.05$) and (**) indicates significance at 99.5% confidence (i.e. $p<0.005$).

CHAPTER III

RESULTS AND DISCUSSION

Two groups of Wistar rats, Control (n=6) and Diabetic (n=7) were used in this study. Blood glucose levels and body weights of both group animals were measured weekly starting after STZ treatment till to eight week. The occurrence of STZ induced diabetes was confirmed by establishing weekly blood glucose levels in diabetic rats, and the animals which maintained its glucose concentration above 200 mg/dL were assumed to be diabetic.

The presence of oxidative stress was shown by thiobarbituric acid reactive substances (TBARS) test. Total protein and total lipid values and their ratios in microsomal membranes were also compared in order to see the effect of the disease on the membrane composition. Furthermore, in this study, protein carbonylation which is another consequence of oxidative damage on proteins was measured and compared in both groups.

The effects of eight week diabetes were evaluated by changes in diabetic rats' antioxidative capacity. Activities of enzymatic antioxidants, superoxide dismutase

(SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione S-transferase (GST) were measured. Also, nonenzymatic antioxidant, reduced glutathione, levels were compared. The conditions of the activity measurements of major antioxidant enzymes were first optimized using control animals, and optimum conditions for the assays (pH, temperature and substrate concentrations) were determined.

3.1 Characterization of Antioxidant Enzyme Activities

3.1.1 Catalase

3.1.1.1 Effect of pH on Catalase Activity

Figure 3.1 shows the effect of varying pH on the catalase activity. The pH of the medium was varied between 3.5 and 9.0 in the presence of acetate, phosphate and Tris-HCl buffers. As shown in the figure, the maximal activity of catalase was observed at pH 7.0 and after that, all the catalase activity measurements were carried out at pH 7.0 with potassium phosphate buffers.

3.1.1.2 Effect of Temperature on Catalase Activity

Figure 3.2 shows the effect of varying temperature on the CAT activity. The temperature of the reaction medium was varied between 0 to 60^{o}C. It has been observed that, activity of catalase did not change drastically between 20 to 40^{o}C. After 40^{o}C, the sharp decrease obtained was probably due to the protein denaturation. As a result, throughout this study, all CAT activity measurements were carried out at room temperature

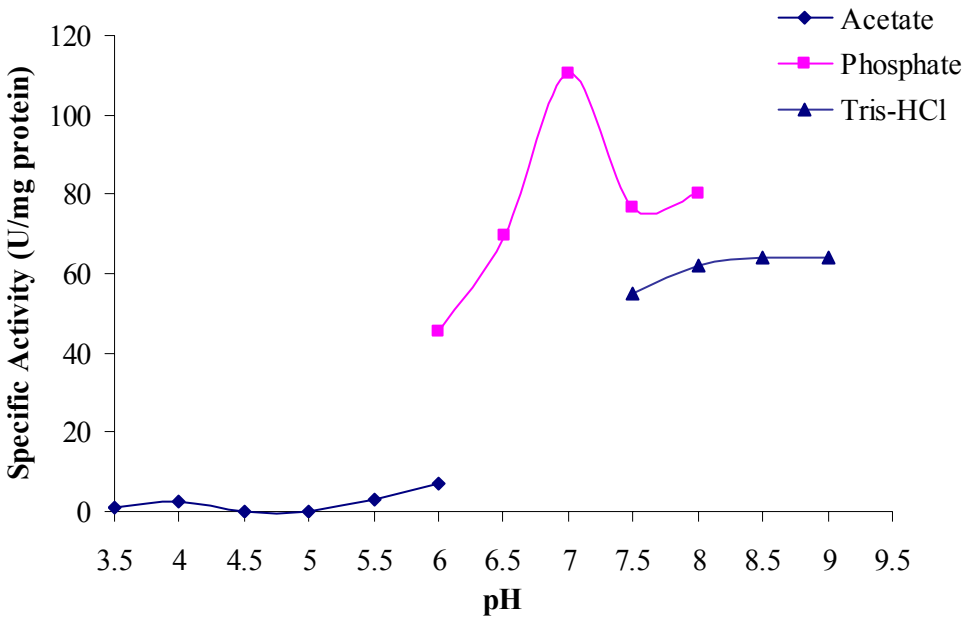


Figure 3.1: Effect of pH on CAT Activity. The points are the means of duplicate measurements.

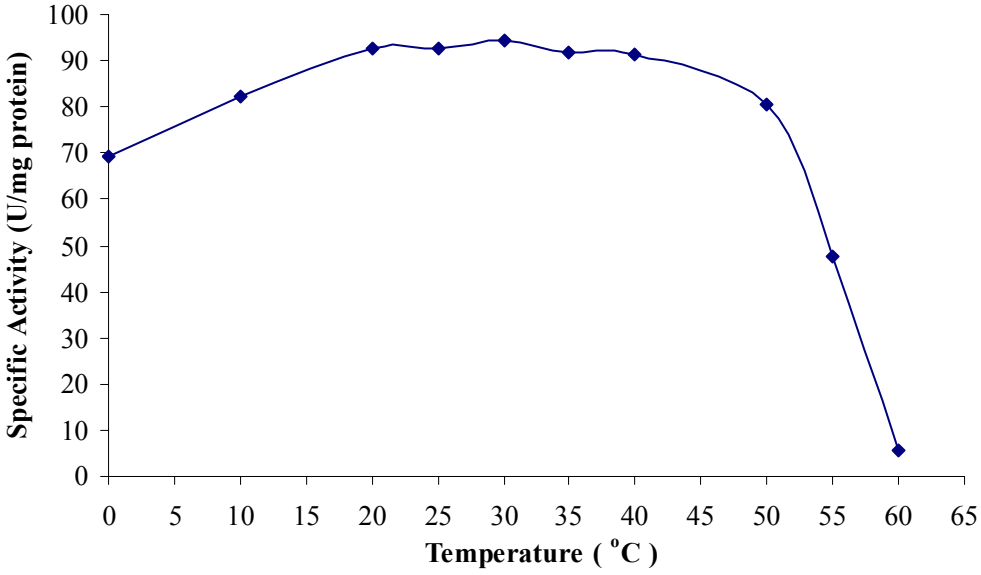


Figure 3.2: Effect of Temperature on Catalase Activity. The points are the means of duplicate measurements.

3.1.1.3 Effect of Substrate Concentration on CAT Activity

Figure 3.3 illustrates the substrate, hydrogen peroxide, saturation curve for the CAT. As it can be seen from the figure, at concentration of approximately 20mM, catalase was fully saturated by hydrogen peroxide.

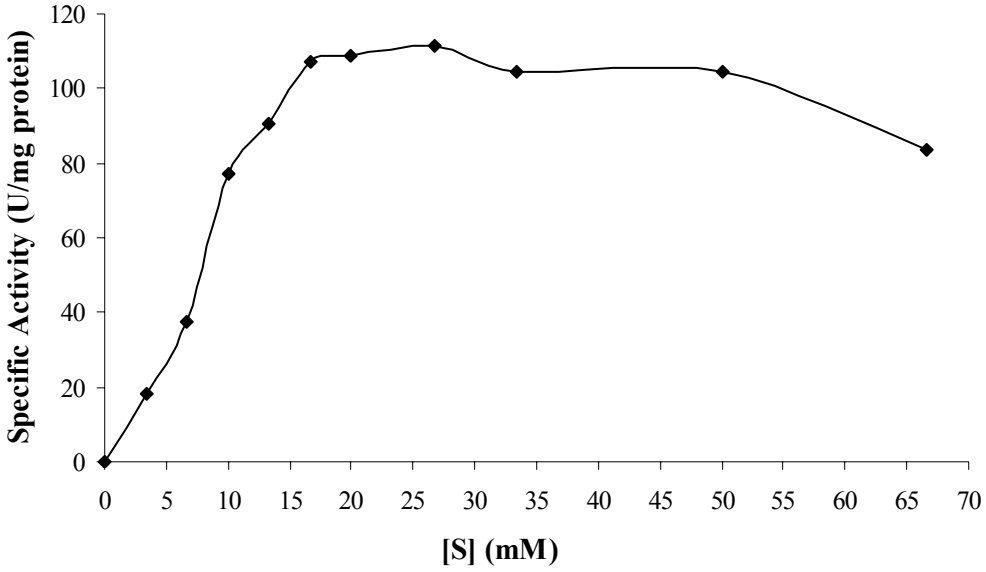


Figure 3.3: Substrate, hydrogen peroxide, saturation curve for rat liver catalase. Reactions were carried out at room temperature and pH 7.0 as described under "Methods". The points are the means of two different sets of data and each point is the mean of duplicate determinations.

By the use of Lineweaver-Burk plot (Figure 3.4), K_m and V_{max} values were determined for the catalase. K_m (hydrogen peroxide) was found as 9mM and V_{max} was calculated as 110 U/mg protein.

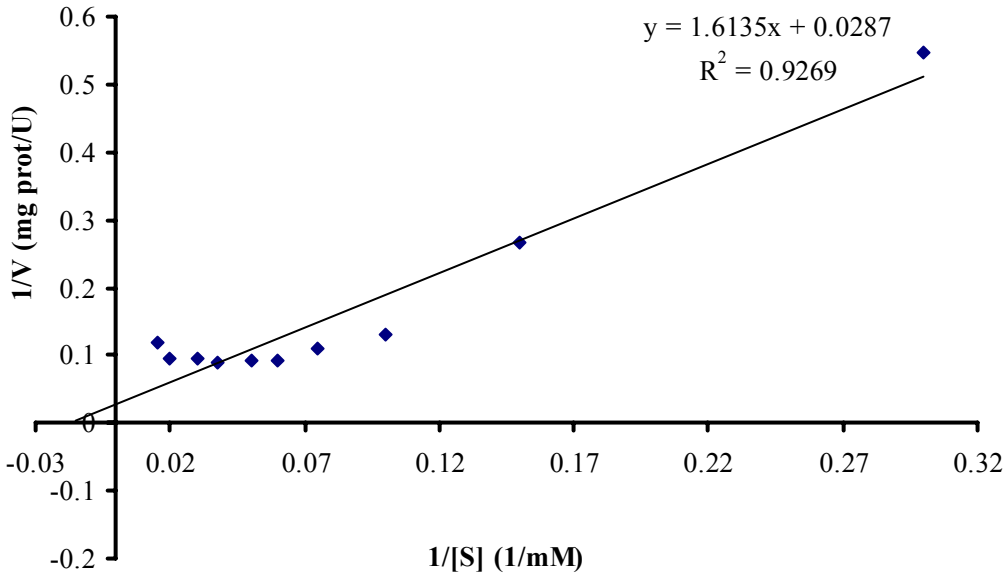


Figure 3.4: Lineweaver-Burk plot of CAT

3.1.2 Glutathione Peroxidase

3.1.2.1 Effect of pH on Glutathione Peroxidase Activity

Figure 3.5 shows the effect of varying pH on the GPx activity. The pH of the medium was varied between 3.5 and 9.5 in the presence of acetate, phosphate and Tris-HCl buffers. The maximal activity of GPx was observed at pH 8.0 and after that all the GPx activity measurements were carried out at pH 8.0.

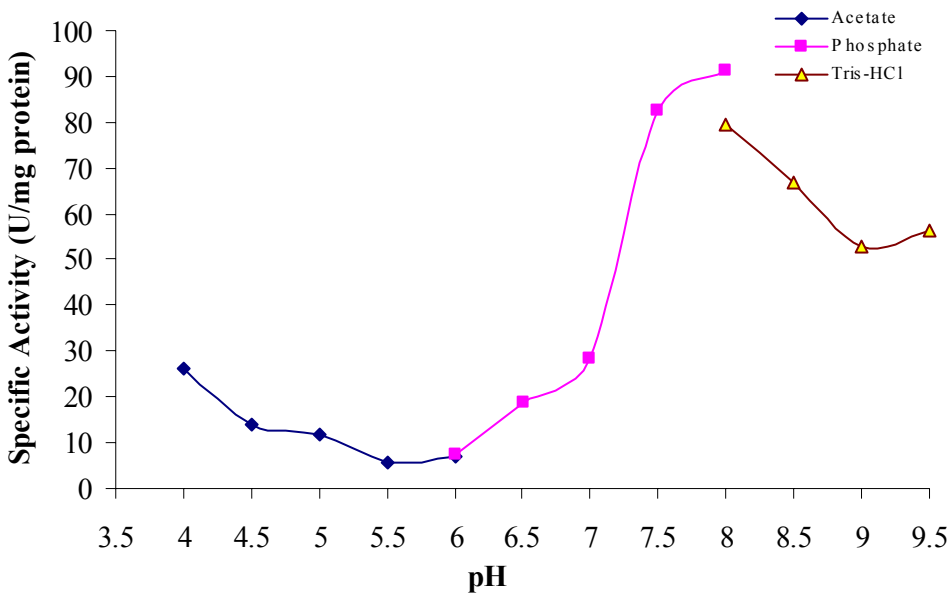


Figure 3.5: Effect of pH on GPx activity. The points are the means of duplicate measurements.

3.1.2.2 Effect of Temperature on Glutathione Peroxidase Activity

Figure 3.6 shows the effect of varying temperature on the GPx activity. The temperature of the reaction medium was varied between 0 to 60^{o}C. It was found that, the activity of GPx was maximum between 35-40 ^{o}C. So, 37 ^{o}C was chosen as the optimum temperature and throughout this study, all GPx activity measurements were carried out at 37^{o}C.

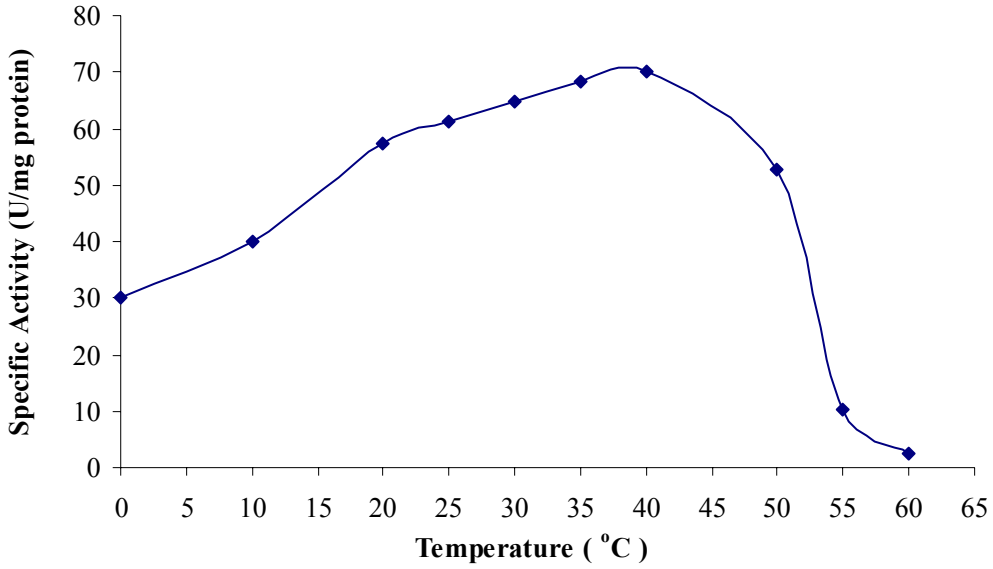


Figure 3.6: Effect of Temperature on GPx Activity. The points are the means of duplicate measurements.

3.1.2.3 Effect of Substrate Concentration on GPx Activity

3.1.2.3.1 Hydrogen Peroxide as a Substrate

Figure 3.7 illustrates the substrate, hydrogen peroxide, saturation curve for the GPx. As seen from the figure, at 0.1mM hydrogen peroxide, GPx was saturated.

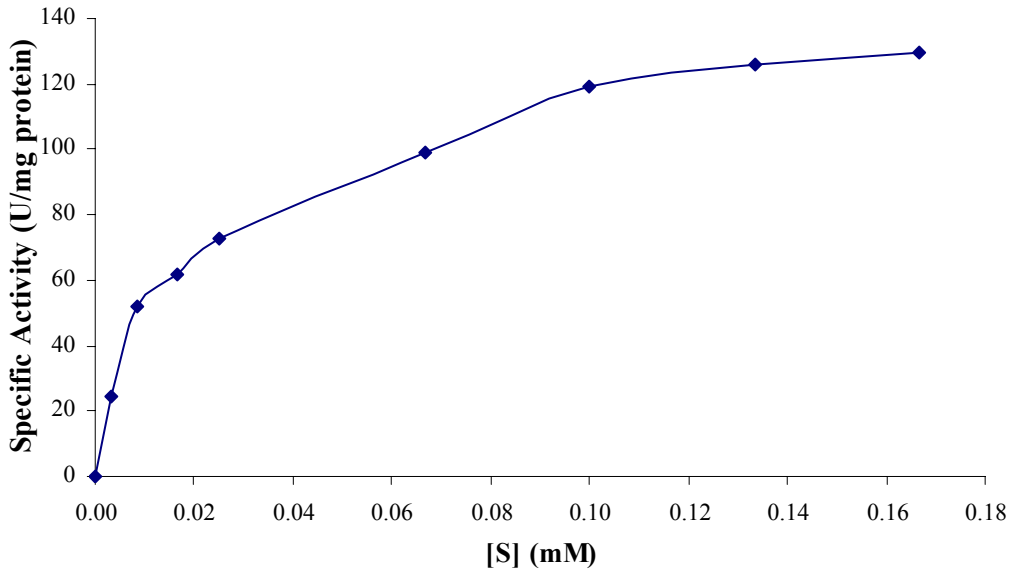


Figure 3.7: Substrate, hydrogen peroxide, saturation curve for rat liver GPx. Reactions were carried out at 37°C and pH 8.0 as described under "Methods". The second substrate, GSH, concentration was kept saturating and the points are the means of two different sets of data and each point is the mean of duplicate determinations.

From the Lineweaver-Burk plot (Figure 3.8), K_m and V_{max} values were calculated for the GPx. K_m (hydrogen peroxide) was found as 9.4 nM and V_{max} was calculated as 127 U/mg protein.

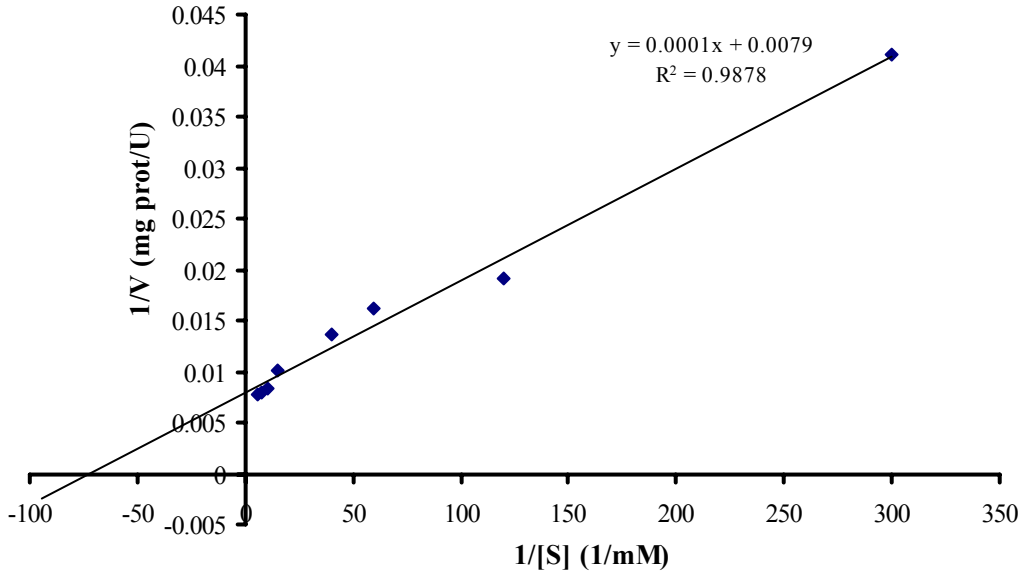


Figure 3.8: Lineweaver-Burk plot of GPx

3.1.2.3.2 Reduced Glutathione (GSH) as a Substrate

Figure 3.9 illustrates the substrate, reduced glutathione, saturation curve for the GPx. As it can be observed from the figure, at concentrations above 2.5mM, GPx was saturated by the substrate, GSH.

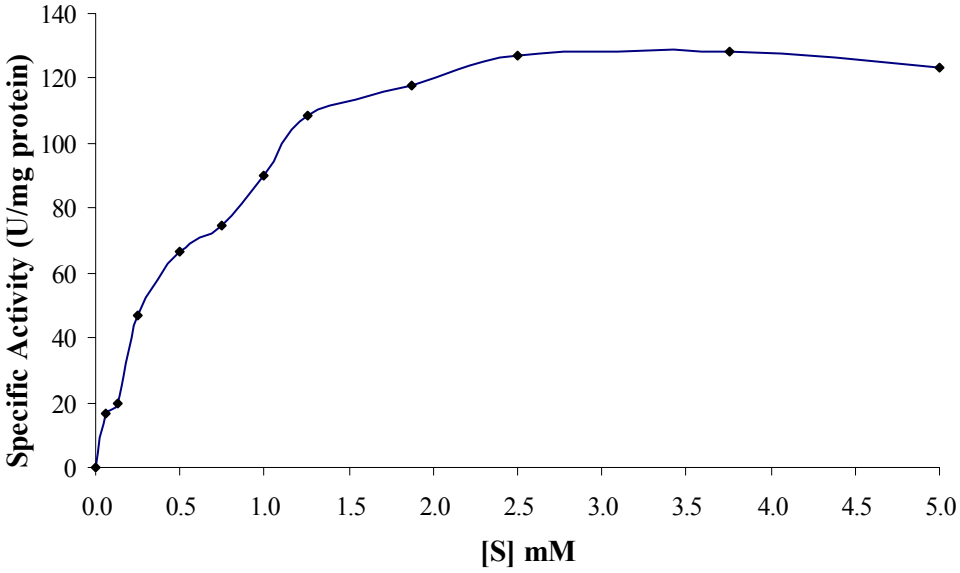


Figure 3.9: Substrate, GSH, saturation curve for rat liver GPx. Reactions were carried out at 37^{o}C and pH 8.0 as described under "Methods". The second substrate, hydrogen peroxide concentration was kept saturating. The points are the means of two different sets of data and each point is the mean of duplicate determinations

By the use of Lineweaver-Burk plot (Figure 3.10), K_m and V_{max} values were determined for the GPx. K_m (GSH) was found as 0.47mM and V_{max} was calculated as 127 U/mg protein.

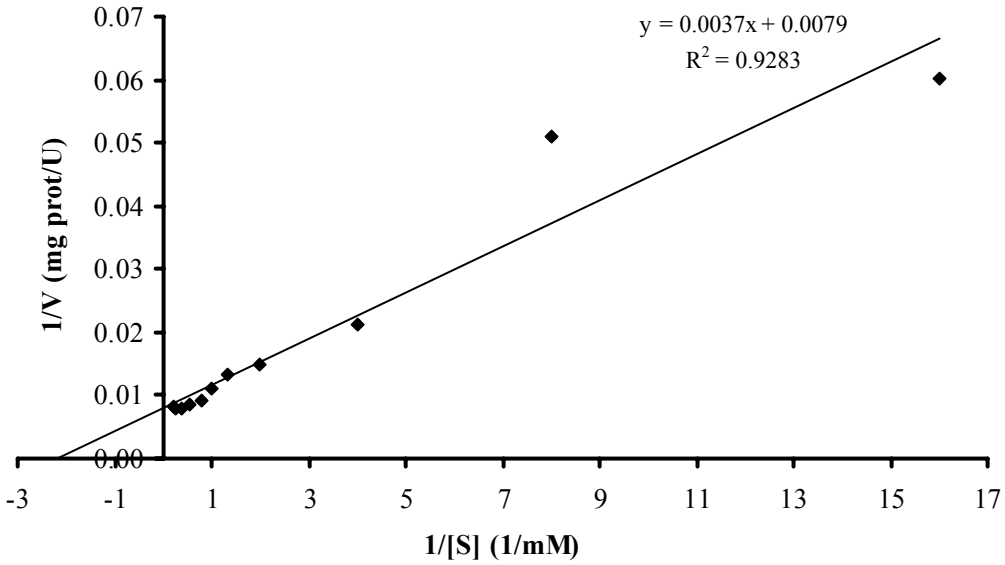


Figure 3.10: Lineweaver-Burk plot of GPx

Glutathione peroxidase, which decomposes hydrogen peroxide into water by using reduced glutathione as a co-substrate has a K_m of 9.4 nM for H_2O_2 and 470 nM for GSH. Catalase which is another enzyme in the neutralization of H_2O_2 into water has a K_m value of 8.0 mM for hydrogen peroxide. According to these results; we think that at low hydrogen peroxide concentrations, glutathione peroxidase can be the primary enzyme for the first step neutralization of the hydrogen peroxide as its affinity for the H_2O_2 is higher. When H_2O_2 concentration increases further, then catalase will be the primary enzyme for the elimination reaction of hydrogen peroxides.

3.1.3 Glutathione S-Transferase

3.1.3.1 Effect of pH on Glutathione S-Transferase Activity

Figure 3.11 shows the effect of varying pH on the GST activity. The pH of the medium was varied between 3.5 and 9.5 in the presence of acetate, phosphate and Tris-HCl buffers. The maximum activity of GST was observed at two pH values, namely, pH 7.0 and pH 9.0. We choosed pH 7.0 as an optimum pH and, all the GST activity measurements were carried out at pH 7.0.

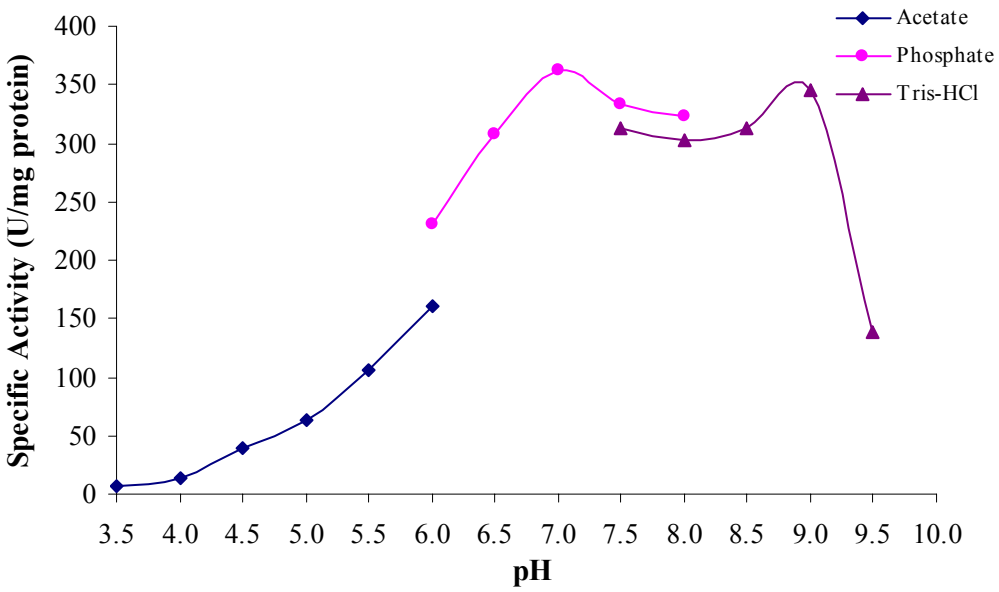


Figure 3.11: Effect of pH on GST activity. The points are the means of duplicate measurements.

3.1.3.2 Effect of Temperature on Glutathione S-Transferase Activity

Figure 3.12 shows the effect of varying temperature on the GST activity. The temperature of the reaction medium was varied between 0 to 60°C. As seen from the figure, the highest activity of GST was observed between 45-50 °C. After 50 °C, there was a drastic decrease in the activity because of denaturation. So, activity measurements were carried out at 45 °C in order to get highest activities.

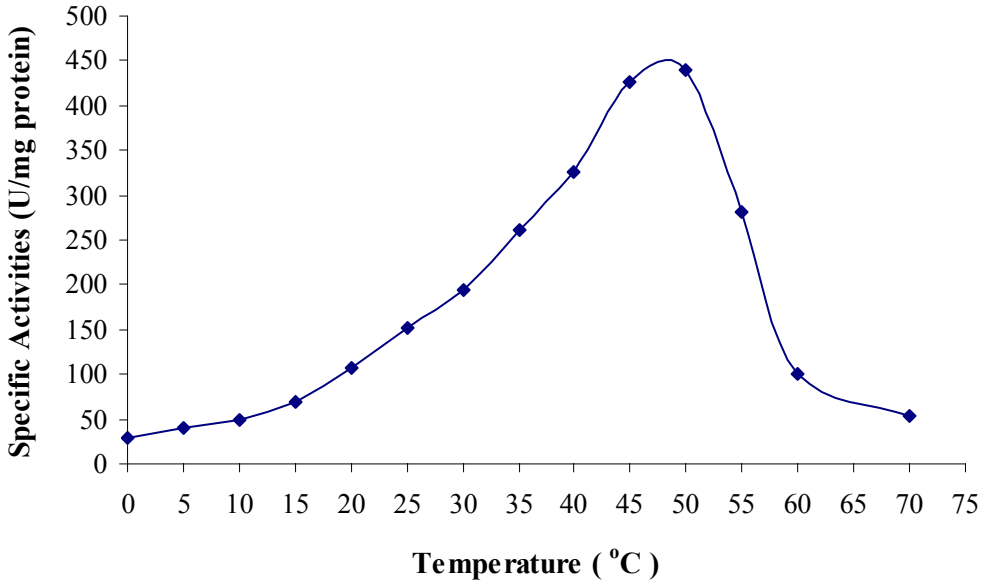


Figure 3.12: Effect of Temperature on GST Activity. The points are the means of duplicate measurements.

3.1.3.3 Effect of Substrate Concentration on GST Activity

3.1.3.3.1 Reduced Glutathione (GSH) as a Substrate

Figure 3.13 illustrates the substrate, GSH, saturation curve of the GSTs. As seen from the figure, enzyme was saturated at around 1.5mM GSH concentration.

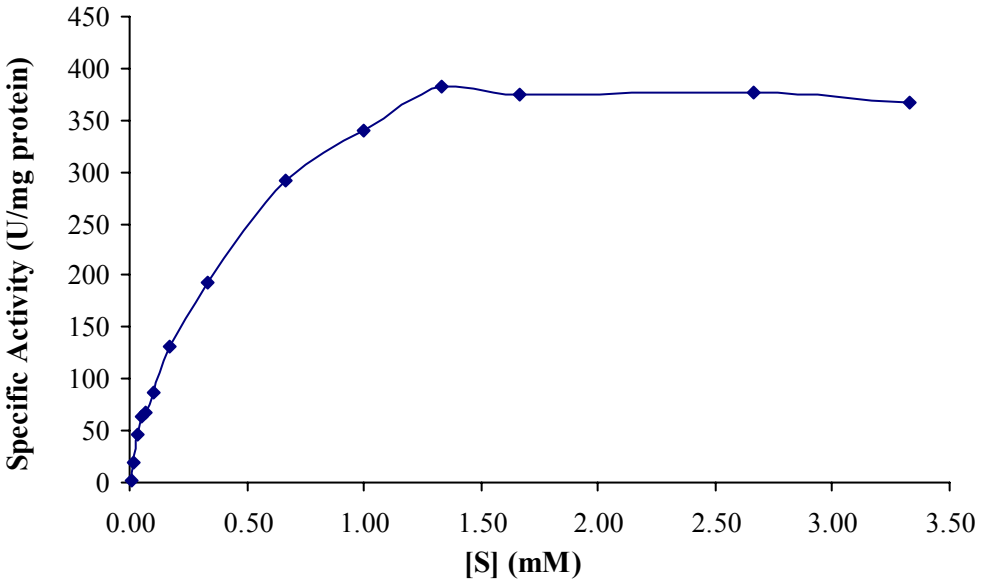


Figure 3.13: Substrate, GSH, saturation curve for rat liver GST. Reactions were carried out at 45°C and pH 7.0 as described under "Methods". The other substrate, CDNB, concentrations were saturating. The points are the means of two different sets of data and each point is the mean of duplicate determinations

Lineweaver-Burk plot (Figure 3.14) was also constructed and the K_m and V_{max} values were determined for the GST. K_m (GSH) was found as 0.25mM and V_{max} was calculated as 454.5 U/mg protein.

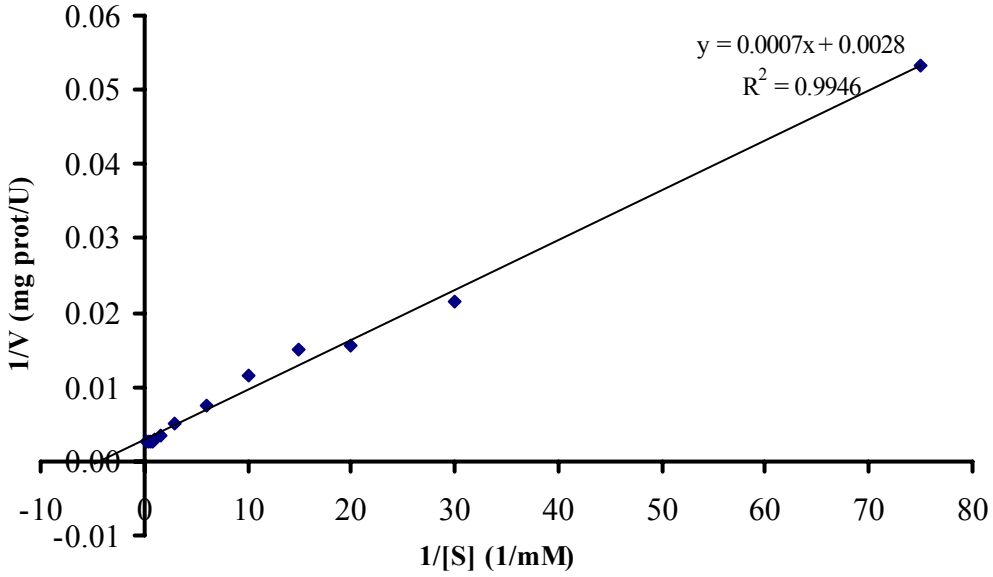


Figure 3.14: Lineweaver-Burk plot of GST

3.1.3.3.2 1-Chloro-2,4-dinitrobenzene (CDNB) as a Substrate

Figure 3.15 shows the substrate, CDNB, saturation curve of the GST. At CDNB concentrations above 1.5mM, GST was fully saturated.

By the use of Lineweaver-Burk plot (Figure 3.16), K_m and V_{max} values were calculated and the K_m (CDNB) was found as 0.36mM and V_{max} was calculated as 454.5 U/mg protein.

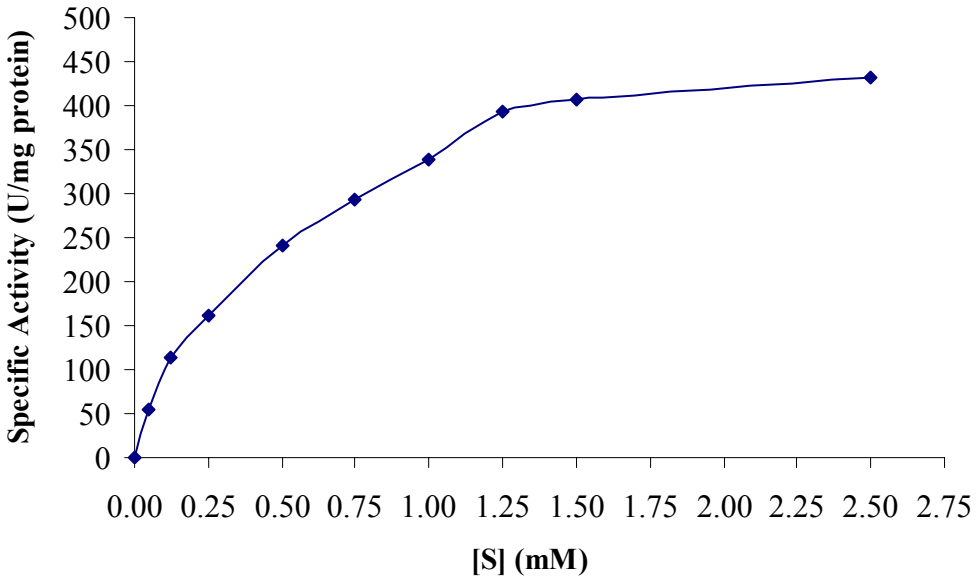


Figure 3.15: Substrate, CDNB, saturation curve for rat liver GST. Reactions were carried out at 45°C and pH 7.0 as described under "Methods". The GSH concentration was saturating. The points are the means of two different sets of data and each point is the mean of duplicate determinations

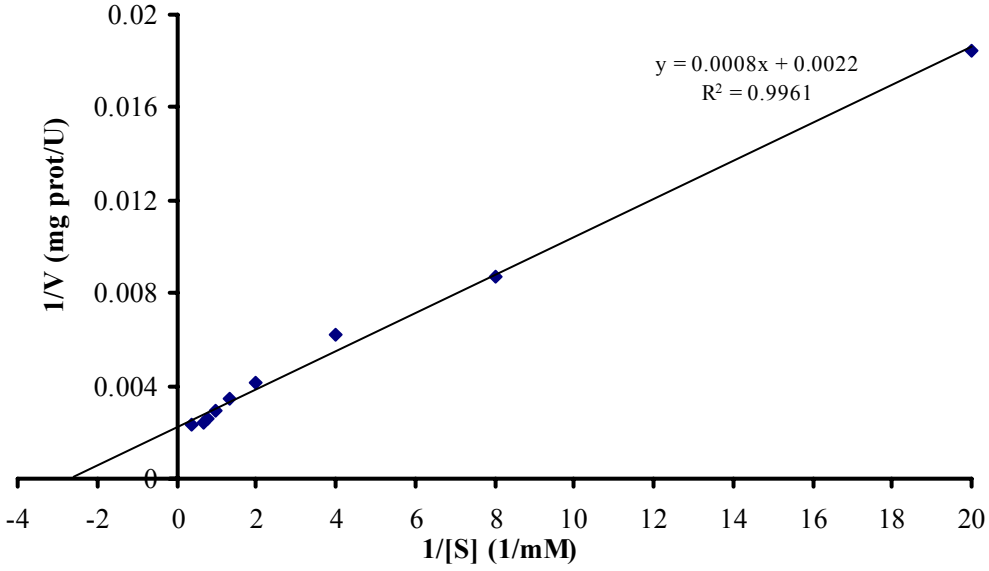


Figure 3.16: Lineweaver-Burk plot of GST

Table 3.1 summarizes the optimum parameters for the activity determinations of the antioxidant enzymes. All the activity measurements in this study were carried out according to these values.

Table 3.1: Summary of the Optimum Parameters for Antioxidant Enzyme Assays

	Optimum Temp (^oC)	**Optimum pH**	**Substrate**	**K_m**	**V_{max} (U/mg)**
CAT	20-40	7.0	H_2O_2	9 mM	110
GPx	35-40	8.0	H_2O_2	9.4 nM	127
			GSH	0.47 mM	
GST	45-50	7.0	CDNB	0.36 mM	455
			GSH	0.25 mM	

3.2 Antioxidant Enzymes Activities of Diabetic and Control Rat Livers

Antioxidant enzymes are considered to be the primary defenses that prevent biological macromolecules from consequences of oxidative damage. SODs rapidly convert superoxide to the less dangerous hydrogen peroxide, which is further degraded by CAT and GPx into water. Thus, the steady-state level of antioxidant enzymes during diabetes may protect some important tissues such as liver against free radical damage.

The change in the antioxidant enzyme activities of diabetic and control groups are given in the following figures, namely for CAT (Figure 3.17), GPx (Figure 3.18), SOD (Figure 3.19) and GST (figure 3.20). The results have shown that the activities of SODs; total SOD, CuZnSOD but not MnSOD, (Figure 3.19) and GPx (Figure 3.18) in the STZ induced diabetic rat liver tissues were significantly increased; whereas, GST activities remained unchanged in diabetic group (Figure 3.20). These observations suggested that except GSTs, overall antioxidant enzyme status augmented during the diabetes as a compensatory mechanism. However, CAT activity of diabetic rats was significantly lower than that of control rats (Figure 3.17). This reduction in CAT activity might change the overall antioxidant enzyme defense system.

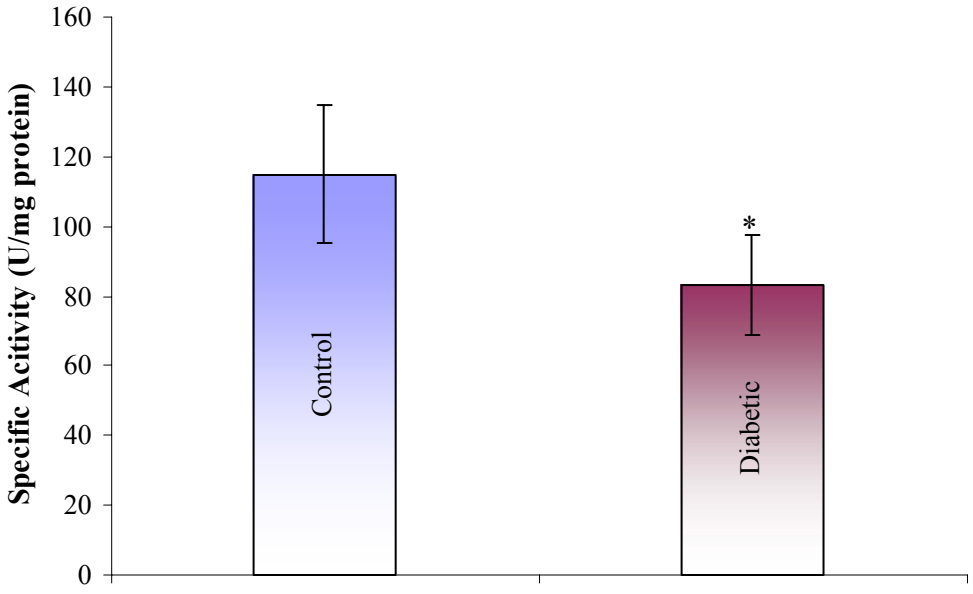


Figure 3.17: CAT activities in Diabetic and Control Rat Liver Tissues

(Values were expressed as mean ± S.E.M).

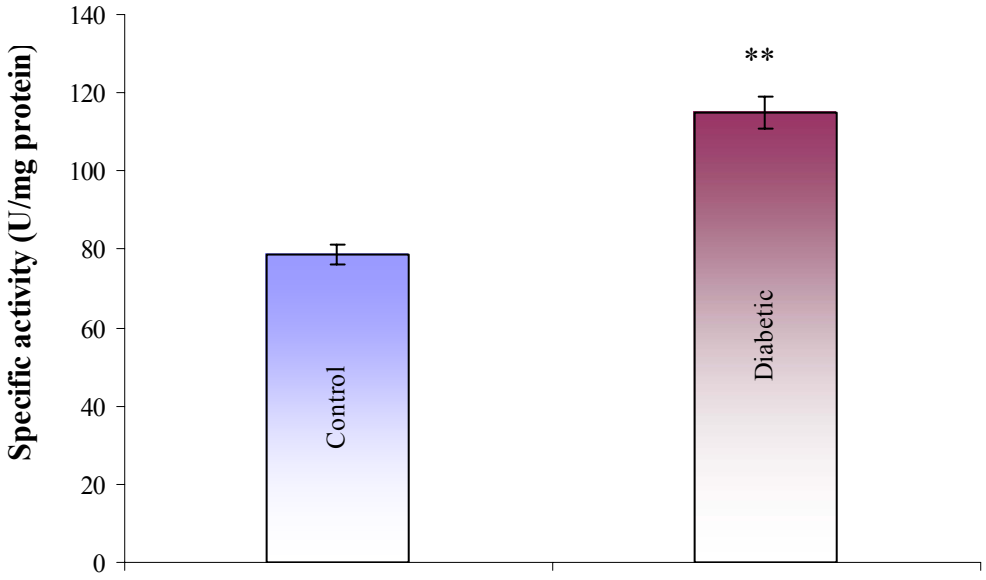


Figure 3.18: GPx activities in Diabetic and Control Rat Liver Tissues

(Values were expressed as mean ± S.E.M).

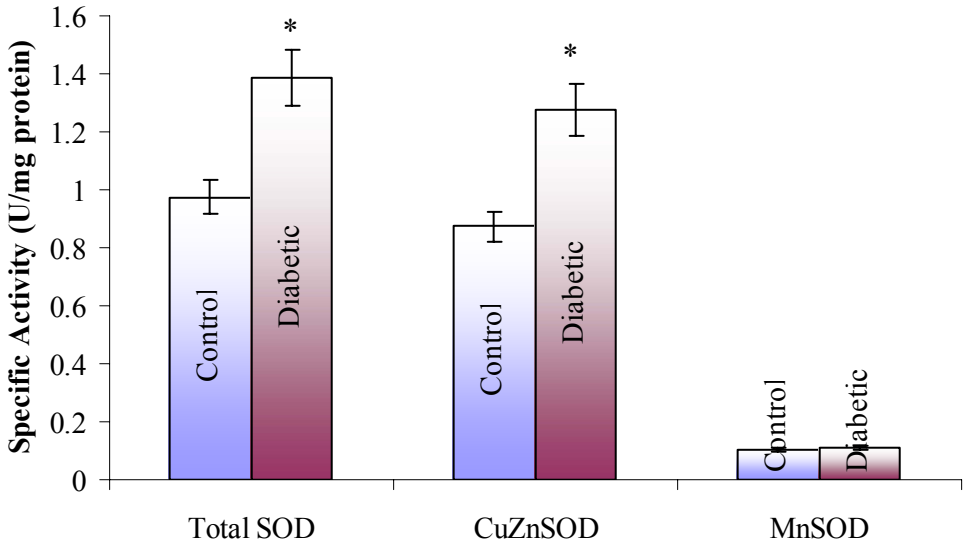


Figure 3.19: SOD activities in Diabetic and Control Rat Liver Tissues

(Values were expressed as mean ± S.E.M).

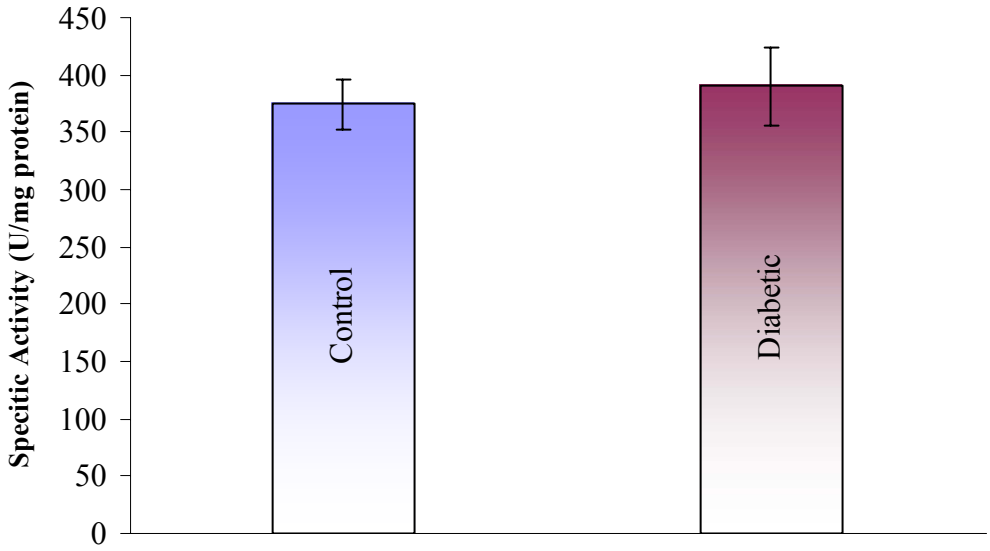


Figure 3.20: Total GST activities in Diabetic and Control Rat Liver Tissues (Values were expressed as mean ± S.E.M).

CAT is a major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to H_2O, sharing this function with GPx. Therefore, both of these enzymes detoxify H_2O_2 derived from SOD activity. In the presence of low H_2O_2 levels, organic peroxides are preferred substrate for GPx. At high concentrations, they are metabolized by CAT (Yu, 1994). Therefore, it is commonly assumed that any significant increase in SOD must be accompanied by comparable increase in CAT and/or GPx to prevent excessive build up of H_2O_2. In our study, SOD activity increased significantly in diabetic rat livers as expected. Also GPx activity showed an increase, but CAT activity followed opposite trend and activity decreased significantly compared to controls. This may be related to the rate of production of H_2O_2 due to the increased SOD activity. Amplified SOD activities

indicated an increase also in superoxide radicals, and this high concentration of superoxide radicals might inhibit CAT activities as shown by Kono and Fridovich (1982). The decreasing trend in CAT activity seemed to be counterbalanced by the increasing trends in SOD and GPx activities in diabetics. Increased H_2O_2 concentrations due to reduced CAT activity could enhance the SOD activities (Mitchelson *et al.,* 1977) for the removal of superoxide radicals which is a potent GPx inhibitor. Because increased concentrations of superoxide radicals can also inactivate GPx (Blum and Fridovich, 1985).

Also, increased total SOD activities are most probably due to increased activities of cytosolic CuZnSOD activities. Mitochondrial MnSOD activitis were not different suggesting that mitochondrial production of superoxide were not as significant as cytosolic production of superoxide anion

Decreased CAT activities were also observed in male and female brains, with size and age, in a study carried out with marine shrimps (Mourente and Diaa-Salvago, 1999). And this decrease seemed to be counterbalanced by the increasing trends in SOD and GPx activities in males and GST and GR activities in females.

The changes in the activities are also seemed to be tissue specific. CAT activity of rats with STZ induced diabetes was increased in liver, kidney, testis and erythrocyte hemolysate but decreased in spleen (Kakkar *et al.* 1995, Oberley, 1988 and D'Aquino *et al*., 1991). But, some other authors have also shown decreased CAT activity in liver, kidney and heart of diabetic rats (Yu, 1994, Asayama *et al*., 1991, D'Aquino *et al*, 1991). In another study carried out with STZ induced diabetic rats,

liver of diabetic rats showed a generalized decrease in CAT, GPx and SOD as well as in the level of GSH. However, diabetic kidney tissues showed decreases in CAT and SOD activities, whereas the activities of GPx were found to be increased. Insulin treatment reversed all of the alterations in tissue antioxidant status (Wohareb and Godin, 1987).

Though there are many studies related with diabetes and antioxidant enzyme activities, the results are contradictory. The length of the diabetes, the presence of insulin treatment for survival or other factors can affect the results. Kakkar and coworkers (1995) observed an increase in all antioxidant enzyme activities including CAT activities in diabetic liver tissues. In another research (Seleh *et al.,* 1987) CAT activity decreased in liver of diabetic rats but they also found decreasing trends in both SOD and GPx activities. Therefore, it is apparent that, no set patterns or trends are obvious with regard to changes in tissue antioxidant enzyme activities in STZ induced diabetes (Oberley, 1988, Yu, 1994).

Glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other oxidant species. It consists of reduced glutathione and an array of functionally related enzymes such as glutathione peroxidase and glutathione S-transferase which are working together with glutathione in the decomposition of hydrogen peroxide or other hydroperoxides (Mak *et al.*, 1996). Also, GSTs are a large, multigene family of proteins and they play an important role in protecting tissues from oxidative damage, because they function in

the transport of cellular components or metabolites (or conjugation of metabolites with glutathione) formed during diabetes.

GST activity, which catalyses both conjugation of GSH with lipophilic electrophiles and reduction of oxidants by GSH showed no significant change due to disease (Figure 3.20). The activity method we have used measured the total GST activity in the tissues. There could be an increase or decrease in the individual isozyme activities, but the total activity seemed to be not changed. These results are in consistent with the study of Girón *et al.,* (1999).

3.3 Reduced Glutathione Levels

As mentioned before, reduced glutathione (GSH), which is the most important biomolecule against chemically induced toxicity, can participate in the elimination of the reactive intermediates by reduction of hydroperoxides in the presence of GPx and GST. GSH also functions as free radical scavenger and in the repair of radical caused biological damage. Previous studies have shown that, hepatic GSH concentration of STZ induced diabetic rats was significantly lower when compared with the normal rats (Loven *et al.,* 1986 and Ewis *et al.,* 1995). We also observed about 1.13 fold decrease in GSH concentration in diabetic rats as shown in Figure 3.21. Most probably, the decrease in GSH in liver during diabetes represents its increased utilization due to oxidative stress.

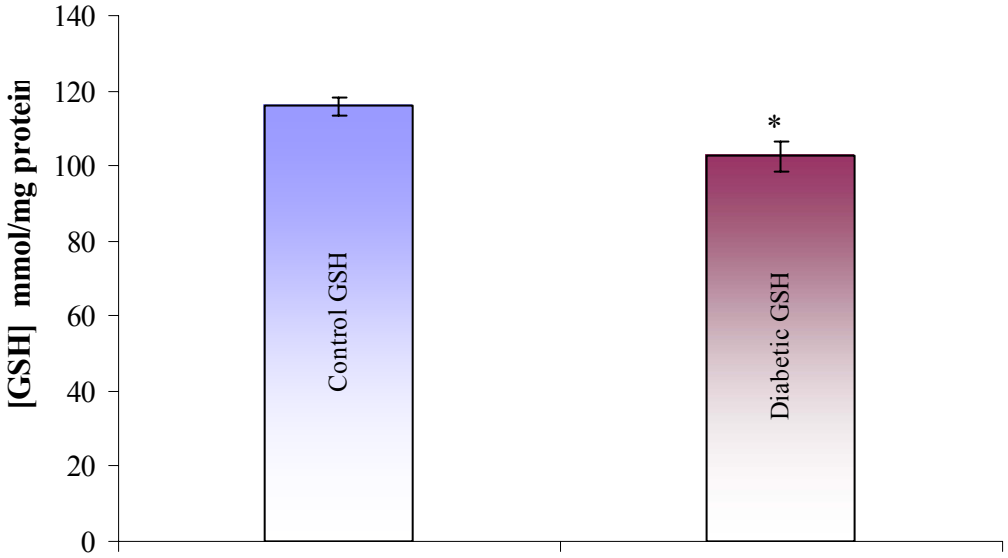


Figure 3.21: GSH concentrations in Diabetic and Control Rat Liver Tissues (Values were expressed as mean ± S.E.M).

3.4 Lipid Peroxidation

Lipid peroxidation is a free radical induced process leading to oxidative damage of unsaturated lipids. Under normal physiological conditions, low levels of lipid peroxides are found in tissues, however, free radicals which react with lipids can cause peroxidative changes that result in enhanced lipid peroxidation. So, it is an important marker of early and reversible tissue damage caused by diabetes mellitus and of decrease in antioxidant defense capacity of tissue.Lipid peroxidation may also bring about protein damage and inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products which are MDA and 4-hydroxynonenal (Halliwell and Gutteridge, 1999).

We found that, lipid peroxidation levels in the liver tissues of diabetic rats were significantly (p=0.004) higher compared to controls as seen in Figure 3.22 and these finding are in agreement with other results (Kakkar et al., 1995, Sato et al., 1981, Malaisse, 1982). According to our results, lipid peroxidation increased approximately 1.23 fold in diabetic group. This marked increased in the lipid peroxidation in membranes in diabetic state suggests an increase in oxygen free radicals that could be due to either their increased production or, decreased elimination.

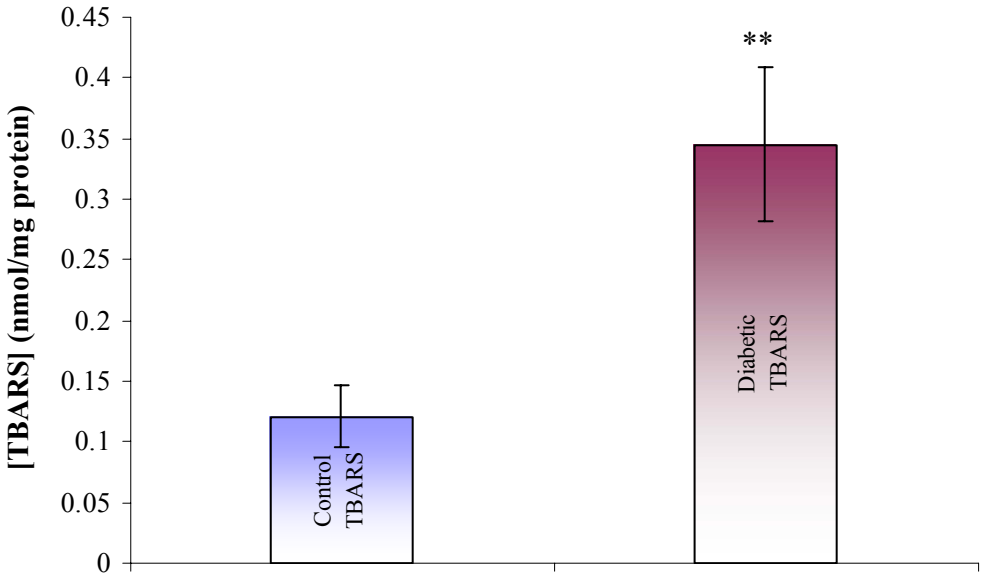


Figure 3.22: Comparison of TBARS Levels in Diabetic and Control Rat Liver (Values were expressed as mean ± S.E.M).

3.5 Lipid to Protein Ratios

Lipids are the major components of the biological membranes. They act as vital substrates for lipid peroxidation and the change in the membrane composition

during diabetes could be monitored by measuring the lipid to protein ratios. This parameter shows an alteration in lipid metabolism in diabetes and this ratio can also be used as an index for the fluidity and degree of macromolecular oxidative damage. According to results as shown in Figure 3.23, in diabetic group microsomes lipid to protein ratio was significantly higher compared to control group. This change in the ratio can be due to the stimulation in lipid synthesis in diabetes or more probably due to the protein oxidation which makes membrane proteins more susceptible to proteolytic attacks.

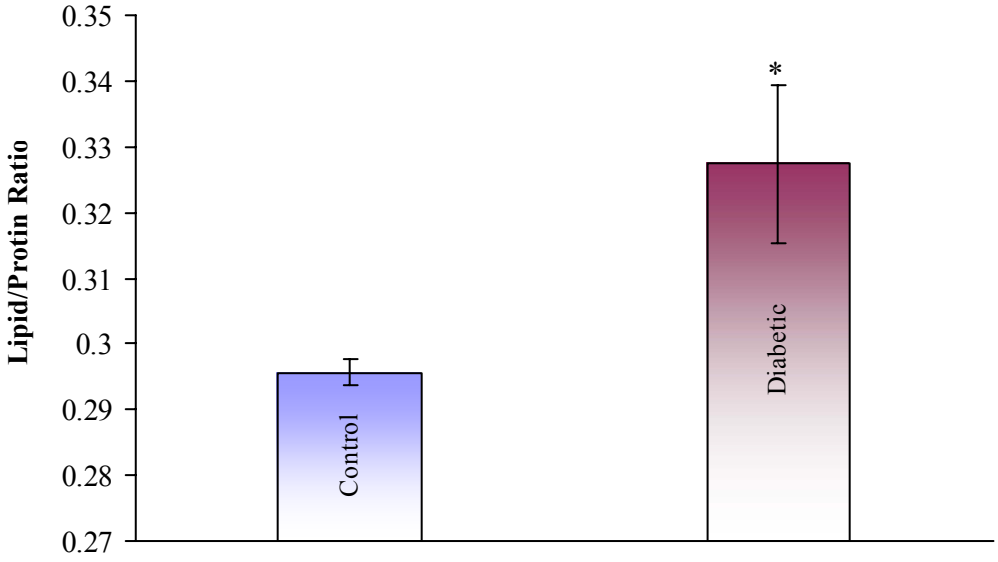


Figure 3.23: Lipid/Protein Ratios in Diabetic and Control Rat Liver Tissues (Values were expressed as mean ± S.E.M).

3.6 Protein Carbonylation

The oxidative modification of proteins has been shown to play an important role in a number of human diseases (Halliwell and Gutteridge, 1999). Protein

carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation (Berlett and Stadman, 1997, and Chevion, 2000) and accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease (AD), diabetes, inflammatory bowel disease (IBD), and arthritis (Chevion, 2000). In our study, we have found that, the carbonyl content of proteins in diabetic group was higher than the control group as seen in Figure 3.24.

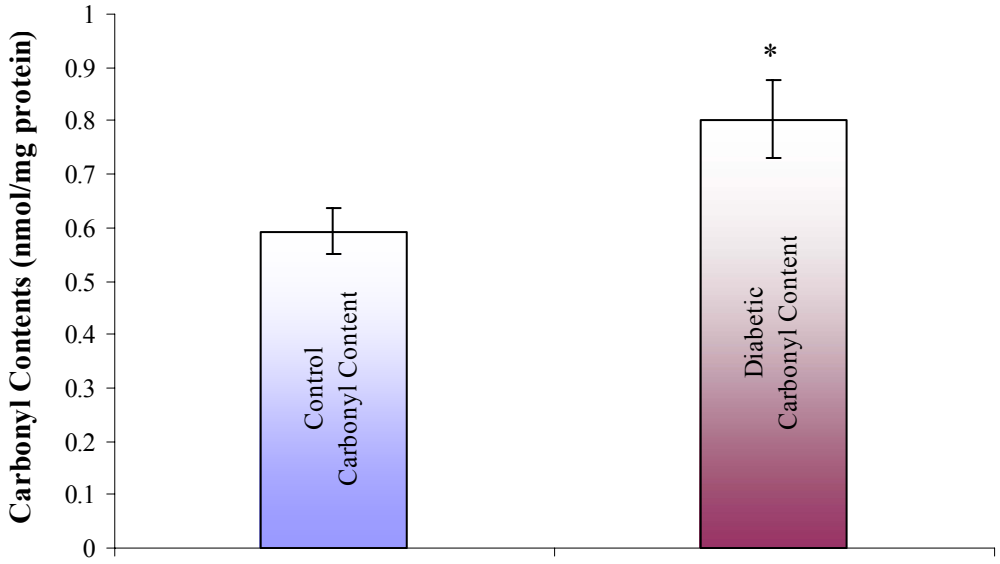


Figure 3.24: Protein Carbonylation in Diabetic and Control Rat Liver Tissues (Values were expressed as mean ± S.E.M).

This increase in carbonylation, most probably, is the result of exposure of proteins to reactive oxygen species which can lead to modifications in amino acid side chains resulting in altered structure and/or functions. The increase in the number of carbonyl groups within a protein correlates well with increased protein damage caused by oxidative stress (Shacter *et al.,* 1994, Telci *et al.,* 2000 and Donne *et al.,*

2003). Owing to the relatively early formation and the relative stability of carbonylated protein, usage of protein carbonyl groups as biomarkers of oxidative stress has many advantages in comparison with the measurement of other oxidation products.

In diabetes, glucose itself can increase reactive oxygen species through autoxidation and nonenzymatic protein glycation. STZ also has been shown to produce oxygen free radicals by stimulating H_2O_2 generation in vitro as well as in vivo in pancreatic β-cells (Takasu *et al.,* 1991).

Antioxidant enzymes can respond to conditions of increased oxidative stress with a compensatory mechanism that increases the enzyme activity in diabetic rats. In our study, the hepatic SOD activities were significantly increased in STZ induced diabetic rats probably to increase dismutation of superoxide anions. Similar changes were also observed for GPx activity in diabetic groups. Whereas CAT activity was markedly decreased in diabetic rats compared to the control group. The increase in the SOD activity should normally protect CAT against enzyme inactivation by superoxide anions as these anions have been shown to inactivate CAT (Kono and Fridovich, 1982). However, CAT activity was found to be low compared to controls. It can be suggested that at the initial stage of diabetes, the oxidative stress in the liver may be counteracted by increased SOD and GPX activities, as CAT was partially inhibited by probably superoxide radicals.

The increase in the TBARS concentration, lipid/protein ratio and protein carbonylation in spite of the increased antioxidant enzyme activities (except for CAT)

could have been due to the overproduction of radicals that exceeded the capacity of antioxidant enzymes during eight week diabetes.

As a summary, Table 3.2 shows, all alterations in total antioxidant capacity and oxidative tissue damage during the eight week STZ induced diabetes. These results thus reveal the occurrence of oxidative stress in the liver during diabetes and suggest its possible involvement in some of the accompanying functional alterations.

Table 3.2: Summary of Biomarkers of Oxidative Stress between Diabetic and Control Rat Liver Tissues

	CONTROL	**DIABETIC**	**% Change in Diabetes**
CAT (mmol/mg.min)	**114.97±19.98**	**83.06±14.21**	**↓ 28 % (*)**
Total SOD (U/mg)	**0.9757±0.039**	**1.3860±0.110**	**↑ 29 % (*)**
MnSOD (U/mg)	**0.1025±0.019**	**0.1121±0.012**	**↑ 8.5 %**
CuZnSOD (U/mg)	**0.8732±0.045**	**1.2739±0.036**	**↑ 31 % (*)**
GPx (µmol/mg.min)	**78.86±3.71**	**114.83±5.99**	**↑ 31 % (**)**
Total GST (nmol/mg.min)	**374.8±22**	**390.0±34**	**↑ 3.8 %**
GSH (mmol/mg protein)	**113.57±5.22**	**88.19±10.39**	**↓ 22 % (*)**
Lipid/Prot Ratio ((mg/mL)/(mg/mL))	**0.2956± 0.0019**	**0.3274± 0.012**	**↑ 9.7 % (*)**
Protein Carbonyls (nmol CO/mg Prot)	**0.593± 0.042**	**0.803± 0.074**	**↑ 26 % (*)**
TBARS (MDA) (nmol/mg protein)	**0.121±0.026**	**0.345±0.063**	**↑ 185 % (**)**

Values were expressed as mean ± S.E.M

(*) indicates significance at 95% confidence interval (i.e. p<0.05)

(**) indicates significance at 99.5% confidence interval (i.e. p<0.005)

CHAPTER IV

CONCLUSION

In conclusion, diabetes mellitus is a disease of increased oxidative stress resulting from enhanced free radical productions leading to changes in activities of antioxidant enzymes and oxidative tissue damage. In general, antioxidant enzymes respond to conditions of increased oxidative stress with a compensatory mechanism that augments the enzyme activities in diabetic rats. In our study, the hepatic SOD activities were significantly increased in STZ induced diabetic rats probably to increase dismutation of superoxide anions. Similar changes were also observed for GPx activity in diabetic groups. Whereas CAT activity was markedly decreased in diabetic rats compared to the control group. Decreased CAT activities may be due to enzyme inhibition with superoxide radicals, because, it has been shown that this radical may inactivate CAT (Kono and Fridovich, 1982). Therefore, it can be suggested that during diabetes, the oxidative stress in the liver may be counteracted by increased SOD and GPx activities, as CAT was partially inhibited by probably

superoxide radicals. GST which catalyses the reduction of oxidants by GSH showed no significant change in terms of activity due to disease.

Increase in protein carbonylation and TBARS concentration, most probably, was the result of exposure of proteins and lipids to reactive oxygen species which can lead to modifications in amino acid side chains and lipid molecules resulting in altered structures and functions. Also, increasing trend in the lipid to protein ratio in diabetes can be due to the stimulation in lipid synthesis in diabetes or more probably due to the protein oxidation which makes membrane proteins more susceptible to proteolytic attacks. And finally, the decline in GSH in liver during diabetes represents its increased utilization due to oxidative stress.

All these results confirm that, during the eight week diabetes in rat liver tissues, enzymatic and nonenzymatic antioxidative mechanisms are in a dynamic interaction trying to minimize consequences of oxidative stress. On the other hand, these interactions were not sufficient to elimininate all produced free radicals leading to oxidative lipid and protein damage.

REFERENCES

Aebi H, Catalase; "Method of enzymatic analysis" New York: Academic Press. 673– 684, 1974

Asayama K, Yokota S, Kato K, Peroxisomal Oxidases in Various tissues of Diabetic Rats, Diabetes Res. Clin. Pract. 11; 89-94, 1991

Ballinger CA, Mendis-Handagama C, Kalmar JR, Arnold RR, and Kinkade JM., Changes in the localization of catalase during differentiation of neutrophilic granulocytes. Blood 83; 2654-2668, 1994

Baynes JW, The role of oxidative stress in the development of complications in diabetes. Diabetes 40; 405-412, 1991

Baynes JW, From life to death - the struggle between chemistry and biology during aging; the Maillard reaction as an amplifier of genomic damage. Biogerontology 1; 235-246, 2000

Beal MF., Oxidatively modified proteins in aging and disease. Free Radic Biol Med. 32(9); 797– 803, 2002

Beisswenger PJ, Howell SK, Touchette AD, Lal S, and Szwergold BS, Metformin reduces systemic methylglyoxal levels in type 2 diabetes. Diabetes 48; 198-202, 1999

Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of ''antioxidant power'': the FRAP assay. Anal Biochem. 239; 70– 76, 1996

Berlett BS, Stadtman ER, Protein oxidation in aging, disease, and oxidative stress. J. Biol. Chem. 272; 20313-20316, 1997

Berry EM, Kohen R, Is the biological antioxidant system integrated and regulated? Med. Hypotheses 53; 397–401, 1995

Best D, O'Neal DN, Diabetic Dislipidaemia. Current treatment recommendations. Drugs 59; 1101-1111, 2000

Betteridge DJ, Diabetic dyslipidaemias. Acta Diabetol. 36; S25-S29, 1999

Blum J, Fridovich I, Inactivation of GPx by superoxide radicals. Arch. Biochem. Biophys. 240; 500-508, 1985

Booth A, Ashley E, Khalifah Raja G., Parvin Todd and Billy G. Hudson, In Vitro Kinetic Studies of Formation of Antigenic Advanced Glycation End Products (AGEs). J. Biol. Chem, Volume 272; Number 9; Issue of February 28; pp. 5430-5437; 1997

Braimer L, Repair of DNA damage induced by reactive oxygen species. Free Radical Res. Comm., 14; 159-171, 1991

Buetler TM and Eaton DL, Complementary DNA cloning, messenger RNA expression, and induction of alpha-class glutathione S-transferases in mouse tissues. Cancer Research. Vol 52; Issue 2; 314-318, 1992

Ceriello A, Is oxidative stress the missing link between insulin resistance and atherosclerosis? Diabetologia, 38; 1484-1485, 1995

Ceriello A, Acute hyperglycaemia and oxidative stress generation (Review). Diabet Med. 14; (Suppl. 3):S45-S49, 1997

Ceriello A, Pirisi M, Is oxidative stress the the missing link between insulin resistance and atherosclerosis? Diabetologia. 38; 1484-1485, 1995

Chance B, Sies H, Boveris A, Hydroperoxide metabolism in mamalian organs. Physiol. Rev., 59; 527-605, 1979

Chaudie`re J, Some chemical and biochemical constraints of oxidative stress in living cells. In: Rice-Evans CA, Burdon RH, eds. Free radical damage and its control. New Comprehensive Biochemistry, Vol. 28. Amsterdam: Elsevier, 25–66. 1994

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA, 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G → T and A → C substitutions. J. Biol. Chem. 267 (1); 166–172, 1992

Chevion M, Berenshtein E, Stadtman ER, Human studies related to protein oxidation: protein carbonyl content as a marker of damage. Free Radic Res; 33; 99–108, 2000

Cominacini L, Garbin U, Pasini AF, Davoli A, Campagnola M, Rigoni A, Tosetti L, Lo-Cascio V. The expression of adhesion molecules on endothelial cells is inhibited by troglitazone through its antioxidant activity. *Cell Adhes Commun*. 7; 223–231. 1999

Cummins I., Cole DJ., Edwards R., A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in black-grass. Plant J. 18; 285-292, 1999

D'Aquino M, Effects of fish oil and coconut on antioxidant defense system and lipid peroxidation in rat liver. Free Rad. Res. Comm. 13, 147-152, 1991

Dandona P, Tnusu K, Cook S, Synder B, Makowski J, Armstrong D, Oxidative damage to DNA in diabetes mellitus. Lancet, 347(17); 444–445., 1996

Davies P, Drath DB, Engel EE, and Huber GL, The localization of catalase in the pulmonary alveolar macrophage. Lab. Invest. 40; 221-226, 1979

DeFronzo RA, Bonadonna RC, and Ferrannini E, Pathogenesis of NIDDM. A balanced overview. Diabetes Care, 15; 318-368, 1992

Dixon DP, Cole DJ, Edwards R, Characterisation of a zeta class glutathione transferase from Arabidopsis thaliana with a putative role in tyrosine catabolism. Arch. Biochem. Biophys. 384; 407-412, 2000

Dixon DP, Lapthorn A, Edwards R, Plant glutathione transferases, Genome Biology, 3(3); reviews3004.1-3004.10, 2002

Drogg FNJ, Hooykaas PJJ, Van der Zaal BJ, 2,4 dichlorophenoxyacetic and related chlorinated compounds inhibit two auxin- regulated type-III tobacco glutathione S-transferase. Plant Physiology. 107; 1139-1146, 1995

Edwards R, Dixon DP, Walbot V, Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci., 5; 193-198, 2000

Elgawish A, Glomb M, Friendlander M, Monnier VM. Involvement of hydrogenperoxide in collagen cross-linking by high glucose in vitro and in vivo. J Bio Chem. 271; 12964-12971, 1996

Epp O, Ladenstein R, and Wendel A, The Refined Structure of the Selenoenzyme Glutathione Peroxidase at 0.2-nm Resolution. Eur. J.Biochem. 133; 51-69, 1983

Ewis SA, Abdef-Rahman MS, Effects of metformin on glutathione and magnesium in normal and STZ induced diabetic rats. J. Appl. Toxico. 15; 387-390, 1995

Flohe L, Günzler WA, Schock HH, Glutathione peroxidase: a selenoenzyme. FEBS Lett. 32; 132-134, 1973

Flohe L, In Glutathione: Chemical, Biochemical and Medical Aspects (Dolphin, D., Poulson, R., and Avramovic, O., eds) Part A, pp. 643–731, John Wiley & Sons Inc., New York, 1989

Flohe L, Aumann KD, Blocker H, Gross G, Kiess M, Kloppel KD, Maiorino M, Roveri A, Schuckelt R and Usani F, Phospholipid-hydroperoxide glutathione peroxidase. Genomic DNA, cDNA, and deduced amino acid sequence. J. Biol. Chem., 269(10); 7342-7348, 1994

Freeman BA, and Crapo JD, Biology of disease: free radicals and tissue injury. Lab. Invest. 47; 412-426, 1982

Fridovich I, Freeman B, Antioxidant defenses in the lung. Annu Rev Physiol. 48; 693-702, 1986

Frings CS, Dunn RT, Colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. Am. J.Clin. Path. 53; 89-97, 1970

Ghe AM, Stefanelli C, Tsintiki P, Veschi G, Influence of metal ions on oxidation of NADH and on the formation of the superoxide anion during enzmatic catalysis by xanthine oxidaise. Talanta 32; 359-362, 1985

Girón MD, Salto R, González Y, Girón JA, Natalia N, José L, Periago M, Suárez D, Hortelano P, Modulation of hepatic and intestinal GSTs' and other antioxidant enzymes by dietary lipids n STZ induced diabetic rats. Chemosphere. 38; 3003-3013, 1999

Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications.Diabetes Care 19; 257-267, 1996

Grill V, Bjorklund A, Dysfunctional insulin secretion in type 2 diabetes: role of metabolic abnormalities. Cell Mol Life Sci. 57; 429–440, 2000

Gutteridge J, Halliwel B, The measurement and mechanism of lipid peroxidation in biological sysems. TIBS 15; 129-135, 1990

Gutteridge JM, Halliwell B, Free Radicals and Antioxidants in the Year 2000: A Historical Look to the Future. Ann. N.Y. Acad. Sci. 899; 136-141, 2000

Habig WH, Pabst MJ, and Jakoby WB, Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249; 7130-7139, 1974

Halliwell B, Chirico S, Lipid peroxidation: its mechanism, measurement, and significance. American Journal of Clinical Nutrition. 57; 715-724, 1993

Halliwell B, Gutteridge J, Free radicals in biology and medicine. NY: Oxford Univ. Press; 1999

Halliwell B, Gutteridge J, Free Radicals in Biology and Medicine. Claredon Press, Oxford 1989

Halliwell B, Gutteridge J, Oxygen toxicity, oxygen radicals, transition metals and disease, Biochem J. 219(1); 1-14, 1984

Halliwell B, Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free Radic. Res.Comm. 31; 261-272, 1999

Hartnett E, Stratton R, Browne R, Rosner B, Lanham R, Armstrong D, Serum Markers of Oxidative Stress and Severity of Diabetic Retinopathy. Diabetes Care. 23(2); 234-240, 2000

Heffner JE, Repine JE, Pulmonary strategies of antioxidant defenses. Am Rev Respir Dis. 140; 531-554, 1989

Hyung-Soon Yim , Sa-Ouk Kang , Yung-Chil Hah , P. Boon Chock , Moon B. Yim, Free Radicals Generated During The Glycation Reaction Of Amino Acids By Methylglyoxal: A Model Study Of Protein-Cross-Linked Free Radicals, J. Biol. Chem. 270(47); 28228-28233, 1995

İşcan M, Modaresi M, Güray T, Production of superoxide radicals by sheep liver nitrofyrantoin reductase: in vitro effects of nitrofurantoin metabolites on DNA. Toxic. In Vitro. 9; 743-750, 1995

Jain SK, and Levine SN, Elevated lipid peroxidation and Vitamin E quinine levels in heart ventricles of streptozoticin-treated diabetic rats. Free Radic. Biol. Med. 18; 337-341, 1995

Jiang ZY, Wooliard ACS, Wolff SP, Hydrogen peroxide production during experimental glycation. FEBS Lett. 268; 69-71, 1990

Junod A, Lanbet AE, Orci L, Pictet R, Gonet AE, and Renold AE, Studies o6f diabetic action of streptozotocin. Proc. Soc. Exp. Biol. Med. 126; 2129-2139, 1967.

Kakkar R, Kalra J, Mantha SV, Prasad K, Lipid Peroxidation and Activity of Antioxidant Enzymes in Diabetic Rats. Mol. Cell. Biochem. 151; 113-119, 1995

Kaur H, Halliwel B, Action of biologically relevant oxidizing species upon uric acid: identification of uric acid oxidation products. Chem. Biol. Interact., 73; 235-247, 1990

Ketterer B, Taylor J, Meyer D, Pemble P, Coles B, ChuLin X, Spencer S, Some functions of glutathione transferases. In: Structure and function of glutathione transferases, ed. Kenneth D. Tew, Cecil B. Pickett, Timothy J. Mantle, Bengt Mannervik, and John D. Hayes. CRC Press, Boca Raton, Florida, 1993

Kirkman H, Gaetani G, Catalase: A Tetrameric Enzyme with Four Tightly Bound Molecules of NADPH, Proc. Natl. Acad. Sci. 81; 4243, 1984

Kneepkens CM, Lepage G, Roy CC, The potential of the hydrocarbon breath test as a measure of lipid peroxidation. Free Radic. Biol. Med. 17; 127–160; 1994.

Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V, Method for the measurement of antioxidant activity in human fluids. J. Clin. Pathol. 54(5); 356 361, 2001

Kono Y, and Fridovich I, Superoxide Radical Inhibits Catalase. The Journal of Biological Chemistry 257(10); 5751-5754, 1982

Lawrence RA, Parkhill LK, Burk RF, Hepatic cytosolic non selenium-dependent glutathione peroxidase activity: its nature and the effect of selenium deficiency. J. Nutr. 108(6); 981-987, 1978

Lebowitz HE, Type 2 diabetes. Clin. Chem. 45; 1339-1345, 1999

Lee AYW, Chung SSM, Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J. 13; 23-30, 1999

Lee Cheolju, Moon B. Yim, P. Boon Chock, Hyung-Soon Yim, and SaOuk Kang, Oxidation-Reduction properties of methylglyoxal-modified protein in relation to free radical generation, J Biol Chem, Vol. 273, Issue 39, 25272-25278, September 25, 1998

Lernmark A, Type I Diabetes. Clin. Chem. 45(8Pt2); 1331-1338, 1999

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz A, Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186; 464–78, 1990

Loft S., Poulsen H.E., Cancer risk and oxidative DNA damage in man. J. Mol. Med. 74:297-312; 1997

Lorenzi M, Montisano DF, Toledo S, Wong HC, Increased single strand breaks in DNA of lymphocytes from diabetic subjects. J. Clin. Invest. 79 (2); 653–656, 1987

Loven D, Schedf H, Wilson H, Daabees TT, Stegink LD, Diekus M, Effects of insulin and oral glutathione on glutathione levels and SOD activities in organs of rats with STZ inductiion. Diabetes. 35; 503-507, 1986

Loyall L., Uchida K., Braun S., Furuya M., Frohnmeyer H., Glutathione and a UV light-induced glutathione S-transferase are involved in signaling to chalcone synthase in cell cultures. Plant Cell. 12; 1939-1950, 2000

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ, Protein measurement with the Folin Phenol Reagent, J.Biol. Chem. 248; 265-275, 1951

Mak DH, Ip SP, Li PC, Poon MK, Alterations in tissue Glutathione Antioxidant System in STZ induced diabetic rats. Mol. Cell. Biochem. 162; 153-158, 1996

Malaisse WJ, Alloxan toxicity to the pancreatic B-cell. Biochem. Pharmacol. 31; 3527-3534, 1982

Marklund SL, Extracellular superoxide dismutase and other superoxide dismutase isozymes in tissues from nine mammalian species. Biochem J, 222; 649-655, 1984

Marklund SL, Human copper-containing superoxide dismutase of high molecular weight. Proc. Natl. Acad. Sci. 79; 7634-7638, 1982

Marklund SL, Marklund G, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47; 469–74, 1974

Marrs KA., The functions and regulation of glutathione Stransferases in plants. Annu. Rev. Plant Physiol. Plant. Mo.l Biol., 47; 127-158, 1996

Matteuci E, Giampietro O, Oxidative stress in families of type 1 diabetic patients. Diabetes Care. 23; 1182-1186, 2000

McCord JM, Oxygen derived free radicals in post ischemic tissue injury. N. Eng. J. Med. 312; 159-163, 1985

McCord JM, Fridovich I, Superoxide Dismutase. An Enzymatic Function for Erythrocuprein. J. Biol. Chem. 244; 6049-6055, 1969

McKersie, B.D. Oxidative Stress.
http://www.agronomy.psu.edu/Courses/AGRO518/Oxygen.htm#contents, 1996

Meister A, Glutathione, ascorbate, and cellular protection. Cancer Res. 54; 1969-1975, 1994

Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A, A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci. 84; 407– 12, 1993

Mitchelson AM, McCord JM, Fridovich I, Superoxide and superoxide dismutases, Eds New York, Academic, 501-515, 1977

Mooradian AD, Thurman JE, Glucotoxicity: potential mechanisms. Clin. Geriatr. Med. 15; 255, 1999

Mossine VV, Linetsky M, Glinsky GV, and Ortwerth BJ, Feather MS, Superoxide free radical generation by Amadori compounds: the role of acyclic forms andmetal ions. Chem.Res.Toxicol. Mar; 12(3); 230-236, 1999

Mourente, Gabriel, Díaz-Salvago, Esther, Characterization of antioxidant systems, oxidation status and lipids in brain of wild-caught size-class distributed *Aristeus antennatus* (Risso, 1816) Crustacea, Decapoda. Comp.Biochem.Phys. 124; 405-416, 1999

Neuefeind T, Reinemer P, Bieseler B, Plant glutathione S-transferases and herbicide detoxification. Biological Chemistry. 378; 199-205, 1997

Nicholls P, Contributions of catalase and glutathione peroxidase to red cell peroxide removal. Biochim. Biophys. Acta. 279; 306-309, 1972.

Nonogaki K, New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia. 43; 533-549, 2000

Oberley LW, Free Radicals and Diabetes, Free Rad. Biol. Med. 5; 113-124, 1988

Onorato JM, Thorpe SR, Baynes JW, Immunohistochemical and ELISA Assays for Biomarkers of Oxidative Stress in Aging and Disease. Ann. N.Y. Acad. Sci. 854; 277-290, 1998

Onorato JM, Jenkins AJ, Thorpe SR, Baynes JW, Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions: Mechanism of action of pyridoxamine. J. Biol. Chem. 275; 21177-21184, 2000

Ortwerth BJ, James H, Simpson G, Linetsky M, The generation of superoxide anions in glycation reactions with sugars, osones, and 3-deoxyosones. Biochem. Biophys. Res. Commun. 245; 161–165, 1998

Oury TD, Chang LY, Marklund SL, Day BJ, Crapo JD, Immunocytochemical localization of extracellular superoxide dismutase in human lung. Lab. Invest. 70(6); 889-898, 1994

Erel Ö, A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clinical Biochemistry. 37; 277– 285, 2004

Erel Ö, A novel automated method to measure total antioxidant response against potent free radical reactions. Clinical Biochemistry. 37; 112– 119, 2004

Öztok U, Yılmaz M, Karakoç A, Çakir N, Karakaya AE, Sarda S, Detection of oxidative damage to DNA in diabetes mellitus by comet assay. Neoplasma. 46; 111–113, 1999

Öztürk Y, Altan VM, and Yildizoglu-Ari N, Effect of experimental diabetes and insulin on smooth muscle cell functions. Pharmacological Reviews. 48(1); 1-44, 1997

Paglia ED, Valentine WN, Studies on the quantitative and qualitative characterization of erythrocytes glutathione peroxides. J. Lab. Clin. Med. 70; 158–69, 1967

Radi R, Turrens JF, Chang L Y, Bush KM, Crapo JD, Freeman BA, Detection of catalase in rat heart mitochondria. J. Biol. Chem. 266; 22028-22034, 1991

Renier G, Desfaits AC, Serri O, Gliclazide decreases low-density lipoprotein oxidation and monocyte adhesion to the endothelium. Metabolism. 49; 17-22, 2000

Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. Methods Enzymol. 234; 279– 293, 1994

Rocher JL, Lalanne A, Chaudiere J, Purification and properties of a recombinant sulfur analog of murine selenium-glutathione peroxides. Eur. J. Biochem. 205; 955-960, 1992

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG, Selenium: biochemical role as a component of glutathione peroxidase. Science. 179; 588-590, 1973.

Roveri A, Maiorino M, Ursini F, Enzymatic and immunological measurements of soluble and membrane bound phgpx. Methods In Enzymol., 233; 202-212, 1994

Rowsey IR., Thomson AM., Flanagan JU., Murdock PR., Moore GBT., Meyer DJ., Murphy GJ., Smith SA., Hayes JD., Mammalian class sigma glutathione S-transferases: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D2 synthases. Biochem J. 359; 507-516, 2001

Roxas VP., Smith RK., Allen ER., Allen RD., Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Nat. Biotechnol. 15; 988-991, 1997

Sakurai T, Tsuchiya S, Superoxide production from nonenzymatically glycated protein. FEBS Letter. 236; 406-410, 1988

Saleh A, David W, Godin V, Alterations in free radical tissue defense mechanisms in STZ induced diabetes in rats. Diabetes. 36; 1014-1018, 1987.

Sandstrom J, Karlsson K, Edlund T, Marklund SL, Heparin-affinity patterns and composition of extracellular superoxide dismutase in human plasma and tissues. Biochem. J. 294; 853-857, 1993.

Sarafian TA, Bredesen DE, Is apoptosis mediated by reactive species? Free Radical. Res. 20; 1-6, 1994

Sardas S, Yilmaz M, Öztok U, Çakir N, Karakaya AE, Assessment of DNA strand breakage by comet assay in diabetic patients and the role of antioxidant supplementation. Mutat. Res. 490; 123–129, 2001

Sato Y, Hotta N, Sakamoto N, Lipid peroxide level in plasma of diabetic patients. Biochem. Med. 25; 373-378, 1981

Schmidt K, Werner ER, Mayer B, Wachter H, Kukoveta K, Tetrahydrobiopterin-dependent formation of endothelial-derived relaxing factor (nitric oxide) in aortic endothelial cells. Biochem J. 281; 297-300, 1992

Scoppola A, Testa G, Frontoni S, Maddaloni E, Gambardella S, Menzinger G, and Lala A, Effects of insulin on cholesterol synthesis in type II diabetes patients. Diabetes Care. 18(10); 1362-1369, 2001

Sedlak J, Lindsay RH, Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissues with Ellman's reagent. Anal.Biochem. 25; 192-205, 1968

Shacter E, Quantification and significance of protein oxidation in biological samples. Drug Metab. Rev. 32; 307– 326, 2000

Shacter E, Williams JA, Lim M, and Levine RL, Differential susceptibility of plasma proteins to oxidative modification: Examination by Western blot immunoassay. Free Radic. Biol. Med. 17; 429–437, 1994

Sheehan D., Meade G., Foley VM., Dowd CA., Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J., 360; 1-16, 2001

Shigenaga MK, Ames BN, Assays for 8-hydroxy deoxyguanosine: a biomarker of in vivo oxidative DNA damage. Free Radic. Biol. Med. 10; 211–216, 1991

Sies H, Carotenoids and tocopherols as antioxidants and singlet oxygen quenchers. J. Nutr. Sci. Vitaminol. 1; 27-33, 1992

Sies H, Oxidative stress: From basic research to clinical application. Am. J. Med. 3; 31-38, 1991

Stadtman TC, Biosynthesis and function of selenocysteine-containing enzymes. J. Biol. Chem. 266(25); 16257-16260, 1991

Takasu N, Komiya I, Asawa T, Nagasawa Y, and Yamada T., Streptozotocin and alloxan induced H2O2 generation and DNA fragmentation in pancreatic islets, Diabetes, 40; 1141-1145, 1991

Telci A, Cakatay U, Kayali R, Erdogan C, Orhan Y, Sivas A, Oxidative protein damage in plasma of type 2 diabetic patients. Horm. Metab. Res. 32(1); 40–43, 2000

Telci A, Cakatay U, Salman S, Satman I, Sivas A, Oxidative protein damage in early stage Type 1 diabetic patients. Diabetes Res. Clin. Pract. 50(3); 213– 223, 2000

Tesfamariam B, Free radicals in diabetic endothelial cell dysfunction. Free Radic. Bio. Med. 16; 383-391, 1993

Tsan MF, Superoxide dismutase and pulmonary oxygen toxicity. Proc. Soc. Exp. Biol. Med. 214; 107-113, 1997

Turrens JF, Freeman BA, Crapo JD, Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. Arch. Biochem. Biophys. 217(2); 411-421, 1982

Turrens JF, Freeman BA, JG, Levitt and Crapo JD, The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch. Biochem. Biophys. 217(2); 401-410, 1982

Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D, Flohe L, Diversity of glutathione peroxidases, Methods Enzymol., 252; 38-53, 1995

Wilce MCJ., Parker MW., Structure and function of glutathione S-transferases. Biochim Biophys. Acta. 1205; 1-18, 1994

Winston GW, Cederbaum AI, Oxyradical production by purified components of the liver microsomal mixed-function oxidase system I: Oxidation of hydroxyl radical scavenging agents. J. Biol Chem 258; 1508-1513, 1983

Wolff SP, Jiang ZY, Hunt JV, Protein glycation and oxidative stress in diabetes mellitus and ageing. Free. Radic. Bio. Med. 10; 339-352, 1991

Wolff SP, Crabbe MJ, Low apparent aldose reductase activity produced by monosaccharide autoxidation. Biochem. J. 226(3); 625-630, 1985

Wolff SP, Dean RT, Aldehydes and dicarbonyls in non-enzymic glycosylation of proteins. Free Radic. Res. Commun. 1; 115-123, 1991

Yu BP, Cellular defence against damage from reactive oxygen species, Physiol.Rev. 74; 139-162, 1994

Zierath JR, Krook A, Wallberg-Henriksson H, Insulin Action and Insulin Resistance in Human Skeletal Muscle. Diabetologia. 43; 821-835, 2000

APPENDIX A: Average body weights of rats

Groups	Average body weights (gr)	
	Before Treatment	**Before Decapitation**
Control (n=6)	225.34±23.04	289.60±41.15
Diabetic (n=7)	252.37±18.75	232.87±18.56

APPENDIX B: Average blood glucose concentrations of rats

Groups	Average blood glucose concentrations (mg/dL)	
	Before Treatment	**Before Decapitation**
Control (n=6)	102.5±16.7	97.9±12.6
Diabetic (n=7)	96±17.25	232.25±32.56