OXIDATIVE DAMAGE AND REGULATION OF ANTIOXIDANT ENZYMES IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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OXIDATIVE DAMAGE AND REGULATION OF ANTIOXIDANT ENZYMES IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

OXIDATIVE DAMAGE AND REGULATION OF ANTIOXIDANT ENZYMES IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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Increased oxidative stress and impaired antioxidant defense mechanisms are believed to be the important factors contributing to the pathogenesis and progression of diabetes mellitus. The products of lipid peroxidation and protein oxidation reactions were all found to be elevated significantly ($p<0.05$) in diabetic animals and supplementing the animals either individually or in combination, with two powerful antioxidants DL-α-lipoic acid (LA) and vitamin C (VC) brought this increment toward the control values.

Considering Cu-Zn SOD, CAT and GST-Mu, there was a significant decrease in all activities in diabetic group as compared with control animals. RT-PCR and Western blot analysis results demonstrated that this decrease in activity is regulated at the level of gene expression, as both mRNA and protein expressions were also suppressed for these enzymes. However, in diabetic animals both the mRNA expressions and the activities of two other antioxidant

enzymes, namely Mn SOD and GPx, did not change, indicating that the control of activities of these two enzymes were not at the level of genes.

Supplementing the diabetic animals with VC increased all CAT, Cu-Zn SOD, GPx, and GST-Mu activities without changing both mRNA and protein expressions suggesting the possible role of post-translational modifications. On the other hand, the effect of VC on Mn SOD was observed at mRNA levels reflecting a transcriptional regulation. Furthermore, supplementing the animals with LA increased the CAT, Cu-Zn SOD, Mn SOD and GPx activities in diabetic rats but different from VC, LA also increased mRNA of CAT and protein levels of CAT, Cu-Zn SOD and Mn SOD suggesting both transcriptional and translational regulation showed by LA. Combined application of antioxidants also increased the CAT, Cu-Zn SOD, Mn SOD and GPx activities toward the control values, but this time there were no statistically significant change in their mRNA expressions even though protein amounts of both CAT and GPx were augmented. That is, when given together, these antioxidants exert their effects mainly at the level of protein synthesis.

As a conclusion, diabetes and the resulting oxidative stress coordinately regulate the activities of the antioxidant enzymes at different regulatory points. LA and VC, two powerful antioxidants affect all antioxidant enzyme activities at different levels of transcription and translation. The results indicated the presence of very intricate control mechanisms regulating the activities of antioxidant enzymes in order to prevent the damaging effects of oxidative stress.

Key words: Diabetes mellitus, Antioxidant Enzymes, Gene Expressions, α-lipoic acid, Vitamin C

ÖZ

STREPTOZOTOSİN İLE OLUŞTURULMUŞ DİYABETİK SIÇANLARDA OKSİDATİF HASAR VE ANTİOKSİDAN ENZİMLERİN REGÜLASYONU

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Artan oksidatif stres ve bozulan antioksidan savunma sistemlerinin diyabetin patojenezinde ve ilerlemesinde önemli rolü olduğu düşünülmektedir. Lipit peroksidasyon ve protein oksidasyon son ürünlerinin diyabette anlamlı ölçüde ($p<0.05$) arttığı belirlenmiş, tekil ya da beraber DL-α-lipoik asit (LA) ve C vitamini (VC) uygulamasının diyabette gözlenen bu artışı kontrol seviyesine düşürdüğü bulunmuştur.

Cu-Zn SOD, CAT ve GST-Mu enzimleri ele alındığında, aktivitelerin diyabette kontrol grubuna oranla anlamı oranda düştüğü bulunmuş, RT-PCR ve Western-blot analiz sonuçları bu enzimlerin ifadelenmelerinin gen düzeyinde kontrol edildiklerini göstermiştir. Çünkü, mRNA ve protein seviyelerinin de diyabette baskılandığı ortaya çıkarılmıştır. Ancak, diyabetik hayvanlarda, diğer iki antioksidan enzimin yani Mn SOD ve GPx'in mRNA seviyeleri ve aktiviteleri değişmemiş, ve bu da Mn SOD ve GPx enzimlerinin aktivite kontrolünün gen ifadelenmesi düzeyinde olmadığını ortaya çıkartmıştır.

Diyabetik hayvanlara VC verildiğinde CAT, Cu-Zn SOD, GPx ve GST-Mu enzim aktivitelerinde anlamlı artış görüldüğü halde, bu enzimlerin mRNA ve protein ifadelenmelerinde önemli bir değişikliğin olmaması VC'nin bu enzimler üzerine olası translasyon sonrası (post-translational) etkisini göstemektedir. Fakat VC uygulaması Mn SOD enzimi üzerine etkisini mRNA düzeyinde göstermiş ve olası transkripsiyonel kontrolü işaret etmiştir. Ayrıca LA uygulaması diyabetik CAT, Cu-Zn SOD, Mn SOD ve GPx aktivitelerini arttırmış olmakla beraber etkisini VC'den farklı olarak mRNA ifadelenmesi ve protein sentezi seviyesinde de göstermiştir. LA uygulamasının CAT'ın mRNA seviyesini ve ayrıca CAT, Cu-Zn SOD ve Mn SOD 'un protein seviyelerini arttırıcı etkisi transkripsiyon ve translasyon düzeyinde de gösterilmiştir. Bu iki antioksidanın beraber uygulaması diyabetik CAT, Cu-Zn SOD, Mn SOD ve GPx aktivitelerini kontrol düzeyine yaklaştırırken bu enzimlerin mRNA ifadelenmelerinde bir değişikliğe neden olmamış, fakat CAT ve GPx protein miktarlarını arttırmış yani etkilerini translasyon düzeyinde göstermişdir.

Sonuç olarak, diyabet ve oksidatif stres antioksidan enzim aktivitelerini değişik seviyelerde koordineli bir şekilde kontrol etmekte, LA ve VC gibi iki kuvvetli antioksidan bütün antioksidan enzim aktivitelerini transkripsiyon ve translasyon seviyesinde etkilemektedir. Bu sonuçlar oksidatif stresin zararlı etkilerini önlemek için, antioksidan enzim aktivitelerini regüle eden, son derece karmaşık kontrol mekanizmalarının varlığını göstermektedir.

Anahtar kelimeler: Diyabet, Antioksidan enzimler, Gen ifadelenmesi, α-lipoik asit, C vitamini

Dedicated to my family…

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CHAPTER 1

INTRODUCTION

Free radicals and reactive oxygen species (ROS) play an important role in living systems through their beneficial and detrimental effects. 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 molecules, making it more reactive than the corresponding non-radical form. They are produced in the cells and tissues by various processes and reactions. For example, high energy ionizing radiation and thermal degradation of organic materials, 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, results in production of molecules having odd number electrons (Halliwell and Gutteridge, 2007).

One of the major free radical produced in the cells is the superoxide radical. It is produced by adding one electron to the oxygen molecule. Several oxidizing enzymes may produce superoxide radical such as diamine oxidase, tryptophan dioxygenase, xanthine oxidase, and cytochrome P450 reductase, while enzymes such as guanyl cyclase and glucose oxidase generate H_2O_2 (Fridovich, 1978; Halliwell, 1978). Furthermore, various Fe-S proteins and NADH dehydrogenase have been implicated as possible sites of superoxide and hydrogen peroxide formation (Turrens *et al.,* 1982). For example, two iron containing protein in the body, hemoglobin and myoglobin is usually in Fe^{+2}

form and when O_2 binds to them, remain in that state, however, some electron delocalization may take place to manufacture superoxide radicals.

$$\text{heam-Fe}^{+2} + O_2 \leftrightarrow O_2^{\cdot} + \text{heam-Fe}^{+3}$$

Many biologically important molecules are also autoxidized by O_2 yielding superoxide radicals. Glyceraldehydes, $FADH_2$, adrenaline, dopamine, and glucose can be autoxidized causing superoxide radical productions (Halliwell and Gutteridge, 2007). Apart from autoxidation reactions, the most important sources of the superoxide radical in aerobic animal cells is the mitochondrial electron transport chain in which electrons can leak directly to the O_2, although passing mainly to the next components of the chain. Some constituents of mitochondrial and other electron transport chains may produce superoxide radical unavoidably.

Endoplasmic reticulum is another site for the superoxide formation. Various oxidative processes, including oxidation, hydroxylation, dealkylations, deaminations, dehalogenation and desaturation, takes place on the smooth endoplasmic reticulum. Mixed function oxygenases containing a heme moiety add an oxygen atom into an organic substrate using NAD(P)H as the electron donor (McKersie, 1996). The reactive oxygen species (ROS) may arise from CYP in two ways. First, the catalytic cycle can short circuit to release superoxide radical and H_2O_2 (Figure 1.1).

Secondly, some CYP family proteins especially CYP2B and CYP2E show high rates of O_2 reduction when using substrates such as 1,1,1-trichloroethane which facilitate the leakage of electrons and leads to ROS release (Halliwell and Gutteridge, 2007).

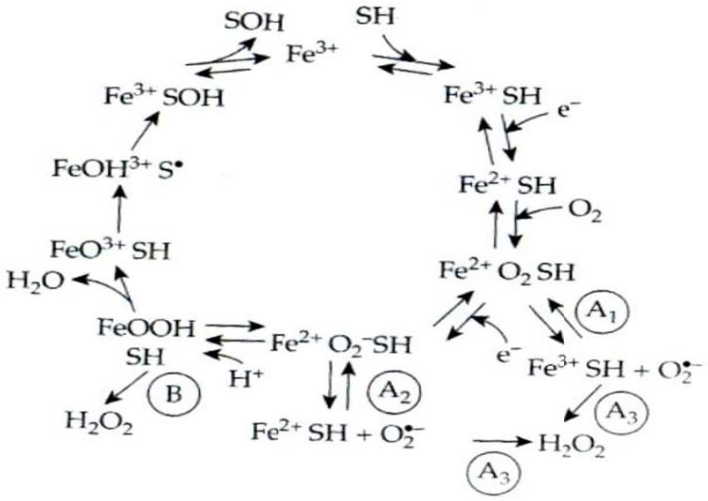


Figure 1.1: Generalized P450 cycle showing where short circuit takes place to release superoxide radical and H_2O_2 (Halliwell and Gutteridge, 2007).

Under normal cellular conditions, superoxide anions are readily converted into hydrogen peroxides enzimatically. Even though hydrogen peroxide is not a free radical, it is the major contributor of the most reactive free radical namely hydroxyl radical in the presence of a transition cations such as iron or copper by the Fenton Reaction; the main source of hydroxyl radicals produced in the cells.

$$H_2O_2 + Fe^{II} \longrightarrow Fe^{III} + HO^{\cdot} + HO^{\cdot} \quad \text{(Fenton reaction)}$$

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. In addition to intracellular metabolism, low-wavelength electromagnetic radiation (e.g., gamma rays) can split water in the body to generate this radical. 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 propagating free-radical chain

reactions. Hypochlorous acid, singlet oxygen, peroxy nitrates and nitric oxide are the other free radicals which are produced continuously in the cells (Halliwell and Gutteridge, 2007).

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. These constituents are summarized in figure 1.2.

1.1 OXIDATIVE STRESS

In healthy cells, production of free radicals is approximately balanced with antioxidant defense systems. If this balance is disturbed or repair or replacement systems to the free radical induced damage fails, the situation having too many radicals in relation to the available antioxidants is called oxidative stress. In 1991, Sies defined the oxidative stress as a “disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage to the cellular macromolecules which is also called oxidative damage.” Increased damage may not only due to increased stress but also due to failure of repair or replacement systems. As a result; consequences of oxidative stress arises such as cell injury, disruption in cellular homeostasis and accumulation of damaged molecules.

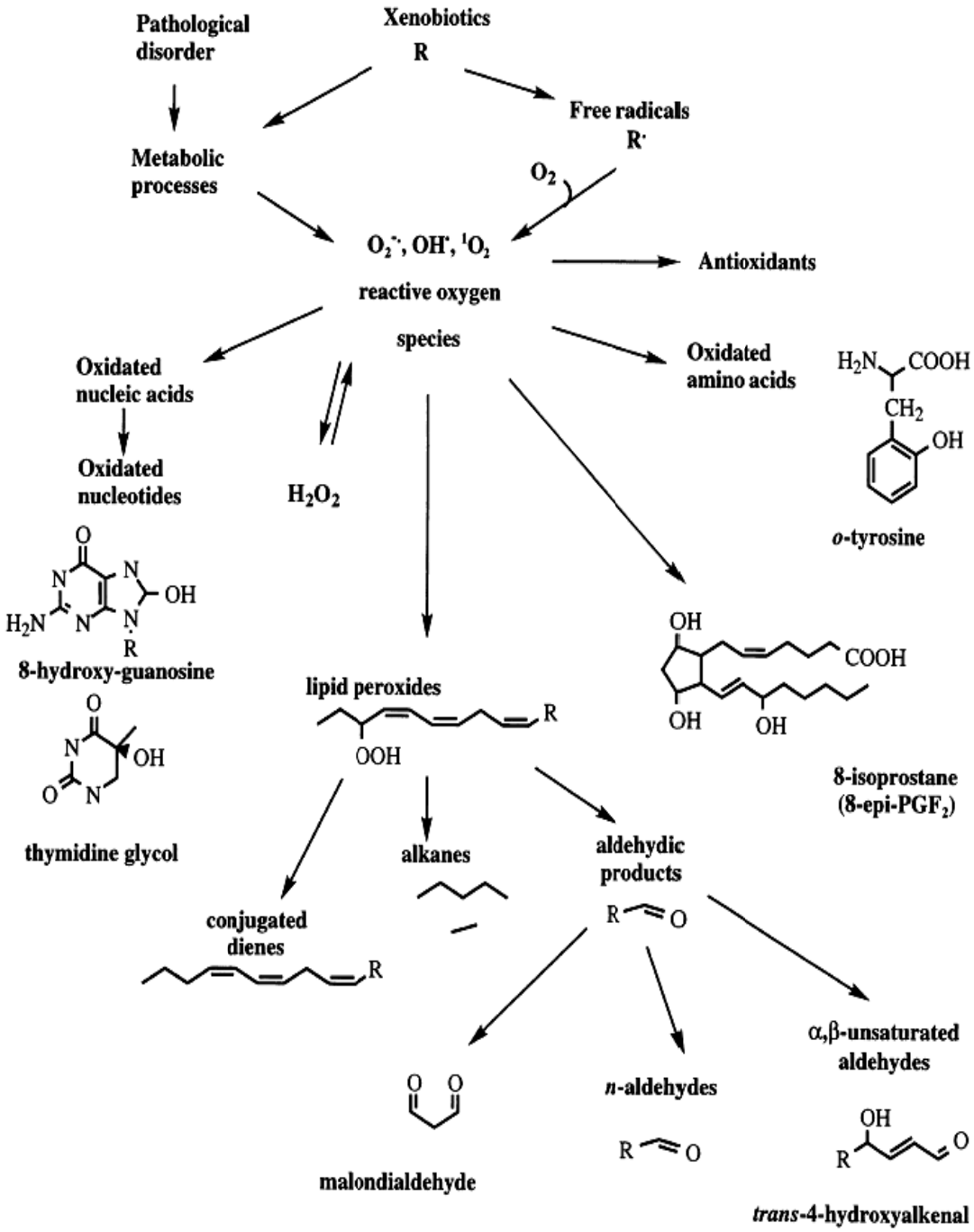


Figure 1.2: Products of free radical damage. Free radicals may react with different cellular macromolecules, such as DNA, components of cell membranes or proteins, which eventually may lead to many different products (De Zwart *et al*., 1999).

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 and glycoxidation reactions have been identified and can be measured in oxidatively modified tissues by some chemical and physical methods.

1.2.1 Total Antioxidant Capacity (TAC)

The accurate assessment of oxidative stress in biological systems is a problem for all investigators working on the role of free radical damage in disease state. Numerous assays have been described to measure various free radical damage products or antioxidant status, but no ideal method is available. The concept of a single test that might reflect total antioxidant capacity (TAC) is an attractive one and low total antioxidant capacity could be a marker of oxidative stress or increased susceptibility to oxidative damage (Young, 2001).

Total antioxidant capacity 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 cells where oxidative stress may occur and protect against reactive oxygen species (ROS). 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 lipoperoxidation

damage. TAC 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)

Oxidative degradation of lipids is referred to as lipid peroxidation. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. Lipid peroxidation is probably the most extensively investigated process which is induced by free radicals. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds that possess especially reactive hydrogens. As with any radical reaction, the reaction consists of three major steps: initiation, propagation and termination (Figure 1.3). A free radical chain reaction propagates until two free radicals destroy each other to terminate the chain. 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. Products resulting from lipid peroxidation are thus attractive parameters to monitor radical damage. 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.

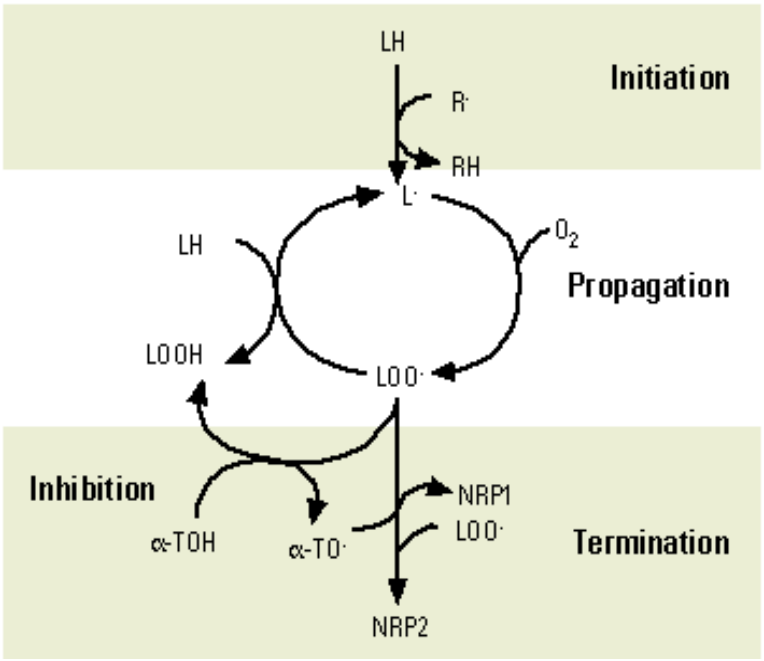


Figure 1.3: Schematic proceed of lipid peroxidation, chain reactions resulting in the formation of many lipid peroxide radicals. Abbreviations: NRP, nonradical product; LOOH, lipid hydroperoxide; α-TOH, a-tocopherol; α-TO, α-TOH radical; LH, lipid substrate; LOO, lipid peroxyl radical (Kelly *et al.,* 1998)

The overall effects of lipid peroxidation are to decrease membrane fluidity, increase the leakiness of the membrane substances that do not normally cross it other than through specific channels and damage to membrane proteins, inactivating enzymes and ion channels (Halliwell and Gutteridge, 2007). Measuring the loss of unsaturated fatty acids and measuring the 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 and antibody techniques can be useful for the detection of lipid peroxidation products. Among the methods, biochemical TBA test is the simplest and oldest one which measures the product of lipid peroxidation, namely malonedialdehyde (MDA).

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 1.4). These moieties are chemically stable, and so their detection and storage can be possible.

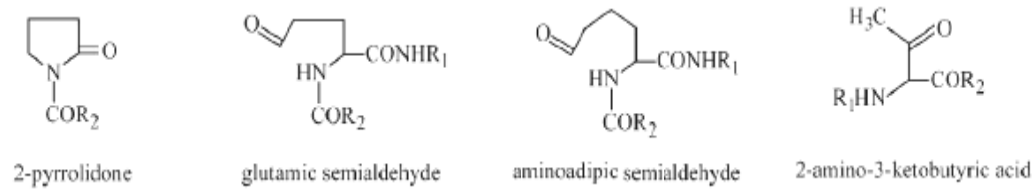


Figure 1.4: Products of Protein Carbonylation which are produced by direct oxidation amino acids side chains. 2-pyrrolidone from prolyl residue, glutamic semialdehyde from arginyl and prolyl residue, a-aminoadipic semialdehyde from lysyl residue, and 2-amino-3-ketobutyric acid from threonyl residue (Donne *et al.,* 2003)

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 (Chevion *et al.,* 2000; Donne *et al.,* 2003; Halliwell and Gutteridge, 2007). The usage of protein CO groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins. Most of the assays for detection of protein CO groups involve reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product. This then can be detected by various

means, such as spectrophotometric assays, enzyme-linked immunosorbent assay (ELISA), and one or two dimensional electrophoresis followed by Western blot immunoassay. 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)

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-dGuo) (Cheng *et al.,* 1992). The 8-oxo-dGuo 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-dGuo can be repaired by base excision repair system. Oxidative damage to DNA has been demonstrated by measuring levels of 8-oxo-dGuo 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 endonuclease III and formamidopyrimidine DNA glycosylase. 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 Oztok *et al.*¸1999) and

one of them reported that Vitamin E supplementation decreases DNA damage in those patients (Sardas *et al.,* 2001).

1.2.5 Tissue Reduced Glutathione (GSH) Content

Glutathione (GSH) is the primary intracellular free radical scavenger, though it also plays a role in the maintenance of plasma antioxidant status and is the cofactor for several enzymes. GSH is synthesized from the amino acids glycine, cysteine and glutamate, and is the cofactor for many antioxidant enzymes, such as GSH peroxidase, GSH reductase and GSH-S-Transferase (Niedowicz and Daleke, 2005). It has very high electron-donating capacity (high negative redox potential) combined with high intracellular concentration (millimolar levels), which generate great reducing power due to free thiol group on it. Its structure is shown in figure 1.5. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Therefore it is a potent antioxidant with powerful enzyme cofactor properties. Therefore, relative amount of intracellular reduced and oxidized GSH is a measure of the cellular redox status.

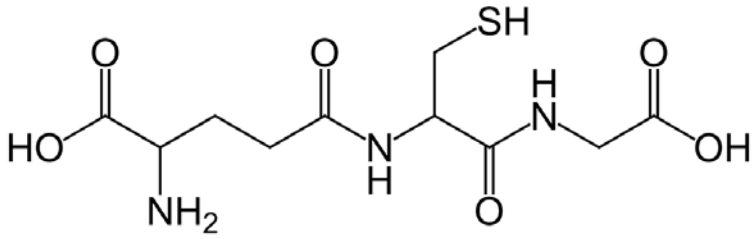


Figure 1.5: Structure of reduced glutathione (GSH), γ-Glu-Cys-Gly

Reduced and oxidized glutathione couple (GSH/GSSG) determines the cellular redox potential and glutathione redox state determines the steady-state value of the intracellular redox potential. 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). 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. The main protective roles of glutathione against oxidative stress are that; (i) it is a cofactor of several detoxifying enzymes such as GPx and GSTs; (ii) it participates in amino acid transport through plasma membrane; (iii) it scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of GPx; (vi) it is able to regenerate the most important antioxidants such as vitamin C and E back to their active forms (Valko *et al.,* 2006).

In conjugation with the ubiquitous free radical generation and radical induced oxidative damage, cells are well equipped with a complex armory of effective defense systems. First of all, 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 ANTIOXIDANT SYSTEMS

Body protects itself by a copious amount of antioxidant molecules such as vitamin C (ascorbic acid), vitamin E (α-tochopherol), α-lipoic acid (thioctic acid), urate, ubiquinol, polyphenols, flavonoids, phytoestrogens, resveratrol and reduced glutathione. These molecules have the capability to take free electrons from the radicals without being as reactive as free oxygen radicals.

1.3.1 Low Molecular Weight Radical Scavengers (Non-enzymatic Antioxidants)

Antioxidants are the compounds that are functioning in the neutralization of the oxidizing effects of the free radicals. Though they accept the free electrons of the radicals, they do not behave like a radical. Since, they have the ability to accommodate free electrons without reacting with other molecules. A number of compounds can act as non-enzymatic antioxidants such as vitamin C and E, and lipoic acid and glutathione. Vitamin E (α-tocopherol) is a hydrophobic antioxidant mainly present in lipid bilayer and ascorbic acid (vitamin C) that is highly water soluble and present mainly in the cytoplasm. Together with Vitamin E, Vitamin C cooperates in cellular defense against ROS in both lipid and aqueous phase at the expense of lipoic acid and dihydrolipoate which serves as a bridge between them.

1.3.1.1 Alpha-Lipoic Acid (Thioctic acid)

Radical quenching, metal chelation, amphiphilic character, bioavailability and safety, interaction with other antioxidants and metabolic regeneration are important criteria to consider a compound as a potent antioxidant. Lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA), meet all the criteria, making lipoic acid an ideal antioxidant (Packer *et al.,* 2001). The molecular structure of α-lipoic acid is shown in figure 1.6.

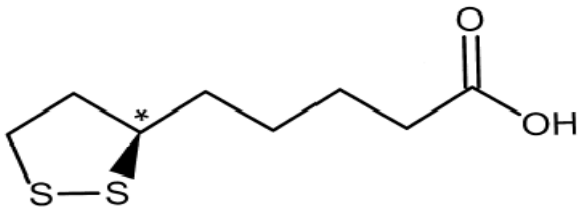


Figure 1.6: Molecular structure of α-lipoic acid.

LA is an essential component of the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase multi-enzyme complexes and serves as driving force for maintaining high concentrations of the other reduced antioxidants; glutathione, thioredoxin and ascorbic acid in the aqueous phase, and vitamin E and ubiquinol in membranes or lipoproteins (Oberley, 1988; Packer *et al.,* 1995). Therefore, lipoic acid and DHLA take central positions in the antioxidant network. Because of its water and also lipid soluble characteristics, it enables to reduce oxidized antioxidants at the interphase between lipid and water (Packer *et al.,* 2001). Figure 1.7 points out to the interaction in between various antioxidants and their relation with lipoic acid network. According to this figure, as vitamin E scavenges a peroxyl radical, produced vitamin E radical may be re-reduced by several antioxidants such as ascorbate, ubiquinol, and reduced glutathione (GSH) and DHLA can reduce all these antioxidants and be regenerated by enzyme lipoamide reductase.

1.3.1.2 Vitamin C (Ascorbic Acid)

Vitamin C (ascorbic acid, ascorbate) is a water-soluble vitamin found widely in plants, quenching reactive oxygen species (ROS) and reactive nitrogen species. It is a part of antioxidant network as described earlier, functioning in the neutralization of free radical damages.

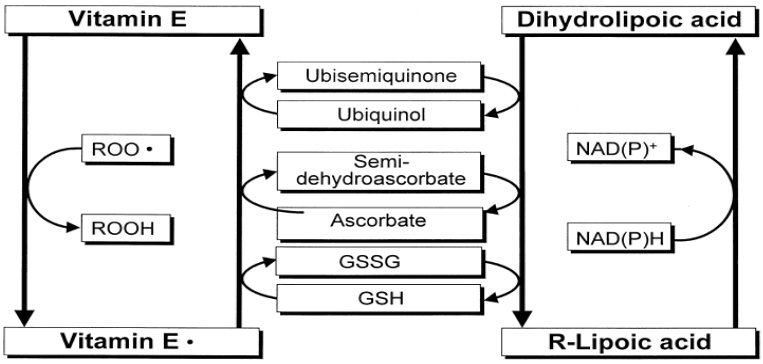
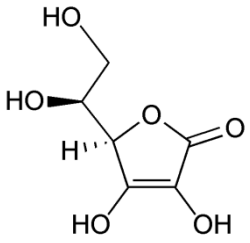


Figure 1.7: The antioxidant network showing the interaction between vitamin E, ubiquinol, vitamin C, glutathione, and R-lipoic acid redox cycles (Packer *et al.,* 2001)

During the process of quenching the radicals, ascorbate donates an electron, becoming the unstable intermediate ascorbyl radical that can be reversibly reduced back to ascorbate. Ascorbyl radical can donate a second electron and be converted to dehydroascorbate (DHA) which may be reduced back to ascorbic acid enzymatically by dehydroascorbate reductase. The molecular structures of ascorbate and DHA are shown in figure 1.8.

a) 

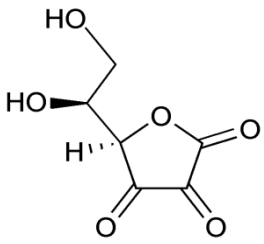
b) 

Figure 1.8: Molecular structure of ascorbic acid (a) and dehydroascorbic acid (b)

Paradoxically, vitamin C is also associated with a pro-oxidant effect at very high concentrations. It was reported that vitamin C acts primarily as an antioxidant at low doses, whereas the pro-oxidant effects of vitamin C are observed at high doses in hepatic ischemia and reperfusion (Seo and Lee, 2002).

1.3.2 Antioxidant Enzymes

Non-enzymatic scavengers are essential in the protection of cellular components from unavoidable damage by the most reactive oxidizing species, but this protection is not adequate to cope with continuously produced radicals such as superoxide, or peroxides and fortunately specific antioxidant enzymes have been designed by nature to destroy these radicals. 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.

Superoxide dismutases (SOD), (Cu-Zn and Mn containing) convert superoxide radicals into hydrogen peroxide. Catalases (CAT) remove hydrogen peroxide by converting it into water. Glutathione peroxidases (GPx) remove hydrogen peroxide by oxidizing the glutathione (GSH) into its oxidized (GSSH) form. Also, xenobiotic metabolizing phase 2 detoxification enzymes, glutathione S-transferases (GSTs), are working in the detoxification reactions of oxidatively modified molecules and peroxides in the cells (figure 1.9).

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^-) into H_2O_2 and O_2 to protect organisms against toxic radicals produced during oxidative processes and it has been shown to play a central role in protecting cells and tissues against oxidant stress (Fridovich and Freeman 1986; Tsan, 1997).

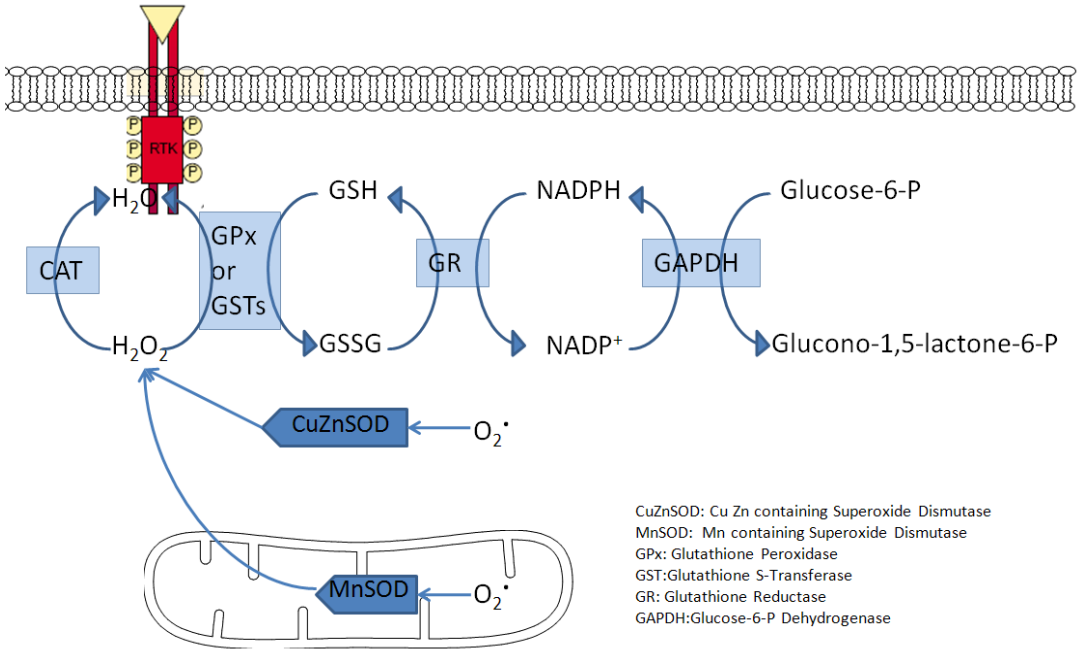


Figure 1.9: Major pathway of antioxidant enzyme network. Superoxide dismutases (SODs), Catalases (CAT), Glutathione Peroxidases (GPx), Glutathione S-Transferases (GSTs), Glutathione Reductases (GR).

Four different isozymes of SODs have been characterized in eukaryotes; a copper and zinc containing form (Cu-Zn SOD) localized in the cytosol, a manganese containing form (Mn SOD) in the mitochondria, iron containing form (Fe SOD) in some prokaryotes and plants' outer mitochondrial membrane, and a copper and zinc containing form in the extracellular matrix (EC SOD) (Marklund, 1982). The SOD catalysed dismutation of superoxide may be written with the following half-reactions:

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

$$M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2.$$

where M = Cu (n=1); Mn (n=2) ; Fe (n=2) and in this reaction the oxidation state of the metal cation oscillates between n and n+1.

Copper zinc superoxide dismutase (Cu-Zn SOD), a homodimer with a molecular weight of 32 kD is mainly localized in the cytosol, but is also found in the nucleus and peroxisomes. Cu-Zn SOD contains both Cu (II) and Zn (II) at its active sites. Manganese superoxide dismutase (Mn SOD), which constitutes approximately 10 to 15% of total cellular SOD activity, is a homotetramer with a molecular weight of 88 kD (Fridovich and Freeman, 1986). MnSOD, having manganese (III) at its active site, is localized in the matrix of the mitochondria, the site important for removal of superoxide produced by the respiratory chain. Iron containing superoxide dismutase (Fe SOD) is present in some bacteria and plants. No animal tissues have yet been found to contain Fe SOD, but some algae, yeast, and higher plants do. The active sites of Mn and Fe superoxide dismutases contain the same type of amino acid side chains. Iron and manganese SODs exhibit a high degree of sequence and structure similarity, strongly suggesting that these enzymes originate from a common ancestry. Extracellular superoxide dismutase is a secretory tetrameric Cu-Zn containing glycoprotein, with a molecular weight of around 135 kD (Marklund, 1982). EC SOD 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).

The Cu-Zn SODs are inhibited by cyanide (CN^-), whereas Fe and Mn containing SODs are not. Therefore inhibition by cyanide can be used to distinguish Cu-Zn SOD activities in tissue homogenates. Moreover, prolonged exposure to H_2O_2 inactivates the Cu-Zn SOD and Fe SOD but not Mn SOD (Halliwell and Gutteridge, 2007). Thus, incubating the homogenates with H_2O_2 inactivates Fe SOD but not Mn SOD allowing the discrimination of these two closely related isoforms.

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. Each subunit contains Fe (III) at its active site. A typical catalase activity is the decomposition of the H_2O_2 into water and oxygen; in addition to that, they have also peroxidative functions, so as to convert peroxides (ROOH) into alcohol (ROH) and water. Catalase catalyses a dismutation reaction in which one H_2O_2 is reduced to H_2O and the other oxidized to oxygen. Below is the representation of catalase catalyzed reaction occurring in two distinct steps.

$$\text{Catalase-Fe(III)} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Catalase} + \text{Fe(III)} + H_2O + O_2$$

$$\text{Net: } 2\,H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is mainly localized in the peroxisomes (Davies *et al.,* 1979), but is also found in the cytosol of human neutrophiles (Ballinger *et al.,* 1994) and in rat-heart mitochondria (Radi *et al.,* 1991). 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. However, some non-peroxisomal catalase may present in the livers of some animals such as guinea pigs (Bulitta *et al.,* 1996). Catalase can be inhibited by azide, cyanide, peroxynitrite and hypochloric acid (Halliwell and Gutteridge, 2007). In mammalian cells, NADPH is bound to catalase and may protect the enzyme from inactivation by H_2O_2 (Kirkman and Gaetani, 1984).

1.3.2.3 Enzymes Working In Glutathione Redox Cycle

Three antioxidant enzymes are important and working in the glutathione redox cycle (figure 1.10). These are Glutathione Peroxidase (GPx), Glutathione S-Transferase (GST) and Glutathione Reductase (GR).

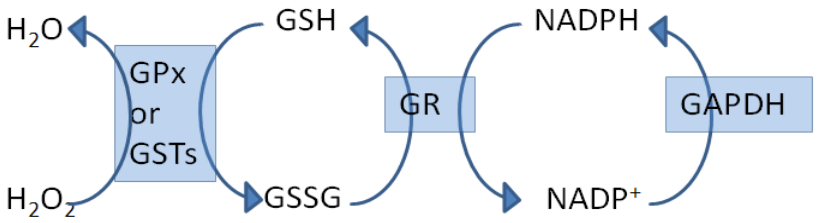


Figure 1.10: Glutathione redox cycle in which antioxidant enzymes Glutathione Peroxidase (GPx), Glutathione S-transferase (GSTs) and Glutatihone Reductase (GR) is functioning.

1.3.2.3.1 Glutathione Peroxidase (E.C: 1.11.1.9)

In animal cells, glutathione peroxidases (GPx) are homotetrameric water soluble selenoenzymes 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.

$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$

$ROOH + + 2GSH \rightarrow GSSG + H_2O + ROH$

GPx active sites contain selenium in the form of a selenocysteine residue, which is incorporated into the polypeptide backbone (Flohe *et al.,* 1973; Stadtman, 1991). In mammals, there are five isoenzymes of GPx and the levels of each isoform vary depending on the tissue type. GPx1 is a cytosolic and mitochondrial enzyme reducing fatty acid hydroperoxides and H_2O_2 at the expense of

glutathione. GPx1 and the phospholipid hydroperoxide glutathione peroxidase (GPx4) are found in most tissues. GPx4 is located in both cytosol and the membrane and directly reduce the phospholipid hydroperoxides, fatty acid hydroperoxides, and cholesterol hydroperoxides that are produced in peroxidized membranes and oxidized lipoproteins (Chance *et al.,* 1979). GPx1 is predominantly present in erythrocytes, kidney, and liver, and GPx4 is highly expressed in renal epithelial cells and testes. Cytosolic GPx2 and extracellular GPx3 are detected mostly in the gastrointestinal tract and kidney, respectively. The final isozyme which is GPx5 is expressed especially in mouse epididymis, and it is selenium-independent (Imai *et al.,* 1998).

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

Glutathione S-transferases (GSTs) are (homo or hetero) dimeric, ubiquitous multifunctional enzymes composed of two polypeptide subunits playing a key role in cellular detoxification (Dixon *et al.,* 2002). The tripeptide glutathione (GSH) involve in the metabolism of xenobiotics (foreign compounds) many of which metabolized by conjugation with GSH by glutathione S-transferases.

$$RX+GSH \rightarrow RSG+HX$$

All eukaryotes have multiple cytosolic and membrane bound GST isoforms, with distinct substrate specificities as well as their catalytic functions. It has been found that some GST isoforms serve as intracellular carrier proteins to heam, bilirubin, bile pigments, thyroid and steroid hormones that bind GSTs non-enzymatically (Halliwell and Gutteridge, 2007). Recent studies have also implicated that GST enzymes can function in the ultraviolet-inducible cell signaling pathways and as potential regulators of apoptosis (Loyall *et al.,* 2000). In most animal cells, hydrogen peroxide is reduced by selenium-dependent GPx,

but there are also some 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. Cytosolic GSTs are encoded by multiple gene families of homo and heterodimeric enzymes whose individual members are composed of various combinations of different monomers. Mamalian GSTs have been segregated in at least seven classes; alpha, zeta, mu, pi, omega, sigma and theta (Smart and Hodgson, 2008).

The alpha, mu and pi class of enzymes are most abundant in mammals and levels are often increased by the exposure to xenobiotics via antioxidant response elements and they have active roles in drug metabolism. Sigma form is functioning in the prostoglandin synthesis (Rowsey *et al.,* 2001). Theta, pi 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). Furthermore some GSTs may be important protectors against lipid peroxidation. Some show activity toward membrane associated lipid peroxides and some metabolize toxic end products of the lipid peroxidation such as 4-hydroxynonenal. 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). Moreover, some GSTs may associate with kinases that are involved in controlling stress responses. For example GST-pi has been shown to bind to Jun kinase and inhibit Jun signaling in nonstressed cells. In response to oxidative or chemical stress, GST-pi releases the Jun and signaling pathway becomes activated. Similarly, GST-mu binds and inhibits ASK1 kinase and in cellular oxidative stress, it is released and induces cellular signaling in response to that stress (Smart and Hodgson, 2008).

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).

$$GSSG+NADPH+H^{+} \rightarrow 2GSH+NADP^{+}$$

This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is important for protection against oxidative stress.

1.3.2.4 Thiol Specific Antioxidants (Thioredoxins and Peroxiredoxins)

Thioredoxins (Trx) are proteins that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Reduced Trx contain two –SH groups that form a disulphide in oxidized thioredoxin. Thioredoxin reductase (TrxR) in conjunction with thioredoxin is a ubiquitous oxidoreductase system with antioxidant and redox regulatory roles. Thioredoxins undergo redox reactions with various proteins with a mechanism described in figure 1.11.

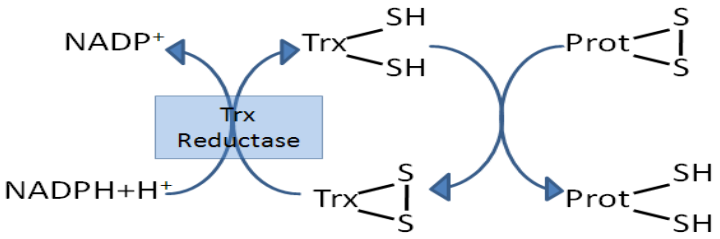


Figure 1.11: Enzymatic reactions of the Trx system. Thioredoxin reductase reduces the active site disulfide in Trx and several other substrates directly under consumption of NADPH. Reduced Trx is highly efficient in reducing disulfides in proteins and peptides, including peroxiredoxins (Prx) and glutathione disulfide (GSSG).

Peroxiredoxins (Prx) are thiol-dependent antioxidants which are family of peroxidases that reduce H_2O_2 and organic peroxides at the expense of $NADPH,H^+$ together with the thioredoxin systems (Figure 1.12). Prx enzymes do not contain any tightly bound metal ions. They have no amino acid sequence similarity with other enzymatic antioxidants like catalases, peroxidases or superoxide dismutases (Aalen, 1999). Peroxiredoxins are slower at catalyzing H_2O_2 removal than GPx and they are readily inactivated by H_2O_2 (Halliwell and Gutteridge, 2007).

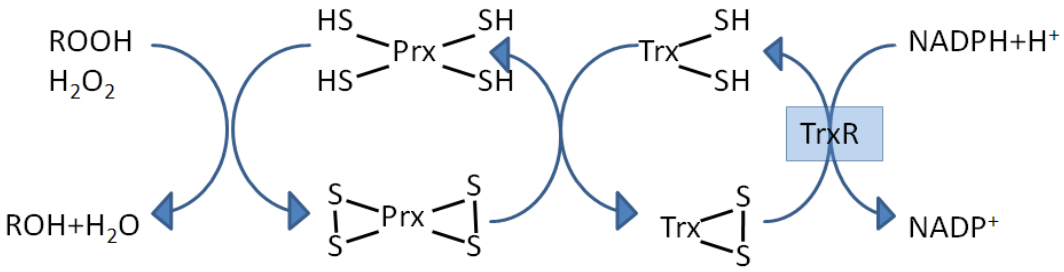


Figure 1.12: Thioredoxin dependent reduction of organic hydroperoxides and hydrogen peroxide with peroxiredoxins.

At low H_2O_2 level, Prx is responsible for removal, but as the cells sense extra H_2O_2, the Prx are partially inhibited to allow gene expression by redox regulation with phosphorylation and dephosphorylation. The Prx proteins are phosphorylated by cyclin dependent kinases that decrease the activity, unlike the CAT and GPx which are phosphorylated by the c-abl and arg kinases which enhance their catalytic activities (Cao *et al.,* 2003a; Cao *et al.,* 2003c)

The tissue level of antioxidants critically influences the susceptibility of various tissues to oxidative stress. Figure 1.13 summarizes the intracellular sources of the oxygen radicals and the downstream antioxidant defense systems developed for the protection against oxidative damage to the tissues.

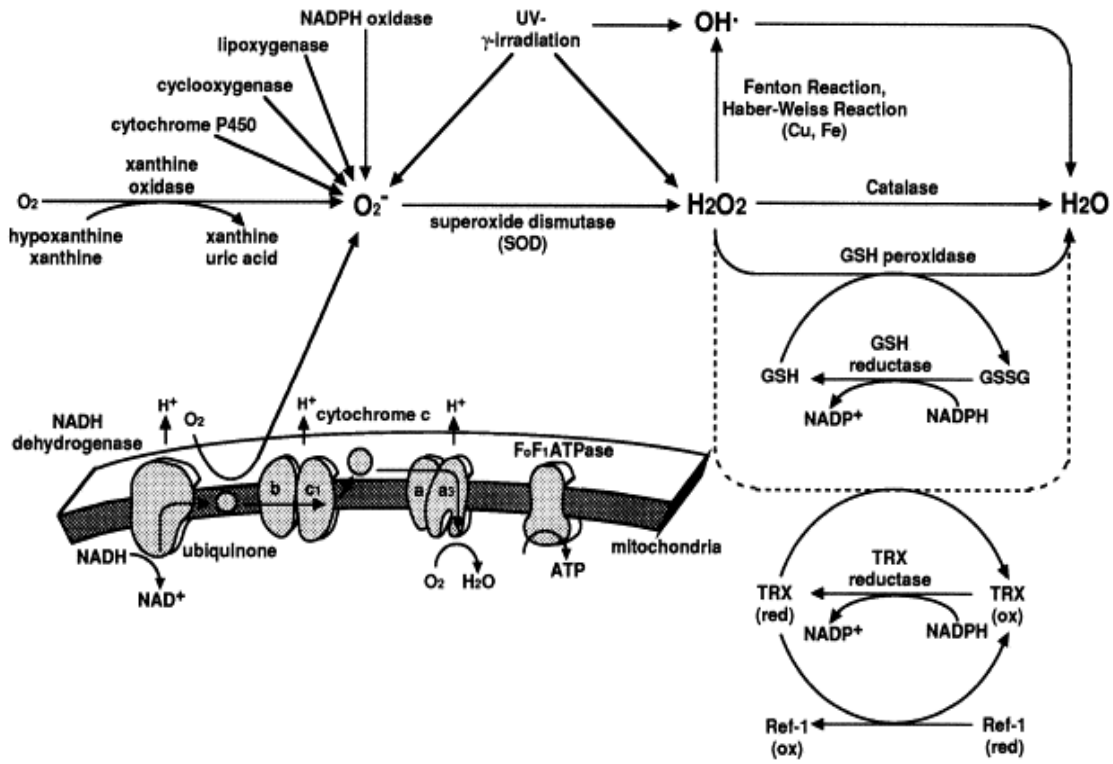


Figure 1.13: Metabolic pathways of reactive oxygen radicals generated in cells by several pathways. Several mechanisms of radical formation and present antioxidant systems eliminating the radical induced damage (Kamata and Hirata, 1999)

1.3.3 Redox regulation of gene transcription

Redox regulation can be defined as the modulation of protein and/or enzyme activity by oxidation and reduction potential differences which control the cellular activities in various ways. Intracellular redox balance is tightly controlled and its disturbance leads to modifications in the pattern of gene expressions of several enzymes (Schoonbroodt and Piette, 2000). Recent studies have investigated the regulation of gene expression by oxidants, antioxidants, and other determinants of the intracellular oxidation-reduction (redox) state (Sen and Packer, 1996; Watai *et al.,* 2007; Surh *et al.,* 2008). It was shown that ROS can oxidize the redox-sensitive proteins directly or indirectly (Storz and Imlay, 1999; Halliwell and Gutteridge, 2007).

Redox sensitive metabolic enzymes can directly modulate cellular metabolism, whereas redox-sensitive signaling proteins execute their function via downstream signaling components, such as kinases, phosphatases, and transcription factors. Therefore, cells may sense, transduce, and translate the oxidant signals into appropriate cellular responses. Redox-sensitive proteins can undergo reversible oxidation and reduction and may switch 'on' and 'off' depending on the cellular redox state. It is known that some proteins (e.g. thioredoxins and peroxiredoxins) have highly conserved cysteine (sulfhydryl) sequence in their active/regulatory sites, which are primary targets of oxidative modifications and thus important components of redox signaling. Oxidative stress possibly via the modification of cysteine residues activates multiple stress kinase pathways (e.g. PK-C) and transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), which differentially regulate the genes for proinflammatory cytokines as well as the protective antioxidant genes. DNA binding sites of the redox regulated transcription factors NF-κB and AP-1 are located in the promoter region of a large variety of genes that are directly involved in the pathogenesis of diseases, e.g., AIDS, cancer, atherosclerosis and

diabetic complications (Holbrook and Fornace, 1991). Moreover, critical steps in the signal transduction cascade are also sensitive to changes in oxidant/antioxidant balance and endogenous glutathione and thioredoxin systems and the exogenous lipoate dihydrolipoate couple can be considered to be effective regulators of redox-sensitive signal transduction and gene expression.

In mammals, NF-κB resides normally in the cytoplasm where it is associated with a transcriptional repressor IκB. ROS stimulate IκB phosphorylation and its dissociation from NF-κB, leading to the nuclear localization of NF-κB (Vranova *et al.,* 2002). NF-κB is a dimeric (p50 and p65) transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response. It is activated by a variety of stimuli ranging from cytokines, to various forms of radiation, to oxidative stress (e.g. exposure to H_2O_2) (Gius *et al.,* 1999). Figure 1.14 summarizes the oxidant stress induced NF-κB activation mechanism. It has been found that antioxidants may diminish or completely eliminate NF-κB activation. For example, H_2O_2, UV, and ionizing radiation have all been observed to stimulate degradation of IκB. Conversely, antioxidants such as α-tocopherol decrease NF-κB activity and translocation (Muller *et al.,* 1997; Gius *et al.,* 1999). In another study, it was found that overexpression of catalase supress the NF-κB activation while overexpression of superoxide dismutase increases the NF-κB activation in TNF cells (Schmidt *et al.,* 1995). These observation leads to understand the role of reactive oxygen intermediates especially the H_2O_2 involved in the activation of NF-κB. The precise mechanism(s) through which oxidants and reductants influence activation of NF-κB is presently unknown; however, there is one evidence that antioxidant enzyme, a redox-sensitive thioredoxin peroxidase, regulates IκB phosphorylation (Jin *et al.,* 1997).

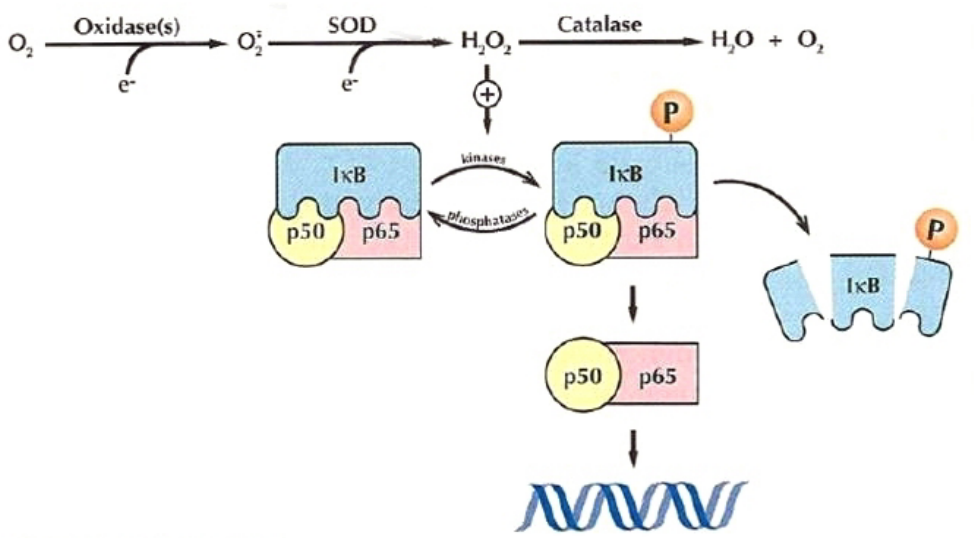


Figure 1.14: A model for oxidative NF-κB activation. Induction of oxidative stress increases the production of superoxide anion ($O_2^{\cdot}$-) and subsequently H_2O_2 which may then either activate kinases or inhibit phosphatases, increasing the phosphorylation of IκB. IκB is then degraded, releasing the NF-κB p50/p65 dimer to bind to DNA (Schmidt *et al*., 1995)

Another redox sensitive mediator of gene expression is the transcription factor called activator protein-1 (AP-1), which couples extracellular signals to gene-activating events associated with growth, differentiation, and cellular stress (Angel and Karin, 1991; Herrlich *et al.,* 1992). AP-1 is composed of the Jun and Fos gene products which form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes. In many cell types, Fos and Jun are expressed at low basal levels, but are induced rapidly and transiently by a variety of extracellular signals, including agents or factors that alter the cellular oxidation reduction status (Abate *et al.,* 1991; Angel and Karin, 1991). Several prooxidant conditions, such as hydrogen peroxide and UV irradiation are shown to induce AP-1 activation (Sen and Packer, 1996; Vranova *et al.,* 2002). The activation of AP-1 is regulated by complex mechanisms consisting of post-transcriptional events acting on preexisting AP-1 molecules and transcriptional activation leading to increased

amounts of AP-1 binding proteins. In addition, redox modification of a conserved cysteine residue in the DNA-binding domain of Fos and Jun may be another mechanism of controlling DNA binding of AP-1 (Schenk *et al.,* 1994)

A distinct difference between the AP-1 complex and NF-κB lies in the mechanism by which a specific signal is passed from the cytoplasm to the nucleus (Baeuerle and Baltimore, 1996; Xanthoudakis and Curran, 1996). In the case of NF-κB, the protein complex itself bridges the cytoplasmic and nuclear compartments by its transport into the nucleus. In contrast, the Fos and Jun family proteins are restricted to the nucleus, and alternative pathways involving protein kinases, such as MAPK, appear to pass messages between these subcellular compartments (Karin and Smeal, 1992; Xanthoudakis and Curran, 1994).

1.3.3.1 The role of redox factor-1 (Ref-1) in the regulation of AP-1

Ref-1 is a hydrolytic endonuclease catalyzing the repair of oxidative lesions (primarily abasic sites) in DNA and stimulating the DNA-binding activity of AP-1 proteins (i.e. Fos and Jun) by a redox dependent mechanism. Ref-1 reduces the redox-sensitive cysteine residues of the transcription factor AP-1 and thereby facilitates its DNA-binding and transcriptional activities. Ref-1 also regulates gene expression by reversibly altering the redox state of specific cysteine residues located in the DNA-binding region of several transcription factors, including such as NF-κB , and Egr-1 (Xanthoudakis and Curran, 1992; Xanthoudakis *et al.,* 1992; Walker *et al.,* 1994). The relationship between the redox and DNA repair activities of Ref-1 is intriguing; both activities have been suggested to play an important role in the cellular response to oxidative stress (Xanthoudakis *et al.,* 1996).

1.3.3.2 Kinases and phosphatases in redox signal transduction

Protein tyrosine phosphorylation and phosphorylation states are governed by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). There is an interaction in between protein phosphorylation and redox dependent signaling, and reactive oxygen species. In cells, both tyrosine phosphorylation and thiol oxidation are reversible and dynamic, and there is a cross-talk between these post-translational protein regulatory mechanisms.

Reactive oxygen species can act as cellular messengers for some cellular processes such as signal transduction and gene expression. Recently it has been shown that H_2O_2 plays a role in intracellular communication that regulates tyrosine phosphorylation on proteins (Chiarugi and Cirri, 2003; Rhee at al., 2005). Unlike other known second messengers, H_2O_2 is not directly recognized by a sensor protein; instead it modulates intracellular protein phosphorylation by the reversible modification of protein cysteine residues in proteins to cysteine sulfenic acid or disulfide, both of which are readily re-reduced to cysteine by various cellular reductants such as thioredoxin system. Proteins with low-pKa cysteine residues, which are vulnerable to oxidation by H_2O_2 include transcription factors such as the NF-κB, AP-1 and protein tyrosine phosphatases (PTPs) (Groen *et al.,* 2005; Rhee *et al.,* 2005).

One of the most important redox regulated proteins in the cell is the protein tyrosine phosphatases (PTPs) and their transient oxidation of thiol groups in active sites leads to their inactivation. Reversible oxidation of the conserved catalytic site cysteine groups is essential for the of PTPs action (Jackson and Denu, 2001) that is emerging as an important regulatory mechanism in cellular signal transduction cascades (Hertog *et al.,* 2004). Figure 1.15 demonstrates the possible effects of oxidized cysteine on PTPs regulating the overall phosphorylation pattern throughout the cell.

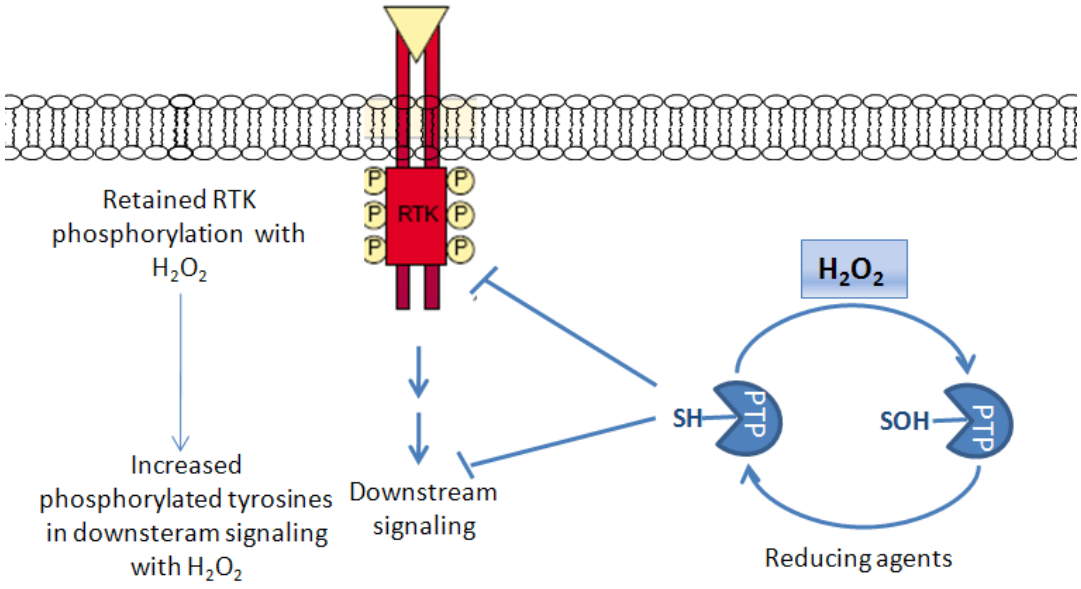


Figure 1.15: Reactive oxygen species (H_2O_2) as modulator of receptor tyrosine kinase (RTK) tyrosine phosphorylation. Protein tyrosine phosphatases (PTPs) are catalytically inactivated by oxidation by H_2O_2 hence allowing the sustained RTK phosphorylation and signal transduction. Decreased ROS concentration leads to RTK dephosphorylation and termination of the signal.

1.3.3.3 Antioxidant Response Elements

Genes encoding various detoxifying enzymes are ubiquitously expressed and coordinately induced in response to antioxidants and xenobiotics (Rushmore *et al*., 1991; Radjendirane *et al.,* 1997). Antioxidant response elements (ARE) are DNA sequences to which transcription factors bind when xenobiotics are present. Binding of transcription factors allows increased transcription of genes encoding xenobiotic metabolizing enzymes that catalyze metabolic detoxification of drugs and carcinogens and, thus, protect the cells against redox cycling and oxidative stress (Matés, 2000). Phase II detoxification enzymes such as NAD(P)H:quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST),

heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL), and multi-drug resistance associated transporter protein (Mrp1) are proved to contain ARE in their promoter regions (Motohashi and Yamamoto, 2004). Moreover, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) genes are considered to be regulated with such kind of antioxidant response elements (Surh *et al.,* 2008) which are subjected to fine transcriptional control by a transcription factor called Nuclear factor-erythroid-2-related factor 2 (Nrf2). Nrf2 is essential for the coordinated induction of those genes and related proteins.

The transcription factor Nrf2 has been implicated as the central protein that interacts with the ARE to activate gene transcription constitutively or in response to an oxidative stress signal. Nrf2 is retained in the cytosol forming an association complex with the cytoskeletal protein Keap1 which is rich in cysteine residues. Upon the oxidation and covalent modifications of critical cysteine residues of Keap1 protein with oxidative elements, Nrf2 is released to be free to accumulate in nucleus and hence induce genes containing ARE elements (Surh *et al.,* 2008). Another activation mechanism is the release of Nrf2 from its repressor by the phosphorylation of Nrf2 with kinases. Cytosolic Nrf2 is phosphorylated with MAPK in response to PK-C activation by cellular stress and translocates into the nucleus. In the nucleus, Nrf2 activate genes through AREs by interacting with other transcription factors (Figure 1.16).

1.4 DIABETES MELLITUS

Diabetes mellitus, Latin meaning is “honey-sweet” is a disease of elevated blood glucose (hyperglycemia) and urinary glucose excretion. In diabetes mellitus the body’s cells experience a relative starvation for glucose because of the lack of insulin which is a pancreatic hormone functioning in the uptake and utilization of glucose by tissues. Insulin also aids in enhancing a number of important enzymes involved in the subsequent metabolism of glucose within the cell.

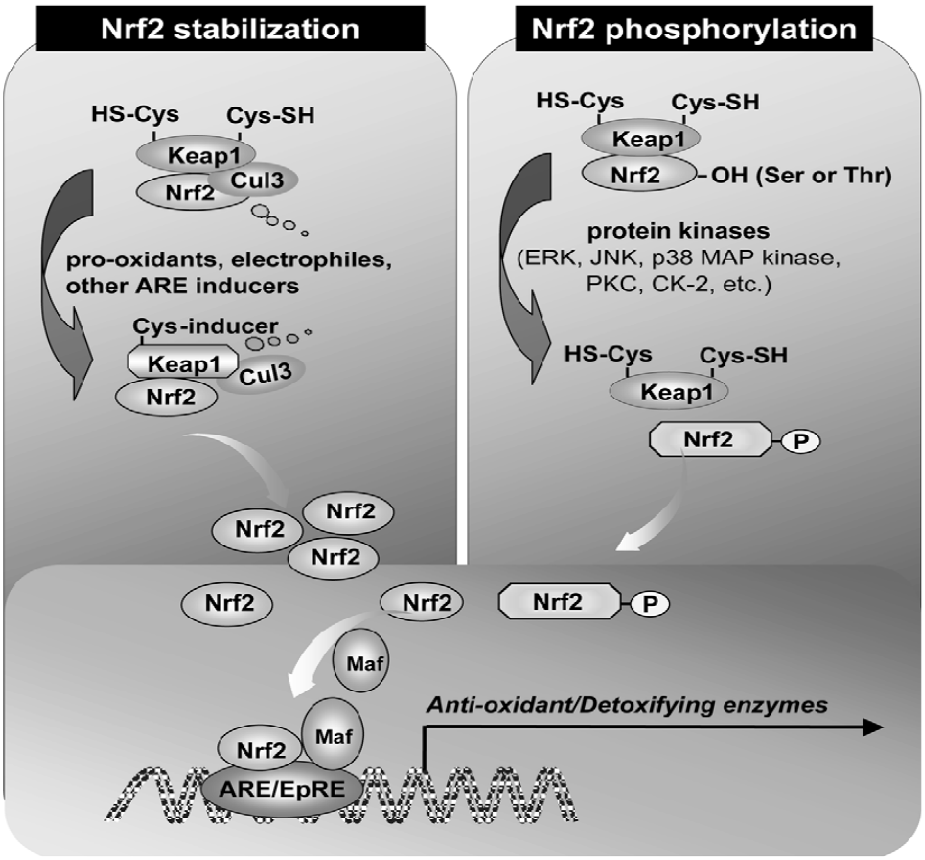


Figure 1.16: Mechanisms of the induction of antioxidant response element with oxidative stress. Nrf2 can be activated by at least two mechanisms that include: (i) stabilization of Nrf2 via Keap1 cysteine thiol modification and (ii) phosphorylation of Nrf2 by upstream kinases (Surh *et al.,* 2008).

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 metabolize the fats and proteins, which are nonpreferred fuels in the cell. This can lead to the accumulation of metabolic by-products (e.g., ketone bodies and ammonia respectively) that can be toxic to the cells when present in higher than normal levels. The starvation sensed by the cells leads to increased food intake

(polyphagia), increased urine volume (polyuria) and increased fluid intake (polydipsia).

In general, two types of diabetes mellitus have been identified according to dependence of disease to the insulin. Type 1 diabetes or insulin-dependent diabetes mellitus is a disease together with 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 requires insulin injections, in addition to careful attention to diet and exercise.

The most common form of diabetes is type 2 (insulin independent) diabetes which is associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity, and ethnicity. 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. Furthermore, this type of diabetes is also characterized by decreased release of insulin from the pancreas. This disease is strongly genetic in origin but lifestyle factors such as excess weight, inactivity, high blood pressure and poor diet are the major risk factors for its development.

Recent researchers have stated that insulin plays a role in the maintenance of brain's nerve cell survival and memory formation other than the role in energy metabolism (Messier and Gagnon, 1996; Steen *et al.,* 2005). Insulin and insulin receptors are found to be expressed in the brain, including the medial temporal regions that support the formation of memory (Schulingkamp *et al.,* 2000). It has been suggested that insulin may contribute to normal cognitive functioning and that insulin abnormalities may exacerbate cognitive impairments, such as those associated with Alzheimer's disease (Stennis and Suzanne, 2003; Steen *et al.,* 2005; Suzanne *et al*., 2005). Recently some scientists

believe that Alzheimer disease is actually the diabetes of type 3. In this disease, brain fails to secrete enough insulin to maintain the brain cell growth and proliferation and also to keep the learning in progress (De La Monte and Wands, 2005). It has been found that brain insulin and its related receptors are lower in individuals with Alzheimer's disease (Hoyer, 2002; Steen *et al.,* 2005). Therefore, Alzheimer's disease may in part be caused by insulin resistance or insulin deficiency in the brain (Watson and Craft, 2003).

Integrated hormonal and metabolic changes are seen in the pregnancy to ensure maternal metabolic efficiency and adequate distribution of nutrients to the fetus. A pregnancy could be associated with a physiologic reduction of insulin sensitivity and the lack of insulin production due to unknown reasons. This situation is termed as gestational diabetes which develops during 5-7% of all pregnancies (Cianni *et al.,* 2003). Gestational diabetes mellitus is defined as glucose intolerance that is first detected during pregnancy. In the presence of predisposing (genetic) and environmental factors, gestational diabetes may be exaggerated.

1.4.1 Experimental Models of Diabetes Mellitus

Animal models have enormously contributed to the study of diabetes mellitus and provided an invaluable insight into the pathogenesis of the human disease. They give researchers the opportunity to control in vivo the genetic and environmental factors that may influence the development of the disease and establishment of its complications. Animal models of diabetes are developed either spontaneously or by using chemical, surgical, genetic or other techniques.

One of the most straightforward ways of studying the effects of hyperglycemia in an animal is to remove the pancreas, either partially or totally. Furthermore, Non-surgical methods of inducing hyperglycemia by damaging the pancreas also exist. These include the administration of toxins such as

streptozotocin (STZ) which is an antibiotic extracted from *Streptomyces achromogenes* and causing diabetes by direct action on the pancreatic β-cells by massive degranulation (Junod *et al.,* 1969). Furthermore alloxan (Lenzen and Patten, 1988) and other toxic substances such as Vacor, Dithizone and 8-hydroxyquinolone (Rees and Alcolado, 2004) induce diabetes by increasing the level of free radicals in pancreas and cause destruction of β-cells.

1.4.1.1 Spontaneous animal models of Type 1 diabetes

The non-obese diabetic (NOD) mouse and bio breeding (BB) rats are the two most commonly used animals that spontaneously develop diseases with similarities to human Type 1 diabetes. These animals have been inbred in laboratories for many generations, by selecting for hyperglycemia.

1.4.1.2 Animal models of Type 2 diabetes

Animal models of Type 2 diabetes are likely to be as complex and heterogeneous as the human condition. As with the NOD mouse and BB rat in Type 1 diabetes, the selective inbreeding of animals (Table 1) that spontaneously develop Type 2 diabetes like phenotype has generated many of the strains are used today (Rees and Alcolado, 2004). Below is the list of commonly used animal models to study Type 2 diabetes mellitus.

- Ob/Ob mouse—monogenic model of obesity (leptin deficient)
- db/db mouse—monogenic model of obesity (leptin resistant)
- Zucker (fa/fa) rat—monogenic model of obesity (leptin resistant)
- Goto Kakizaki rat
- KK mouse
- Diabetic Torri rat
- New Zealand obese mouse

1.4.1.3 Gene targeting and transgenic techniques for diabetes

Gene targeting refers to the process by which a single gene may be disrupted in an embryonic stem cell and then transmitted along the germ cell line. This produces 'knockout' animals. Furthermore incorporation of modified genes (transgenes) into the pronucleus of a zygote allows researchers to produce animals which are deficient in or overexpress important genes in insulin and/or glucose metabolism. Table 1 summarizes the some transgenic (knocked out) animals showing diabetes like features or phenotypes (Rees and Alcolado, 2004).

Table 1.1: Transgenic animals in diabetes research

Knock Out Gene Name	Gene Copy Number	Diabetes Like Phenotype
Insulin receptor	0	Severe hyperglycemia, death from ketoacidosis
Insulin receptor	1 (in muscle)	Mild insulin resistance
IRS1	0	Mild insulin resistance, Growth retardation
IRS2	0	Hyperglycemia, Insulin resistance
Glucokinase	0	Severe hyperglycemia
Glucokinase	1	Non-progressive glucose intolerance
GLUT4	>1	Increased insulin sensitivity

1.4.2 Oxidative stress in diabetes mellitus

It is unclear whether oxidative stress causes diabetes, but it is apparent that diabetes does cause oxidative stress (Halliwell and Gutteridge, 2007). Hyperglycemia has been accepted as principle actor for the development of diabetic complications most probably due to the oxidative stress. Several articles have indicated that some biochemical pathways, strictly associated with hyperglycemia, can increase the production of free radicals (Halliwell and Gutteridge, 2007). These include direct autoxidation of glucose, increased protein glycation and production of advance glaycation end products (AGEs), stimulation of PK-C activity, increased flux to polyol pathway, and amplified hexosamine pathway (Brownlee, 2001). These are the major contributor for the

production of free radicals and diabetic complications observed in diabetes mellitus.

In all these pathways, there is a common element linking the hyperglycemia and oxidative damage: overproduction of superoxide by mitochondrial electron transport chain by overfeeding with hyperglycemia associated production of NADH (Brownlee, 2001). Figure 1.17 demonstrates how superoxide radical is produced with increased hyperglycemia derived NADH. According to this figure, increased hyperglycemia derived electron donor (NADH) generate high membrane potential by pumping the protons. Overfeeding the system with NADH inhibits the electron transport at complex III (Brownlee, 2001) causing the ubiquinone to pass its electrons to the oxygen generating superoxide radical.

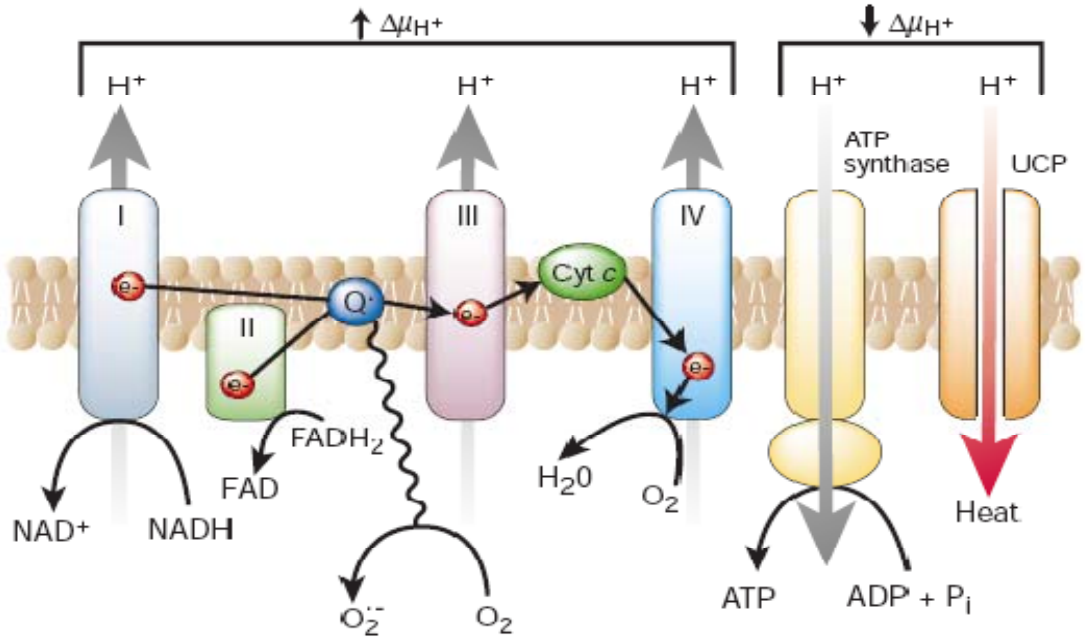


Figure 1.17: Production of superoxide by the mitochondrial electron transport chain (Brownlee, 2001).

1.4.2.1 Free radicals generated by polyol pathway

Aldose reductase is the first enzyme in the polyol pathway and catalyses the NADPH dependent reduction of a wide variety of carbonyl compounds, including glucose. It has low affinity (high Km) to glucose and under normal conditions; glucose is not a preferred substrate for this enzyme. However as glucose concentration rises in a cell, with the concomitant decrease in NADPH, it converts glucose to sorbitol which is further oxidized to fructose with the reduction of NAD^+ to NADH as shown in figure 1.18 (Baynes, 1991). It has been found that decrease in $NADPH/NADP^+$ ratio also ameliorates the reduced glutathione renewal by decreasing the NADPH dependent glutathione reductase. Furthermore, increase in $NADH/NAD^+$ ratio may rise the triose phosphate concentrations increasing the formation of diacylglycerol (DAG) activating the PK-C (Brownlee, 2001).

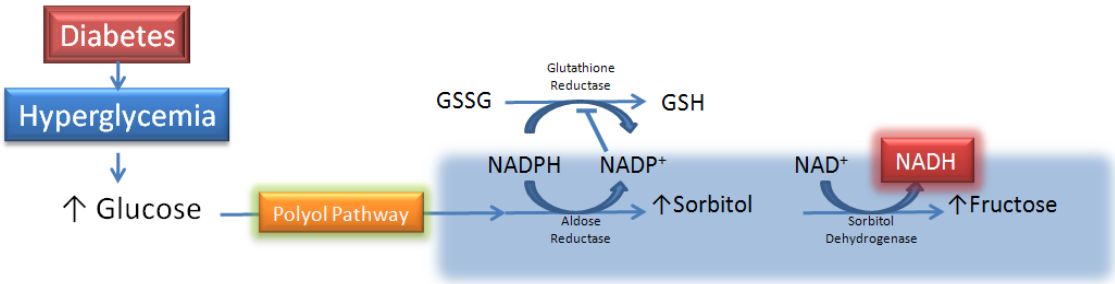


Figure 1.18: Hyperglycemia induced increased influx to the polyol pathway leading to excess NADH production which causes formation of superoxide.

1.4.2.2 Non-enzymatic glycation and glycoxidation

Aldehyde form of the glucose can combine with the amino ($-NH_2$) groups of proteins and modify them. This process is called non-enzymatic glycation (also called glycosylation) which increase the formation of free radicals through a

multistep mechanism (Figure 1.19). Initially, glucose can slowly condense nonenzymatically with proteins' amino groups forming a Schiff base which may rearrange to form the Amadori product.

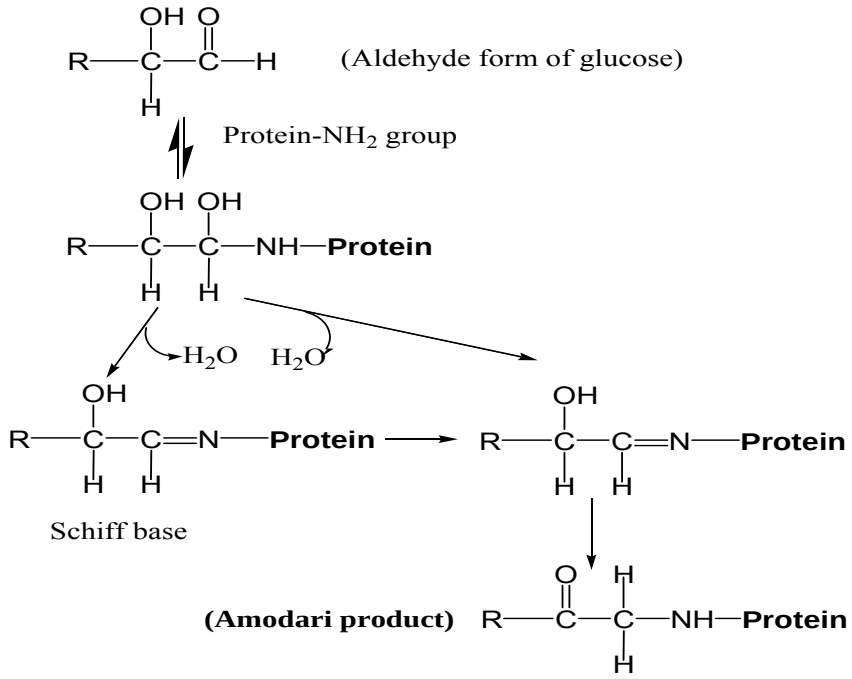


Figure 1.19: Non-enzymatic glycosylation of proteins amino group. The addition of glucose to protein is followed by rearrangements and dehydrations producing Amodari product (Wolff *et al.,* 1991).

The Amadori product subsequently degrades into heterogeneous group of molecules such as carboxymethyllysine, methylglyoxal derivatives, and deoxyglucasones by the help of the series of subsequent reactions, including successions of dehydrations, oxidation-reduction reactions, and other arrangements leading to the formation of compounds named as advance glycation end products (AGEs) (Figure 1.20). In the presence of a suitable electron acceptor (oxygen), enolization reactions may produce superoxide radical and hence H_2O_2.

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 named as diabetic complications. Once produced, AGEs may bind the AGE receptors present on the cell surface and initiate a ROS signaling cascade inside the cell and leading to NF-κB activation (Brownlee, 2001).

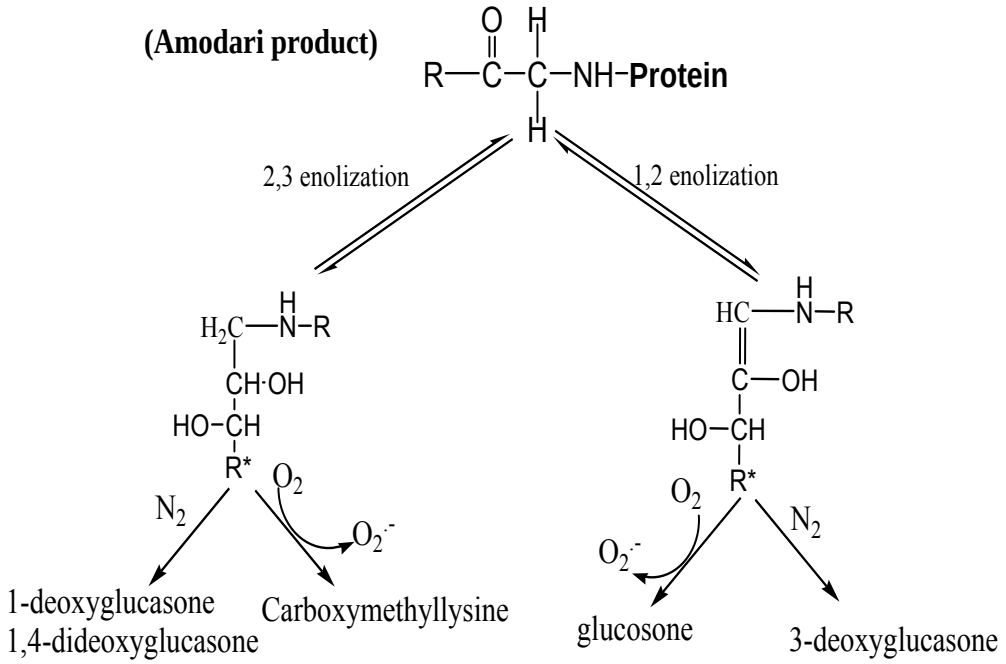


Figure 1.20: Degradation of Amadori product and formation of AGEs leading to formation of superoxide radical and hence H_2O_2 (Elgawish *et al.,* 1996).

1.4.2.3 Activation of Protein Kinase C

Protein Kinase C (PK-C) family proteins are activated by the lipid second messenger diacylglycerol (DAG) and it has been observed that intracellular hyperglycemia can increases the amount of DAG in cultured microvascular cells and in the retina and renal glomeruli of diabetic animals (Brownlee, 2001). DAG is synthesized *de novo* from the glycolytic intermediate dihydroxyacetone

phosphate (DHAP), through the reduction of glyceraldehyde-3-phosphate (Koya and King, 1998) as demonstrated in figure 1.21. Hyperglycemia may also activate PK-C by AGEs as they bound to their cognate receptors on cell surface (Brownlee, 2001). Increase in PK-C may leads to induction of proinflamatory genes through NF-κB, and activates HAD(P)H oxidases producing reactive oxygen spesies.

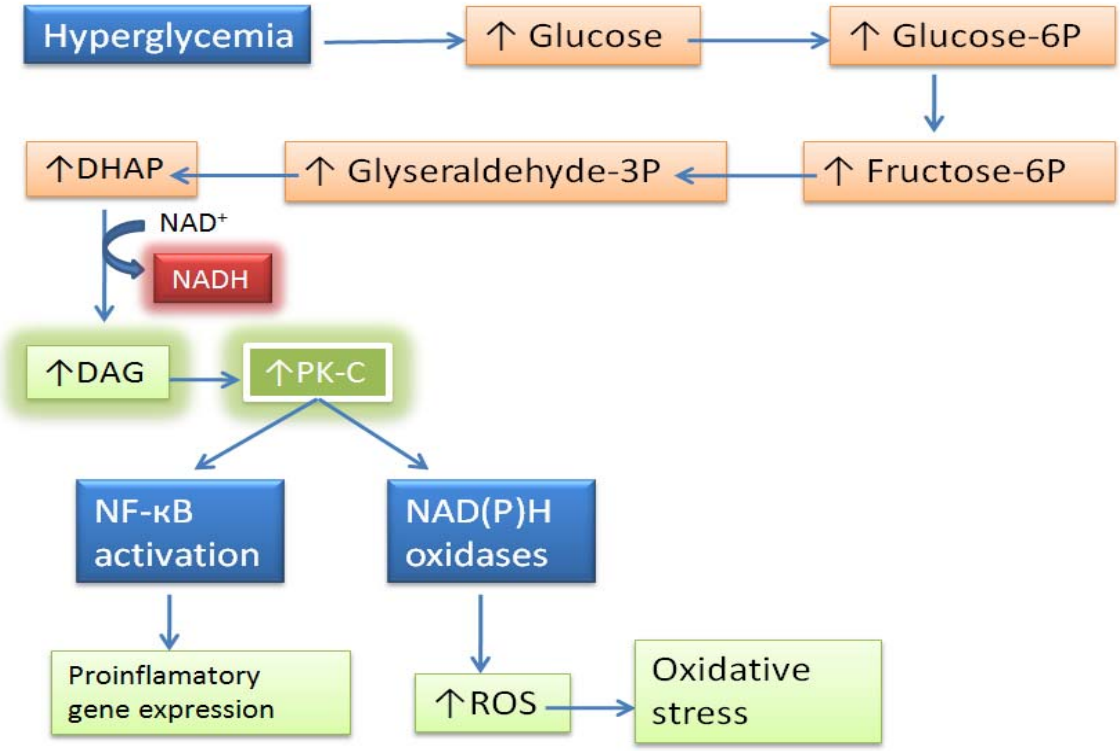


Figure 1.21: Consequences of hyperglycemia-induced activation of protein kinase C (PK-C). Hyperglycemia increases *de novo* synthesis of diacylglycerol (DAG), which activates PK-C, activating NF-κB and NAD(P)H oxidases (Brownlee, 2001).

1.4.2.4 Increased flux through the hexosamine pathway

The hexosamine biosynthesis pathway is an additional pathway of glucose metabolism that may mediate some of the toxic effects of glucose (Du *et al.,* 2000 and Brownlee, 2001). Under usual metabolic conditions, 2–5% of glucose

entering cells is directed into the hexosamine pathway, beginning with the conversion of fructose 6-phosphate to glucosamine 6-phosphate. During hyperglycemia, because of the increased nutrient availability, much of the excess glucose is shunted into the hexosamine pathway. The end product of this pathway, UDP-*N*-acetylglucosamine, is the substrate for the glycosylation of important intracellular factors (McClain and Crook, 1996) including transcription factors, thereby affecting the expression of many genes including plasminogen activator inhibitor-1 (PAI-1) and leads to the development of the complications of diabetes (Edelstein *et al*., 2000; Goldberg *et al.,* 2002).

1.4.2.5 Increased 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 1.22). This process entails the reduction of oxygen, producing oxidizing intermediates, such as superoxide and hydroxyl radical and H_2O_2 (Hunt *et al.,* 1988). 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.

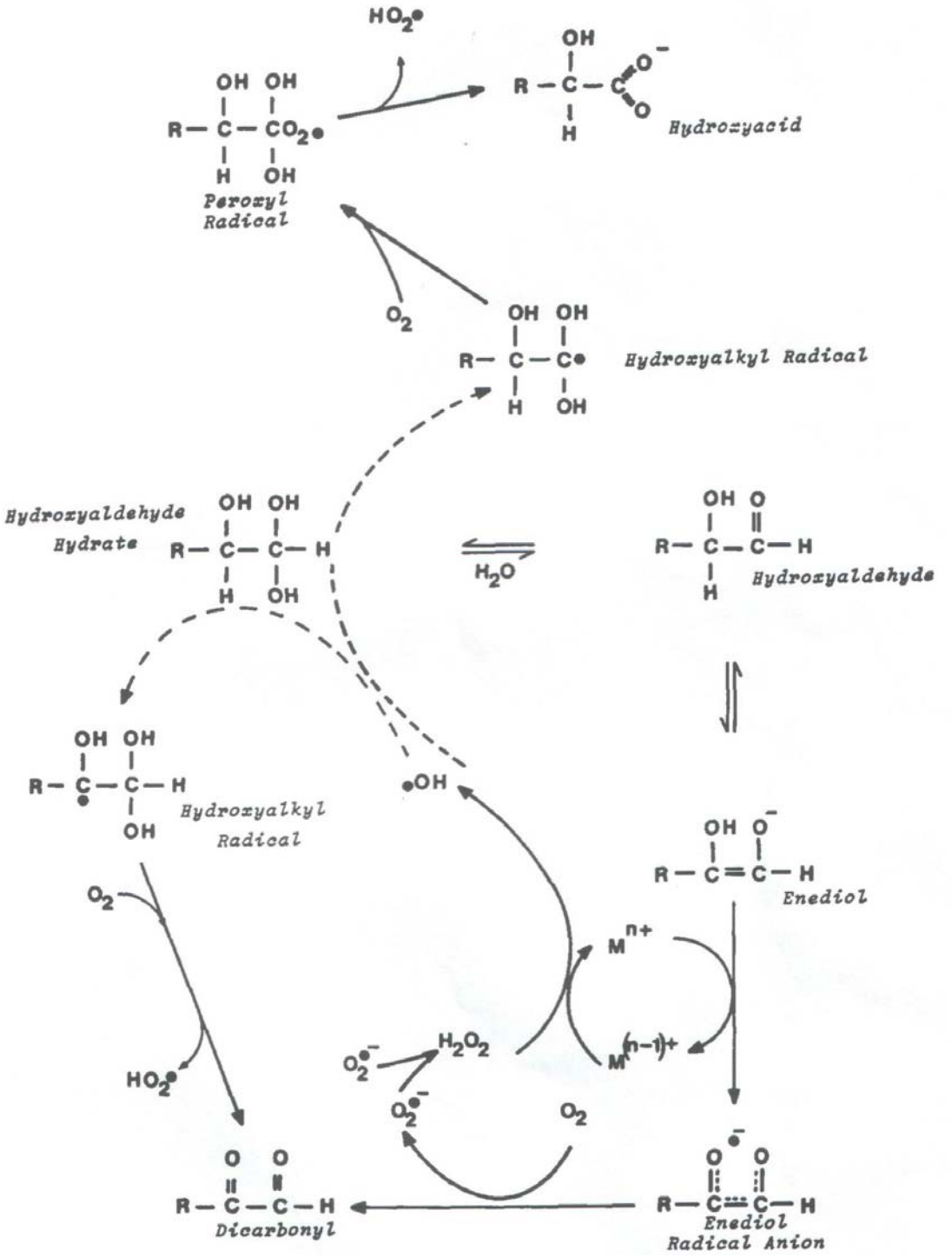


Figure 1.22: The process of autoxidation of a monosaccharide, showing how free radicals are produced when excess glucose is present (Wolff and Dean, 1988).

1.5 AIM OF THE STUDY

Increased oxidative stress can be related with the pathogenesis and the chronic complications associated with the disease, diabetes mellitus (Baynes, 1991). Numerous studies proposed that, reducing oxidative stress by using antioxidants might be an effective strategy for reducing diabetic complications. These articles showed the beneficial effects of the antioxidants for the treatment of diabetes (Dincer *et al.,* 2002; Maritim *et al.,* 2003b; Seven *et al.,* 2004). On the other hand, while roles of oxidative stress in diabetic complications are widely studied, the molecular mechanisms playing role in the regulations of antioxidant enzymes in the presence of antioxidants have not been clearly established because of the complexity of the pathways. The present study was planned in order to determine the extent of oxidative stress in which the liver tissues were exposed during diabetes, and to examine the accompanying changes in antioxidant enzyme status of the tissue, to understand their role in the pathogenesis of the disease. Oxidative stress in the liver during the diabetes will be determined by measuring the extent of oxidative damage, namely by quantifying the oxidation products of the lipids and proteins, measuring the MDA content and protein carbonylation levels, respectively. Furthermore, the concentration of major antioxidant, GSH will also be determined.

The study would emphasize the regulation of the expression of antioxidant enzyme systems under oxidative stress conditions. So the nature of regulation of the primary antioxidant enzymes will be explored by measuring their mRNA levels using RT-PCR, their protein amounts by Western analysis and also by assessing their catalytic activities. In order to understand whether hyperglycemia selectively up or down regulates the gene expressions of liver Cu-Zn SOD, Mn SOD, CAT, GPx and GST-Mu isoform, the effect of two antioxidants, α-lipoic acid, vitamin C and combination of both antioxidants will also be studied and whether antioxidant treatments normalizes the changes observed in diabetic

rat liver tissues will be reported. Any change observed in the levels of expression of antioxidant enzymes may provide new insights for analyzing cellular mechanism for protection of liver cells against oxidative damage. Therefore in this study we will work on the molecular nature of the regulation of the antioxidant enzymes in diabetic state at three different levels: gene expression, translation and control of activity and also the effects of antioxidants on the biomarkers of oxidative stress will be evaluated in diabetes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Streptozotocin (STZ), DL-α-lipoic acid, ascorbic acid, isopropanol, dithiothreitol (DDT), bovine serum albumin (BSA), ethyldiaminetetraaceticacid (EDTA), sodium-potassium tartarate, phenylmethylsulfonylfloride (PMSF), 1,4-Dithiothreitol (DTT), Folin-Ciocalteu-Phenol reagent, guanidium isothiocyanate, sarcosyl, sodium acetate, phenol, chloroform, isoamyl alcohol, sodium citrate, pyrogallol, reduced glutathione (GSH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), dinitrophenylhydrazine (DNPH), thiobarbutiric acid (TBA), agarose and AP-conjugated anti Sheep IgG were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, U.S.A.

Potassium chloride (KCl), dipotassium hydrogen phosphate and 2-mercaptoethanol were purchased from Merck Chemical Company, Germany. Sodium carbonate, sodium hydroxide, cuppersulfatepentahydrate, hydrogen peroxide, methanol and ethyl alcohol were purchased from Riedel de-Haen Chemical Company, Germany. Diethyl pyrocarbonate (DEPC), deionized formamide, Tris-(hydroxymethyl)-aminomethane (Tris) and morpholinopropane sulfonoic acid (MOPS), nitro blue tetrazolium chloride (NBT), 5-Bromo-4-chloro-3-indolyl phosphate (BCIP), Phenazine metabisulphate, were purchased from

AppliChem Chemical Company, Darmstadt, Germany. 1,2-dichloro-4-nitrobenzene (DCNB) was purchased from Fluka Chemical Company, U.S.A.

Taq Polymerase, dNTPs, Ribolock^{TM}, Mu-MLV Reverse Transcriptase were obtaind from Fermentas, Germany and primers were acquired from lontek Company, Merter, Istanbul. Primary antibodies for CAT, GPx and GST-Mu were obtained from Abcam (Cambridge, USA), MnSOD, GAPDH, AP-conjugated Anti Rabbit IgG were from Santa Cruz (USA) and Cu-ZnSOD were from Calbiochem (Darmstadt, Germany).

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

2.2. Animals and Induction of Diabetes

Male Wistar rats at the same age were randomly divided into five groups at the start of the experiment. In four groups diabetes was induced by single intraperitonal injection of Streptozotocin (50 mg/kg body weight) dissolved in 0.05M citrate buffer (pH 4.5) and blood glucose concentrations were checked by Accu-check-go blood glucose analyzer (Roche) and animals having blood glucose concentration higher than 200 mg/dl was considered as diabetics. After one week of diabetes, antioxidants were given by intraperitoneal injection. Experimental groups comprised the following groups: control group (n=9), untreated diabetic group (n=9), diabetic groups supplemented with DL-α-lipoic acid (50mg/kg body weight/day) (n=8), diabetic groups supplemented with vitamin C (50mg/kg body weight/day) (n=12) and diabetic groups supplemented with both DL-α-lipoic acid+ vitamin C (50+50 mg/kg body weight/day) (n=7). All experiments were carried out with the approval of local animal use ethical committee (Fırat University-Appendix F). The procedures involving animals and their care are conformed to the institutional guidelines (Giles, 1987). At the end of the 4 week growing period, rats were decapitated and livers were removed

and quickly frozen in liquid nitrogen and kept at -85^{o}C for subsequent biochemical analysis. For each tissue, an appropriate portion was homogenized in ice cold homogenization solution containing 1.15% (w/v) KCl , 5mM EDTA, 0.2mM PMSF, 0.2mM DTT, in 25 mM phosphate buffer, pH 7.4 using teflon glass homogenizer. Four ml homogenization buffer is added to each gram wet weight of tissue. All the 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. The homogenates were centrifuged at 1.500*g* and the supernatants were sampled for the determination of enzyme activities, reduced glutathione (GSH), lipid peroxidation (MDA), protein carbonylation, protein concentration and for western blot analysis.

Protein concentrations of homogenates were determined according to Lowry *et al.,* (1951). 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. Samples 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 (BSA) solutions (0.02, 0.05, 0.10, 0.15, and 0.20 mg/ml) were also prepared. In separate duplicate tubes, 0.5 ml diluted samples and standard BSA solutions were added.

Then to each tube, 2.5 ml Lowry ACR reagent [Reagent I (2% w/v $CuSO_4.5H_2O$) Reagent II (2% w/v Na-K Tartarate) and Reagent A (2% w/v Na_2CO_3 in 0.1 N NaOH) were mixed respectively with a ratio of 1:1:100 (v/v/v)] was added, mixed and incubated for 10 min at room temperature for copper reaction in alkaline medium. After that, 0.25 ml Folin Reagent (1N) was added to each tube and mixed immediately. 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 (Appendix C).

2.3. Determination of Antioxidant Enzyme Activities

2.3.1. Determination of Catalase Activity

The decomposition of hydrogen peroxide (H_2O_2) was followed directly by the decrease in absorbance at 240nm (ε_{240}=0.00394 L/mmol.mm), and the difference in absorbance per unit time was the measure of catalase activity. Aebi's method (1974) was used to determine catalase activities. Cytoplasmic fractions were diluted tem times with 1% (v/v) Triton X-100 and then, further 300 fold dilutions were done with 50 mM phosphate buffer (pH 7.0). After that, 2ml of enzyme solution was mixed with 1 ml 30 mM H_2O_2 and the rate of decomposition was followed at 240nm for 1 minute with Shimadzu 1601 UV/Vis double-beam spectrophotometer. One unit of catalase activity was defined as the amount of substrate (µmol) consumed in one min by 1 mg total protein.

2.3.2. Determination of Total, Cu-Zn SOD and Mn SOD Activities

Marklund and Marklund's (1974) method was used to determine the SOD activities. According to this method, superoxide radical, which is the substrate for SODs, 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.

In 3 ml plastic spectrophotometer cuvettes, 2.8 ml TE buffer (50mM-Tris, 10mM-EDTA, pH 8.2) and varied volumes (5, 10, 15, 20, 25, 30, 40, 50µL) of cytosolic fractions (1.500*g*) were added. Then, final volume was completed to 2.9 ml with TE buffer. Pyrogallol autoxidation was started with the addition of 100 µL pyrogallol (15 mM) and illumination, and the rate of autoxidation was followed at 440 nm for 1 min. Enzyme blank readings were measured (without cytosolic fractions) to determine uninhibited autoxidation. In all readings, reference cuvette contained only TE buffer.

Same procedures for the same samples were repeated but this time, TE buffer also contain 1 mM KCN, so that Cu-Zn SOD activities were inhibited and only Mn SOD activity could be determined.

Percent inhibition of pyrogallol autoxidation was calculated according to the equation given below for each protein amount that was added to the reaction medium.

$$\%inhibition = 100 \times \frac{\left(\frac{\Delta \mathrm{OD}}{\mathrm{min}}\right)\mathrm{blank} - \left(\frac{\Delta \mathrm{OD}}{\mathrm{min}}\right)\mathrm{sample}}{\left(\frac{\Delta \mathrm{OD}}{\mathrm{min}}\right)\mathrm{blank}}$$

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. After calculating total SOD activities by using TE buffer, and calculating the Mn SOD activities by using KCN containing TE buffer, Cu-Zn SOD activities were found by subtracting Mn SOD activities from total SOD activities.

2.3.3. Determination of Glutathione Peroxidase (GPx) Activity

GPx activity measurement was carried out by using Paglia and Valentine's method (1967) which was based on the measurement of degree of NADPH oxidation at 340nm with glutathione reductase which use oxidized glutathione and NADPH as substrates. Since oxidized glutathione is produced by GPx, the degree of NADPH oxidation is directly proportional to GPx activity.

In 3 ml quartz cuvettes, 2.525 ml Tris buffer (0.1M, pH 8.0), 75 µL GSH (80 mM), 100 µL glutathione reductase (0.24 U), 100 µL 40 fold diluted cytosolic fraction, 100 µL NADPH (2 mM) were mixed and incubated for 3 min at room temperature. Then, enzymatic reaction was initiated with the addition of 100 µL hydrogen peroxide (1.5 mM), and the rate of disappearance of NADPH at 340nm (ε_{340}=6220 $M^{-1}.cm^{-1}$) was followed for 3 min. Glutathione peroxidase activity was then described as the amount of NADPH consumed in one minute by 1 mg protein containing cytosolic fraction.

2.3.4. Determination of Glutathione S-Transferase (GST) Mu Class Isozyme Activity

1,2-Dichloro nitrobenzene (DCNB) is a substrate for GST-Mu isozyme and GST-Mu catalyzed GSH oxidation was monitored by the increase in the absorbance at 345nm due to the colored adduct formation (Habig *et al.,* 1974).

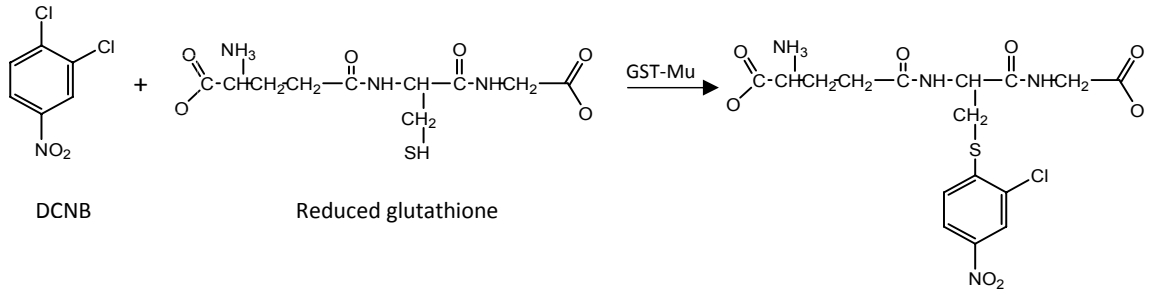


Figure 2.1: Mechanism of GST-Mu catalyzed reaction

Into the 3 ml quartz cuvette, 2500 µL potassium phosphate buffer (100 mM, pH 7.5), 200 µL GSH (75 mM), and 150 µL10 fold diluted cytosol were added and the reaction was started with the addition of 150 µL 20 mM DCNB. Then, thioether formation was followed at 345 nm for 2 min. Each time, there were enzyme blank readings (reaction with no cytosol) for subtracting non-enzymatic product formation from the GST assay. Then, the enzyme activity was calculated as the amount of thioether (nmol) formed by 1 mg total protein in one minute by using 0.0085 $\mu M.cm^{-1}$ as an extinction coefficient of thioether formed by GST.

2.4. Determination of Cellular GSH Levels and Oxidative Modifications of Lipids and Proteins

2.4.1. Determination of Reduced Glutathione (GSH) Concentration

GSH determination was carried out 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 (Sedlak and Lindsey, 1968). According to method, the ability of SH groups to reduce DTNB to form 1 mole of 2-nitro-5-mercaptobenzoic acid (yellow) per mole of SH group were evaluated. To the 0.25 ml appropriately diluted cytosolic fractions, or GSH standard solutions ranging between 0.1 to 1 mM, 0.75 ml TE buffer (0.2M Tris, 20 mM EDTA, pH: 8.2) 0.05 ml 10 mM DTNB and 3.95 ml methanol were added and thoroughly mixed. Then, tubes were incubated at room temperature for 30 min with occasional shaking. After incubation, the samples were centrifuged at 3.000*g* for 15 min to remove proteins having SH group which reacts with DTNB. GSH standard calibration curve was drawn and the slope was used to determine GSH concentration. The results were expressed as nmol GSH per mg protein.

2.4.2. Determination of Lipid Peroxidation (MDA Measurement)

Malonedialdehyde (MDA), which is the end product of lipid peroxidation reactions, gives reaction with thiobarbituric acid (TBA) to form a colored complex whose absorbance can be read at 532 nm (Jain and Levine, 1995). Two hundred microliters of microsomal suspension was mixed with 0.8 ml PBS (18 mM NaCl, 18 mM Na_2HPO_4, pH 7.4) and 0.025 ml of BHT (0.04 M in ethanol) to prevent artificial increase of MDA during the experiment. Then, 0.5 ml TCA (30%) was added and tubes were allowed to stand in ice for at least two hours with occasional shaking. After incubation, tubes were centrifuged at 2.000*g* for 15 min and 1 ml of each supernatant was transferred to another tube containing 0.075 ml EDTA (0.1 M) and 0.25 ml TBA (1% (w/v) in 0.05N NaOH). 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 532 nm. Finally, MDA concentrations were determined by using extinction coefficient of colored complex as 1.56×10^5 $(M.cm)^{-1}$ and expressed as nmol MDA for one mg of protein containing supernatant.

2.4.3. Determination of Carbonyl Contents on Proteins

A spectrophotometric assay (Levine *et al.*, 1990) 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 360 nm.

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 280 nm to that at 260 nm is greater than one (Levine *et al.*, 1990). Whenever the ratio of 280/260 nm is less than one, samples were incubated with 10% (w/v) streptomycin sulfate until a final concentration of 1% streptomycin sulfate is reached. Test tubes were left at room temperature for 15 min and then centrifuged at 6.000*g* for 10 min to remove insoluble nucleic acid precipitates.

After the removal of nucleic acid contaminants, to the 200 µL protein solution, 800 µL DNPH solution (10 mM in 2M HCl) 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 min. After incubation, 500 µL of 30% (w/v) Trichloroacetic acid were added and samples were incubated in ice for further 10 min, then tubes were centrifuged at 11.000*g* for 5 min and supernatant was discarded. Resulting pellets were washed 3 times with 1.5 ml ethanol-ethyl acetate (1:1) in order to remove unbound reagent (by mixing and centrifugation at 11.000*g* for 5 min and discarding the supernatant each time). Then, the precipitated proteins were redissolved in 1.5 ml guanidine hydrochloride (6 M in 20mM phosphate buffer, pH 2.5) solution. Proteins usually redissolve within 45 min at room temperature. Any insoluble materials were removed by centrifugation in the microcentrifuge for 5 min at 11.000*g* and absorbances at 360nm were read against blanks. Then, carbonyl contents of the proteins were calculated by using a molar absorption coefficient of $2.2x10^4$ lt/mol.cm.

2.5. Determination of Antioxidant Enzyme mRNA Expressions

2.5.1. Isolation of Total RNA from Rat Liver Tissues

Total RNAs were isolated from the liver tissues according to Chomczynski and Sacchi's (1987) method based on differential extraction of RNAs by organic solvents. Proteins are denaturated by strong denaturating agent, Guanidine isothocyanide, and DNAs are selectively fractionated from RNAs by phenol at acidic pH.

Before starting RNA isolation, to decrease the possibility of RNA degradation during the procedure, all glassware and plastics were treated by 0.1% DEPC (Diethyl pyrocarbonate) solution overnight and then autoclaved and dried in oven which converts DEPC into CO_2 and ethanol. Furthermore, all solutions were DEPC treated or prepared by using 0.1% DEPC treated water.

According to the method, approximately 100 mg liver tissue were minced on ice and homogenized (at room temperature) with 1 ml of solution D (4M Guanidine isothiocyanide, 25 mM sodium citrate,pH 7.0, 0.5% (w/v) L-lauryl sarcosine, 0.1 M 2-mercaptoethanol) in a glass-teflon homogenizer and subsequently transferred into a 15 ml polypropylene tube. Sequentially, 0.1 ml of 2M sodium acetate (pH 4.0), 1 ml of phenol (water saturated, acidic pH), 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension were shaken vigorously for 10 s and cooled on ice for 15 min. Then, samples were centrifuged at 10.000*g* for 20 min at 4°C, and after that, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 volume of ice cold isopropanol, and then placed at -20°C for at least 30 min to precipitate RNA. Sedimentation at 10.000*g* for 20 min was again performed and the resulting RNA pellet was redissolved in 0.3 ml of

solution D, transferred into a 1.5 ml eppendorf tube, and precipitated with 1 volume of isopropanol at -20°C for 30 min. After centrifugation at 10.000*g* for 10 min at 4°C, the RNA pellet was resuspended in 1 ml 75% ethanol, sedimented, dried in oven (60-65^{o}C, 15 min), and dissolved in 50 µL DEPC treated pyrogen free water or deionized formamide (for long term storage) at 65^{o}C for 10 min. Formamide provides a chemically stable environment that also protects RNA against degradation by RNases. Purified, salt-free RNA dissolves quickly in formamide up to a concentration of 4 mg/ml. At such concentrations, samples of the RNA can be analyzed directly by gel electrophoresis and RT-PCR, saving time and avoiding potential degradation (Sambrook and Russell, 2001). If necessary, RNA can be recovered from formamide by precipitation with 4 volumes of ethanol as described by Chomczynski (1992).

2.5.2. Spectrophotometric Analysis of Total RNA

Seven microliters of total RNA isolate was diluted with 693 µL of TE buffer,pH 8.0 (10mM Tris, 1mM EDTA pH 8.0) in a quartz cuvette. Then, the absorbance of the solution was measured at 260 and 280 nm using TE buffer as blank. The purity of the isolated RNA was determined by taking the ratio of A_{260} and A_{280} readings. The optimal value for RNA purity is accepted to be between 1.9-2.2 (MacDonald *et al.,* 1987). Forty µg/ml solution of single stranded RNA gives absorbance of 1 and we can calculate the concentration of the RNA in our sample as follows:

$$RNA\ conc.(\mu g/ml) = (OD\ 260)\ x\ (dilution\ factor)\ x\ (40\ \mu g\ RNA/\mu l)$$

Results of the spectrophotometric measurement of total RNAs were given in Appendix D.

2.5.3. Electrophoresis of RNA

Integrity of the RNA isolates was checked with denaturing agarose gel electrophoresis. RNA requires different techniques from DNA for electrophoretic separations. Because single-stranded RNA can form secondary structures, electrophoresis should be carried out in a denaturing system. RNA must be denatured prior to loading into the gel and the electrophoresis conditions should maintain the denatured state.

As in all work with RNA, it is important to minimize RNase activity when running agarose gels. Electrophoresis apparatus should never be directly exposed to DEPC because acrylic is not resistant to DEPC. Therefore in order to sterilize electrophoresis apparatus, we cleaned it with detergent solution first, then rinsed with distilled water and dried with ethanol. After that, gel apparatus was filled with 3% hydrogen peroxide and soaked for further 10 min and then finally rinsed thoroughly with DEPC-treated water.

Formaldehyde agarose gels with MOPS buffer (200 mM MOPS pH 7.0, 50 mM Sodium acetate, 10 mM EDTA) in which RNAs are strongly denatured are the most commonly used system for RNA electrophoresis to check the RNA integrity (Sambrook and Russell, 2001). Formaldehyde forms unstable Schiff bases with the single imino group of guanine residues. These adducts maintain RNA in the denatured state by preventing intrastrand Watson-Crick base pairing. Because the Schiff bases are unstable and easily removed by dilution, RNA can be maintained in the denatured state only when formaldehyde is present in the buffer or gel.

One percent of agarose gel containing 0.5 µg/ml EtBr was prepared in 1X MOPS buffer and 6% Formaldehyde to have denaturating conditions. A preelectrophoresis before loading samples was carried out for 20 min. Ten microliters of total RNA was mixed with 2 µl of loading solution (50% glycerol,

10mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF) and loaded onto the gel. Electrophoreses was carried out at a maximum of 5V per 1 cm of distance between electrodes until bromophenol blue has traveled at least 80% of the way through the gel. The RNA bands were visualized under UV transilluminator and were photographed. Typical markers of RNA quality were observed as sharp and intense bands of 18S (~1900bases) and 28S (~4800 bases) rRNA subunits. DNA contamination of the RNA migrated as high molecular weight ethidium-bromide staining material. Degradation of the RNA was evidenced by smearing of the ribosomal RNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band (Figure 2, lane 3). The 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA showed a smeared appearance, lacked the sharp rRNA bands, or did not exhibit a 2:1 ratio. Completely degraded RNA appeared as a very low molecular weight smear (Figure 2.2, lane 2).

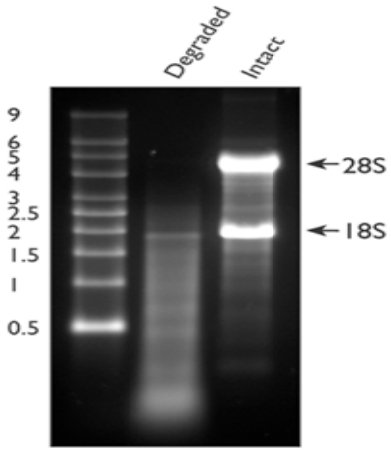


Figure 2.2: Intact vs. Degraded RNA. The 18S and 28S ribosomal RNA bands were clearly visible in the intact RNA sample. The degraded RNA appeared as a lower molecular weight smear (Ambion, USA)

2.5.4. Complementary DNA (cDNA) Synthesis and Multiplex RT-PCR Amplification of Antioxidant Enzymes

cDNA synthesis was carried out by using M-MuLV Reverse transcriptase (MBI Fermentas, USA). In the reverse transcription reaction, 1 µL oligo$(dT)_{15}$ primer was added for 1 µg total RNA used. Then, the volume was completed to

12 µL with DEPC-treated water. Afterwards, the mixture was gently mixed and incubated for 5 min at 70^{o}C and chilled on ice. Then 4 µL of 5X M-MuLV reaction buffers and 1 µL of RiboLock^{TM} (20u/µL) (MBI Fermentas, USA) were added. After the addition of 2 µL 10 mM dNTP mix, tubes were incubated at 37^{o}C for 5 min. Finally, 1 µL of M-MuLV RT (200U/µL) is added and reaction was carried out at 42^{o}C for 1 hour and stopped at 70^{o}C for 10 min with denaturation of reverse transcriptase. Finally, cDNA mixture was chilled on ice and stored at ambient temperature until subsequent PCR reactions.

Multiplex PCR was performed in order to amplify the gene of interest (Cu-Zn SOD, Mn SOD, CAT, GPx and GST-Mu) and the internal control gene (β-actin) simultaneously. It employs different primer pairs in the same amplification reaction so, each time more than one gene could be amplified. The primer pairs used for amplification of cDNAs of antioxidant enzymes and β-actin, are shown at Table 2.1.

Table 2.1: Primer sequences and expected product sizes of antioxidant enzymes and internal standards

cDNA	Forward primer sequence	Reverse primer sequence	Expected Product Size (bp)
β-actin	5′-CCTGCTTGCTGATCCACA	5′-CTGACCGAGCGTGGCTAC	500 bp
CuZnSOD	5′-GCAGAAGGCAAGCGGTGAAC	5′-TAGCAGGACAGCAGATGAGT	450 bp
MnSOD	5′-GCACATTAACGCGCAGATCA	5′-AGCCTCCAGCAACTCTCCTT	240 bp
GPx	5′-CTCTCCGCGGTGGCACAGT	5′-CCACCACCGGGTCGGACATAC	290 bp
CAT	5′-GCGAATGGAGAGGCAGTGTAC	5′-GAGTGACGTTGTCTTCATTAGCACTG	670 bp
GST-Mu	5′-AGAAGCAGAAGCCAGAGTTC	5′-GGGGTGAGGTTGAGGAGATG	450 bp

Cu-Zn SOD, CAT, GPx, β-actin (Limaye *et al.,* 2003), Mn SOD (Han *et al.,* 2005), and GST-Mu (Hoen *et al.,* 2002) primer pairs were taken from the literature. Specificity of the products of the RT-PCR amplification was confirmed with DNA sequence analysis shown in Figure 2.3.

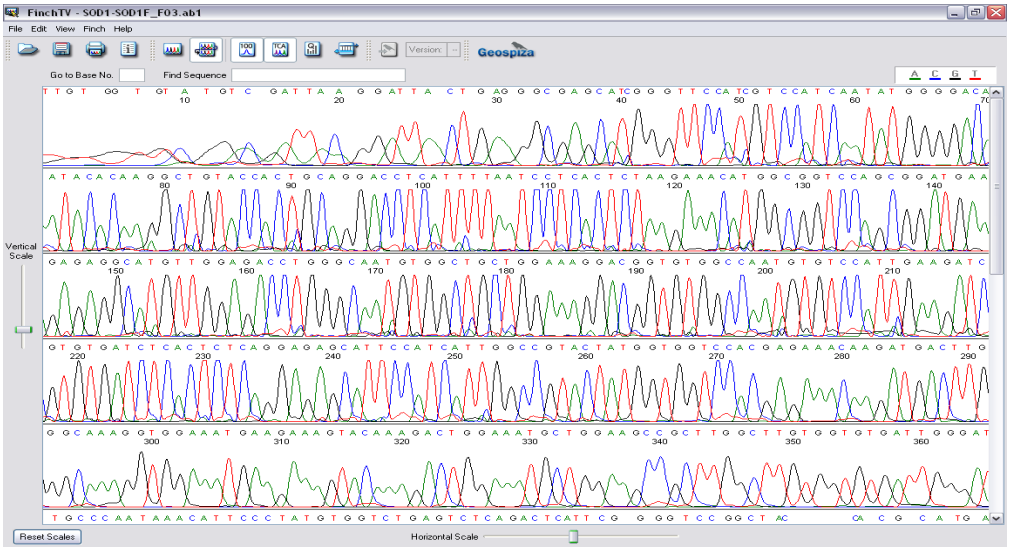


Figure 2.3: An example chromatogram obtained from the sequence analysis of Cu-Zn SOD.

After the DNA sequence analysis, the obtained sequences were cross checked with Bioinformatics Tools for Similarity & Homology EMBL-EBI Database Searching and specificity of the PCR products was validated. Obtained sequences and the results of the comparisons of these sequences with the sequences published before are shown in Appendix E. According to results, we obtained at least 96% homology for all enzymes in between sequences obtained and sequences published before.

For the RT-PCR amplification of antioxidant enzymes, 1 µL of cDNA mixture was amplified in a 25 µL of PCR reaction mixture containing 1X reaction buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTP (each), 1.0 mM of each primer and 2.5 Unit Taq polymerase. PCR program was set for initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 45 sec (28 times repeated) and final extension at 72°C for 5 min. The template concentration and the cycle number were all optimized to ensure linearity of response and to avoid saturation of the reaction. After the reaction was completed, PCR products were mixed with 6X loading dye and run on 2 %

agarose gel. After agarose gel electrophoresis, intensities of bands shown in Figure 2.4 were measured with Image J software (Rasband, 2008). The intensities of the bands were converted into peaks by the software and antioxidant enzyme gene expressions were calculated from the area under these peaks.

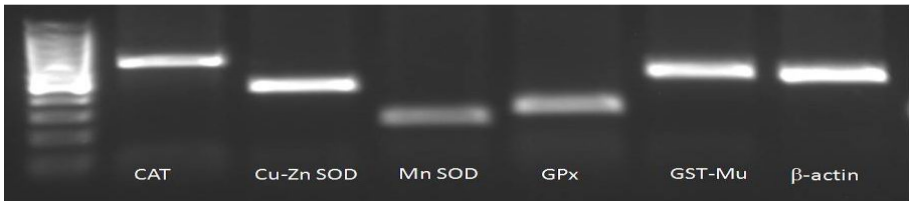


Figure 2.4: Agarose gel electrophoresis bands obtained from the products of the RT-PCR amplification reaction of the antioxidant enzymes from a control tissue.

2.6. SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis of Antioxidant enzymes

Polyacrylamide gel electrophoresis was performed with anionic detergent SDS on 4% stacking gel and 12% separating gel. EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, USA) was used for gel preparation and electrophoresis. Preparation of the stacking and separating gels are summarized at Table 2.2.

Table 2.2: Formulations for SDS-PAGE separating and stacking gels of EC120 Mini Vertical Gel System.

	Seperating Gel	**Stacking Gel**
Monomer Concentration	**12 %**	**4 %**
Acrylamide Solution*	2 ml	0.415 ml
Distilled water	1.65 ml	1.7 ml
1.5 M Tris-HCl, pH 8.8	1.25 ml	----
0.5 M Tris-HCl, pH 6.8	----	0.315 ml
10% (w/v) SDS	50 µL	10 µL
10 % APS	25 µL	10 µL
TEMED	2.5 µL	2.5 µL

**Acrylamide solution (4M Acrylamide+0.05M N,N'-methylenebisacrylamide) was prepared by dissolving 30gr acrylamide and 0.8gr N,N'-methylenebisacrylamide in a final volume of 100mL. Then, solution is filtered through a filter paper and stored at +4^{o}C in a dark bottle.*

Samples were diluted with 1 % Triton X-100 prepared in 0.5 M Tris buffer at pH 6.8 and mixed with sample dilution buffer (0.25 M Tris buffer, pH 6.8, 8 % SDS, 40 % Glycerol, 20 % 2-mercaptoethanol, 0.004 % Bromophenol Blue) with a ratio of 3 to 1 respectively. Then 10 µg of total protein (except 20 µg for GST-Mu and 50 µg for GPx) was loaded into each well. Then, samples were run in stacking gel at 50 V and 100 V in separating gel. Electrophoresis was carried out till the dye Bromphenol Blue reached 1 cm above the bottom of the gel. For Western Blot Analysis, after electrophoresis the proteins were transferred from the gels onto PVDF membranes (Towbin *et al*., 1979) by using EC140 Mini Blot Module of the EC120 Mini Vertical Gel System (E-C Apparatus Corp., NY, U.S.A.) through electroblotting. The membranes were then washed with TBST buffer (500 mM NaCl, 20 mM Tris buffer at pH 7.4, 0.05 % Tween-20) several times and blocked with 5% non-fat dry milk (dissolved in TBST) for 1 hour at RT or overnight at +4^{o}C. Then membranes were washed with TBST again and incubated with primary antibodies for antioxidant enzymes and/or internal standard (GAPDH) for 2 h with constant shaking. After primary antibody incubation, the membranes were washed with TBST 5x5 min and alkaline phosphatase conjugated secondary antibodies are applied for 1 h in 5% milk TBST with constant shaking. After secondary antibody incubation, the membranes were washed with TBST 5x5min. The information about the primary and secondary antibodies used in the experiments is summarized at Table 2.3.

Table 2.3: Antibodies used in the experiments, their brands and the optimum dilutions for Western Blot Analysis.

	Primary AB	Primary AB Dilution	Brand	Secondary AB	Secondary AB Dilution	Brand
Catalase	Rabbit Anti-CAT	6.000	Abcam	Anti-Rabbit IgG-AP Conj.	10.000	Abcam
CuZnSOD	Sheep Anti CuZnSOD	2.000	Callbiochem	Anti-Sheep IgG-AP Conj.	30.000	Sigma
MnSOD	Rabbit Anti MnSOD	750	Santa Cruz	Anti-Rabbit IgG-AP Conj.	10.000	Abcam
GPx	Rabbit Anti GPx	2000	Abcam	Anti-Rabbit IgG-AP Conj.	10.000	Abcam
GST-Mu	Rabbit Anti GST-Mu	10.000	Abcam	Anti-Rabbit IgG-AP Conj.	10.000	Abcam
GAPDH	Rabbit Anti GAPDH	2000	Santa Cruz	Anti-Rabbit IgG-AP Conj.	10.000	Abcam

For visualization, BCIP/NBT solution [40 ml of solution A (0.1 M Tris buffer at pH 8.8, 0.1 M NaCl, 0.0024 % diethanolamine, 2 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.3 mg/ml NBT) was mixed with 0.268 ml phenazine methosulfate (2.0 mg/ml) and 0.136 ml of BCIP (40 mg/ml)] was overlaid on the membranes for 15 min at dark. Then, the membranes were washed with distilled water several times and air dried. Intensities of bands corresponding to antioxidant enzymes and reference protein were measured with Image J software (Rasband, 2008). Intensities were proportional to the amount of desired protein present.

2.7. Statistical Analysis

Differences in measured parameters between normal, diabetic and antioxidant supplemented animals were assessed by the Student T-test with the help of MINITAB 12.1 statistics software. Data were expressed as mean $\pm$ standard error of mean (SEM). The relationships between oxidative parameters characterizing diabetic and control rat liver status were analyzed and a probability of 0.05 and 0.005 was set as the level of statistical significance.

CHAPTER 3

RESULTS

Increased oxidative stress and impaired antioxidant defense mechanisms are important factors contributing to the pathogenesis and the progression of diabetes mellitus. For this reason, we studied the effects of the diabetes on the gene expressions of antioxidant enzymes which play important roles in the determination of the oxidative status of the organisms. Furthermore we studied the effects of two strong antioxidants, water soluble, vitamin C and lipid soluble, DL-α-lipoic acid on the system in order to understand the regulation of the molecular mechanisms.

Animal models of the type 1 diabetes mellitus were produced by single intraperitonial application of streptozotocin (STZ) at a dose of 50 mg/kg body weight. This increased the blood glucose concentration of the animals above the 200 mg/dl at which we accept that diabetes was induced. Blood glucose concentrations and weights of animals throughout the 4 weeks of diabetic period were measured weekly and given in Appendix A and B. According to our results, it is clear that diabetes reduced the average body weights of the animals below the control values and application of neither antioxidant restored this decrement. Decrease in body weight seen in diabetes could be due to use of metabolic fuels other than glucose such as fats and body proteins, as diabetic animals could not use glucose due to lack of insulin and/or its action. Antioxidant treatments were done with single dose of DL-α-lipoic acid, 50 mg/kg body

weight/day and Vitamin C, 50 mg/kg body weight/day and combination 50+50 mg/kg body weight/day of LA and VC. Dose response curves were studied in a previous study and this concentration of both antioxidants were found to be effective in regenerating at least some of the negative effects of diabetes.

Before measuring the oxidative stress parameters (like MDA, protein carbonyls, GSH) and gene and protein expressions of the antioxidant enzymes, namely, Superoxide Dismutase (Cu-Zn and Mn containing), Glutathione Peroxidase (GPx), Catalase (CAT), Glutathione S-Transferase Mu isoform (GST-Mu), several optimization studies for the RT-PCR experiments and Western analysis were carried out by using control rat liver tissues. Results of the optimization studies are given in the following paragraphs. Antioxidant enzyme activities were measured according to the previously optimized conditions (Sadi, 2004).

After optimizing the conditions, antioxidant enzyme mRNA and protein expressions and enzymatic activities were determined in all control, diabetic, LA treated diabetic, VC treated diabetic and combination (LA+VC) treated diabetic rat liver tissues. Also the oxidative parameters were determined and the results are given in separate Tables.

3.1 Optimization studies for the determination of mRNA expressions of antioxidant enzymes

3.1.1 Isolation of total RNAs from rat liver tissues.

After isolation of total RNAs by the GTC method, denaturing agarose gel electrophoresis was carried out in order to check the quality and intactness of purified RNAs. As shown in Figure 3.1, upper band represents 28S large ribosomal subunit, and second band represents 18S ribosomal small subunit. The band present at the bottom of the gel corresponds to the mixture of tRNAs and

5S ribosomal RNAs. A smear appearance instead of intact bands indicates the degradation of RNA by the contaminated RNAse during the isolation procedure. Hence, intact bands shown in the figure indicate that total RNAs were isolated successfully from the liver tissues without any sign of degradation.

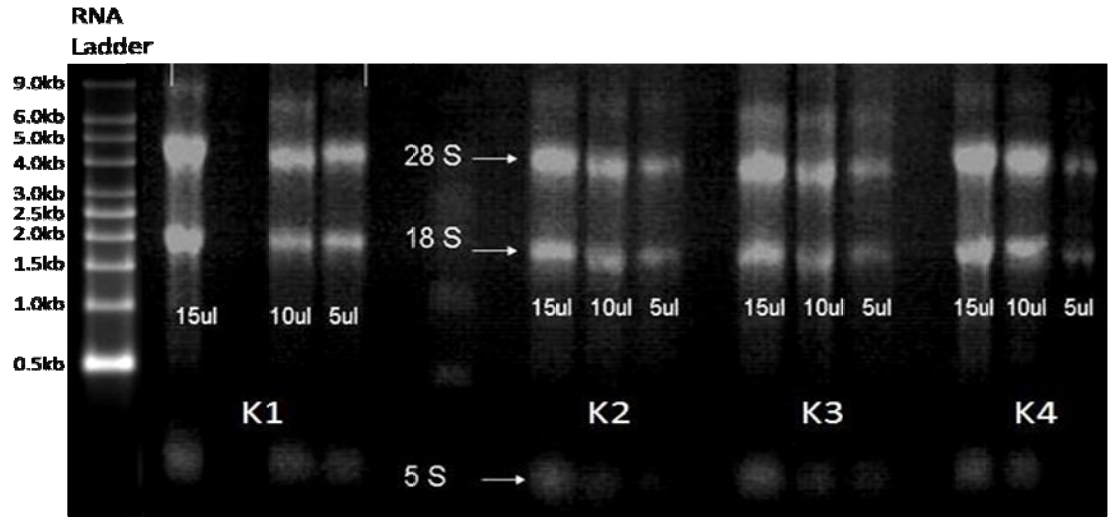


Figure 3.1: Isolated total RNAs from control rat liver tissues. Samples were run on 1.0 % formaldehyde agarose gel electrophoresis.

3.1.2 Determination of RNA purity and concentration

After formaldehyde agarose gel electrophoresis, the concentrations and the purity of the isolated total RNAs were determined by spectrophotometric measurements at 260nm and 280nm (Appendix D). The ratio of OD_{260}/OD_{280} should be between 1.8-2.0 to ensure the lack of protein or DNA contamination.

3.1.3 Optimization of total RNA amount for the cDNA synthesis and PCR reactions

The results of the optimization studies showed that the amount of RNA template strongly influenced the outcome of the RT-PCR reactions. When the amount of RNA available was very low, we adapted the reaction or cycling conditions and modified them in order to allow reaction to work efficiently. For the reverse transcription reactions, three different amounts of starting total RNAs (0.5, 1.0, 2.0 µg) were tried and the amplification with 30 cycle was found to be sufficient to observe the corresponding bands on agarose gels as shown in Figure 3.2.

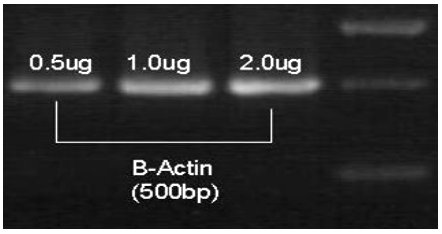


Figure 3.2: Optimization of total RNA concentrations for cDNA synthesis and subsequent RT-PCR reactions.

As seen from the gel images starting with 1ug of total RNA for the β-actin gene was sufficient in PCR amplification reactions at 30 cycles. So, 1 µg of starting total RNA amount was used for the rest of the experiments for the determination of mRNA expressions of all antioxidant enzymes.

3.1.4 Cycle optimizations for cDNA amplification of antioxidant enzymes

Cycle optimizations are important in RT-PCR experiments since in order to make quantification, PCR products should be in linear phase of amplification. At the plateau, no matter how much initial mRNA is present, all the intensities would be the same. This non-proportionality is due to a so-called *plateau effect*, which is the attenuation in the exponential rate of product accumulation in late

stages of a PCR. The effect is shown in Figure 3.3. This may be caused by degradation or depletion of reactants (dNTPs, enzyme); former a problem with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated product (Innis and Gelfand, 1990).

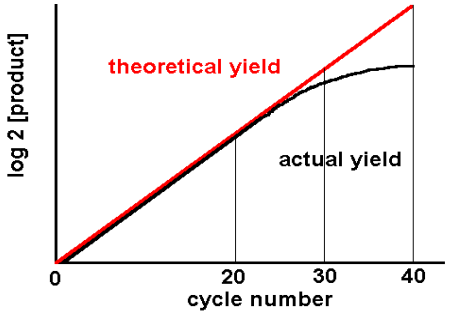


Figure 3.3: Plateau effect in PCR amplification

Cycle optimization experiments for the amplification of antioxidant enzyme mRNAs from the cDNAs synthesized from 1µg of total RNA were carried out and the PCR cycles ranging from 20 to 45 were tried for all primer pairs given previously in Table 2.1. After making serial amplification reactions and agarose gel electrophoresis of the products, densitometric analysis of the bands to measure their intensities were carried out by using imageJ software. Figure 3.4 shows how antioxidant enzyme amplification reactions responded to each PCR cycle.

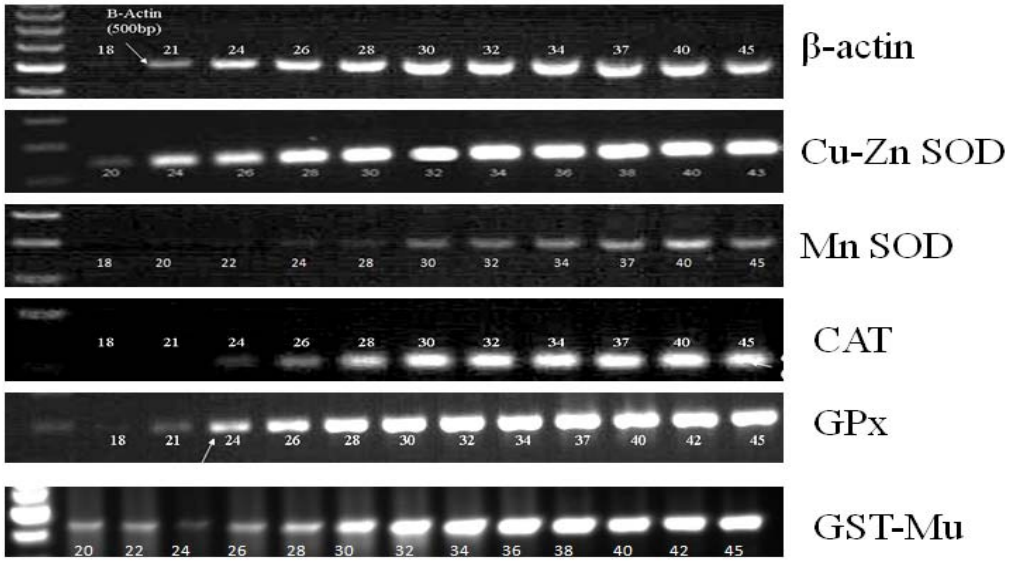


Figure 3.4: Optimization of PCR cycles. The amplification of a control cDNA which was synthesized by using 1µg total RNA and all antioxidant enzyme specific primers at different PCR cycles to find the cycle at which amplification is in linear phase.

Cycle number versus band intensity curves are given in Figure 3.5. As seen from the figure, at cycle number 28, amplification reactions of all antioxidant enzymes and the internal standard, β-actin were in the linear phase of the amplification and the bands could easily be detected by agarose gel electrophoresis.

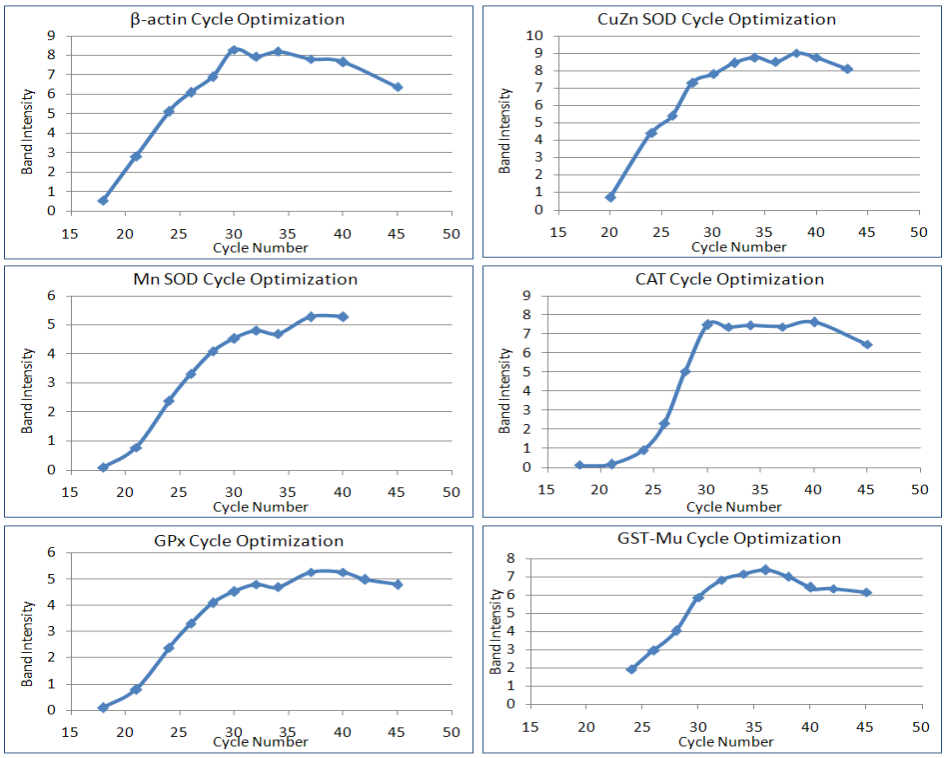


Figure 3.5: Cycle number versus band intensities curves obtained for all antioxidant enzymes to find the cycle number at which intensity is directly proportional to starting cDNA and hence mRNA amounts.

For the ease of application, in our experiments we chose 28 as the common cycle number for all PCR amplification reactions for all enzymes. In the rest of the experiments the amplification of each antioxidant enzyme and the internal standard carried out simultaneously, in the same multiplex reaction mixture. The changes in the mRNA expressions of antioxidant enzymes were calculated by using the internal standard used in the multiplex reactions.

3.1.5 Optimization of annealing temperatures for PCR reactions

One of the important parameter in PCR amplification reactions is the annealing temperature. Since formation of DNA/DNA or DNA/RNA complexes, e.g., binding of a primer to a template, requires a temperature which should be lower than the extension temperature 72^{o}C. Moreover, the flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients (especially in multiplex PCR). Since primer sequence of each antioxidant enzymes are different from each other, the efficiency of primers to anneal to the cDNA sequence of interests can vary with temperature. Also the optimization of the annealing temperature is especially critical when long products are synthesized or when total genomic DNA is the substrate for PCR. The optimal annealing temperature depends on the melting temperature of the primer-template hybrid. If the temperature is too high the primers will not anneal efficiently, and if the annealing temperature is too low the primers may anneal nonspecifically. For this reason the annealing temperatures for PCR reactions of all antioxidant enzymes were determined separately. Figure 3.6 shows the results of the amplification reactions of the all antioxidant enzymes at different annealing temperatures ranging between 55 to 65^{o}C. According to this Figure, it is clear that changing the annealing temperature has subtle effect on amplification reactions of all enzymes amplified by using control cDNAs with 28 cycles. We chose 58^{o}C as common annealing temperature for the amplification reactions of all antioxidant enzymes to make the multiplex reactions possible.

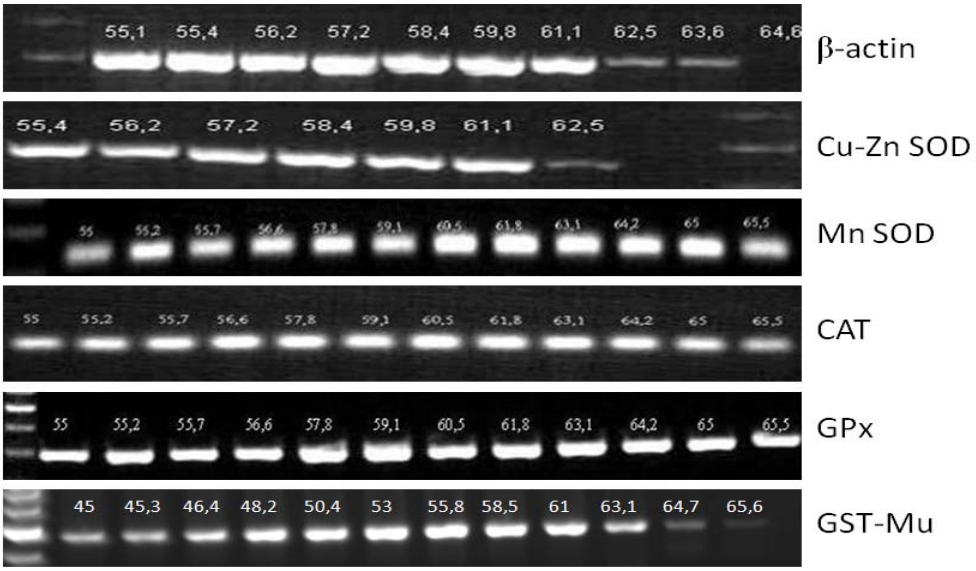


Figure 3.6: Optimization of annealing temperatures. Control cDNAs were synthesized by using 1µg total RNA and antioxidant enzyme specific primers at different PCR annealing temperatures were used for amplifications.

3.1.6 Optimization of annealing times for PCR reactions

Annealing time is another important parameter which should be controlled. So for each antioxidant enzyme and housekeeping gene 20, 30, 45 and 60 seconds annealing time was tried. We observed very little variation in the efficiency of annealing depending on time (Figure 3.7). As it can be seen from the figure, 30 seconds annealing time was sufficient and used as optimum annealing time for all antioxidant enzymes. Increasing the annealing time to 1 minute did not appreciably influence the outcome of the PCR reactions. Since, longer annealing times may increase the likelihood of unspecific amplification products; 30 sec was used as an annealing time for all antioxidant enzymes.

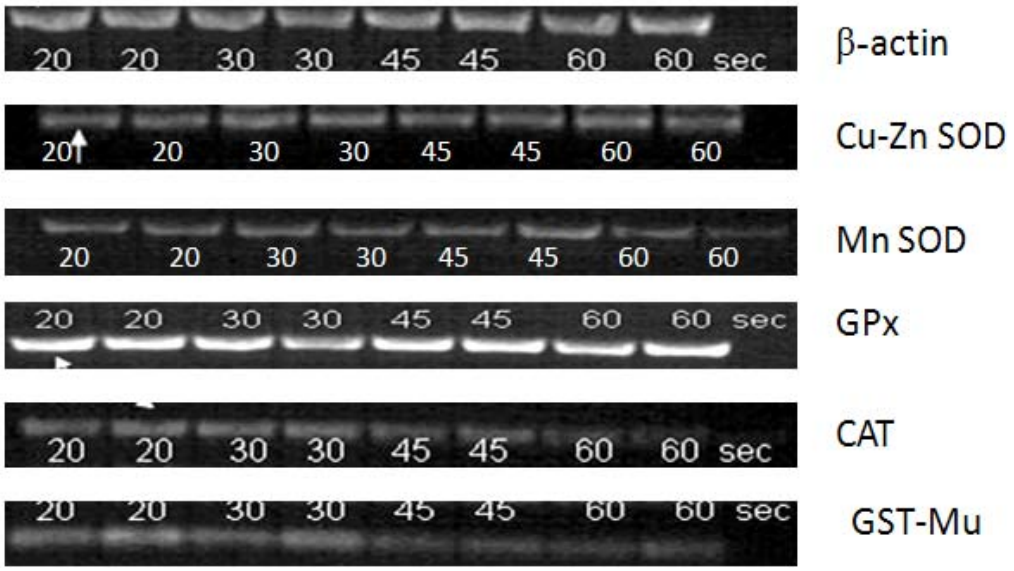


Figure 3.7: Annealing time optimizations. Different annealing times of 20, 30, 45 and 60 seconds were used for the PCR reactions of all antioxidant enzymes.

3.1.7 Optimization of primer concentrations for PCR reactions

The amount of DNA primer(s) available during the PCR reaction may influence efficiency of the amplification such that; too high primer concentrations may inhibit the reactions whereas too low amounts may not be sufficient enough to carry out the PCR reaction. In order to optimize primer amount, different primer concentrations between 0.3-2.0mM were tested for only Cu-Zn SOD and β-actin gene amplifications. The results of the experiments showed that, changing primer concentration did not affect the quality of amplicons significantly (Figure 3.8). Only 0.3 mM Cu-Zn SOD primer was most probably not sufficient for the PCR reaction as no visible band was observed. Therefore, 1 mM which was used also in literature was selected as optimum primer concentration for all antioxidant enzymes and the housekeeping gene.

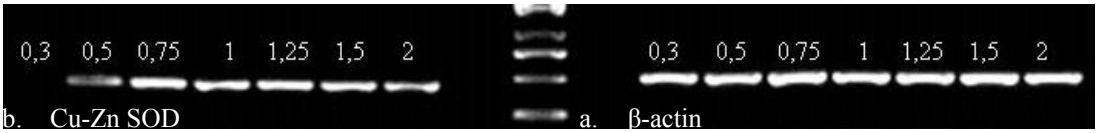


Figure 3.8: Primer concentration optimization for (a) Cu-Zn SOD (left), and b) β-actin (right), carried out at different primer concentrations ranging from 0.3mM to 2mM final concentrations.

3.1.8 Optimization of $MgCl_2$ concentration for PCR reactions

Mg^{+2} ions in PCR reactions have two important functions; promoting DNA/DNA interactions and form complexes with dNTPs which are the substrates of Taq Polymerase enzyme. In literature, optimum Mg^{+2} concentration curve had a broad maximum. But when Mg^{+2} was too low, primers were failed to anneal to the target DNA. Furthermore, when Mg^{+2} concentrations were too high, the base pairing became too strong so that the amplicon failed to denature completely during the denaturation step at 94°C (Henegariu *et al*., 1997). Higher concentrations of $MgCl_2$ also inhibit the polymerase activity, decreasing the amount of all products. So, in order to obtain sufficient amount of PCR products, magnesium concentration was tested between 0.5 to 4mM. Except 0.5mM concentration for β-actin, the amount of PCR products did not change significantly with different $MgCl_2$ concentrations. Like the literature, 2.5mM $MgCl_2$ was selected as the working concentration for all antioxidant enzyme and β-actin gene amplifications (Figure 3.9).

Figure 3.9: $MgCl_2$ concentration optimization for (a) Cu-Zn SOD (left) and (b) β-actin (right). 0.5 to 4 mM Mg^{+2} concentrations were tested.

After the optimization reactions, as a result, in all the RT-PCR experiments, cDNAs were synthesized from 1 µg of total RNA by using Mu-MLV reverse transcriptase and amplified in a 25 µl reaction mixture containing 1X reaction buffer (75mM Tris-HCl pH:8.8 , 20mM $(NH_4)_2SO_4$, 0.01% Tween-20), 2.5mM $MgCl_2$, 0.1mM dNTP (each), and 1.0mM of each primer. Into the reaction mixture 2.5 Unit Taq polymerase was added and the PCR program was used as follows; initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 45 sec (28 cycle) and final extension at 72°C for 5 min. After using these optimized conditions in the reactions, the PCR products of all antioxidant enzymes are visualized as shown in Figure 3.10.

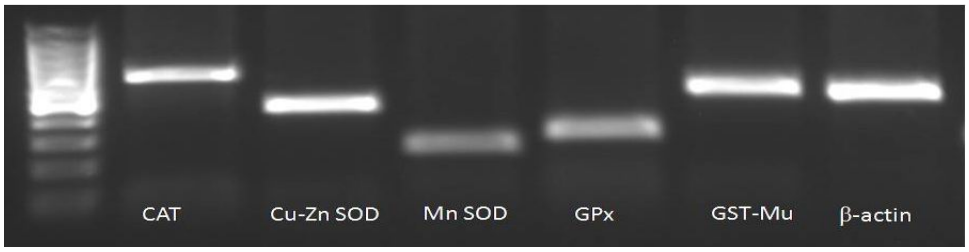


Figure 3.10: The gel images of the RT-PCR products of antioxidant enzymes after serial optimization studies.

3.2 Optimization of Western-blot analysis

Western-blot analysis was carried out in order to detect the differences in the production of an individual antioxidant enzyme under specific conditions. Before the analysis, all Western-blotting protocols including the antibody concentrations were optimized. Optimization allows us to achieve maximum sensitivity and consistency. Three parameters, primary antibody concentration, secondary antibody concentration, and total protein amounts that could be applied to the gel for the electrophoresis are very important parameters which can affect the results of the whole analysis. Appropriate primary and secondary

antibody concentrations depend on each antibody's specific activity and specificity for its antigen, as well as the amount of antigen present in the sample. The specific activity of secondary antibodies varies also among manufacturers. Re-optimization is necessary when one or more of the experimental variables such as the antigen, primary or secondary antibody, or substrate is changed.

3.2.1 Primary antibody concentrations

Primary antibodies vary widely in quality, affinity, and concentration. The correct working range for antibody dilution depends on the characteristics of our primary antibody and the amount of target antigen to be detected. Suggested dilutions are written on the product sheets brought by manufacturer so, we have made multiple dilutions around these recommended dilutions. Thus, from the observed results the primary antibody dilution was selected to achieve maximum performance with lowest amount of antibody consumption.

3.2.1.1 Determination of optimum Catalase primary antibody concentration

For the determination of protein amount of catalase by Western-blot, primary antibody (anti-Catalase Rabbit IgG; Abcam: Cambridge, USA), concentrations, ranging from 1000 to 6000 dilutions were used in the experiments by using control rat liver S1 fractions of different protein contents, as indicated in Figure 3.11.

As it can be seen from the Figure 3.11, for the detection of catalase which is present in 10 µg of total protein containing S1 fractions, 1:6000 diluted primary antibody was sufficient and gave visible bands. Therefore, 10 µg of total protein incubated in 1:6000 dilution (in 5% non-fat dried milk prepared in TBST buffer) was selected as optimum protein and primary antibody concentration for catalase determination.

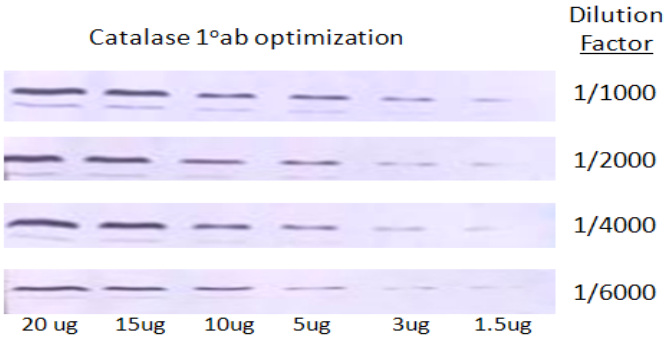


Figure 3.11: Western-blot analysis of control rat liver S1 fractions containing different amount of proteins for catalase, by using serially diluted primary antibodies (Anti-Catalase Rabbit IgG, Abcam: ab16731)

3.2.1.2 Determination of optimum GPx primary antibody concentration

In order to determine GPx protein amount in control, diabetic and antioxidant treated groups, anti GPx Rabbit IgG (Abcam: Cambridge, USA) was used at two different dilutions, namely, 2000 and 4000 fold dilutions. As seen from Figure 3.12, 2000 fold diluted antibody solutions was found to be sufficient; visible bands were obtained at 50 µg of total protein.

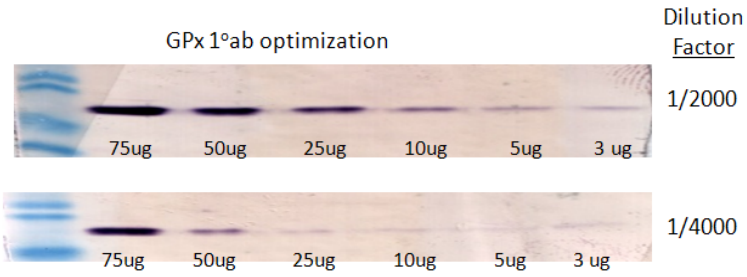


Figure 3.12: Western-blot analysis of control rat liver S1 fractions containing different amount of proteins for GPx, by using different dilutions of primary antibodies (Anti GPx Rabbit IgG, Abcam: ab16798).

3.2.1.3 Determination of optimum Cu-Zn SOD primary antibody concentration

For the determination of Cu-Zn SOD protein, primary antibodies (Anti-SOD1-Sheep IgG, Calbiochem: Darmstadt, Germany) was used at four different dilutions, namely, 500, 1000, 2000 and 4000 fold. Among them 2000 fold diluted antibody solutions was found to be sufficient which produced visible bands at 10 µg of total protein (Figure 3.13).

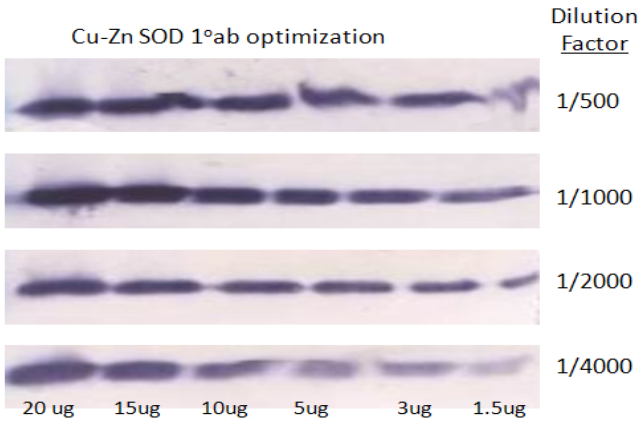


Figure 3.13: Western-blot analysis of control rat liver S1 fractions containing different amount of proteins for determination of Cu-Zn SOD by using different dilutions of primary antibodies (Anti-SOD1-Sheep IgG, Calbiochem: 574597).

3.2.1.4 Determination of optimum MnSOD Primary Antibody concentration

Mn SOD protein amount was determined by Western-blot analysis and primary antibody (Anti-Mn SOD Rabbit IgG, Santa Cruz, USA) concentrations ranging from 500 to 4000 fold dilutions were tried. It was found that, 500 fold dilution was enough to observe sharp bands by using 10 µg protein of control rat liver S1 fractions as shown in Figure 3.14.

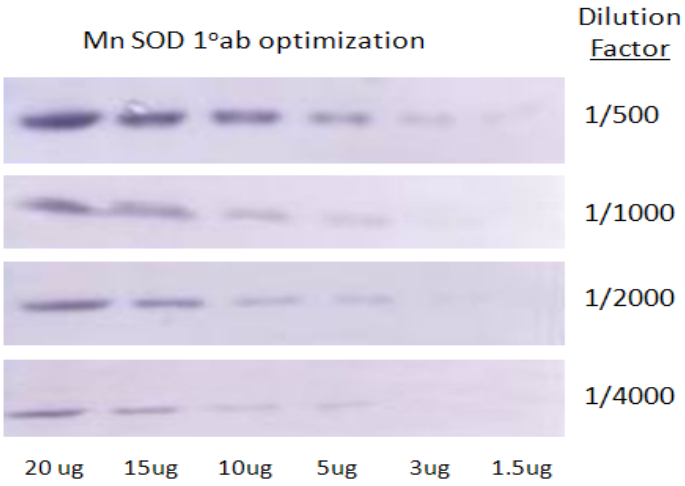


Figure 3.14: Western-blot analysis of control rat liver S1 fractions containing different amount of proteins for determination of Mn SOD by using different dilutions of primary antibodies (Anti MnSOD Rabbit IgG, Santa Cruz: sc-30080).

3.2.1.5 Determination of optimum GST-Mu Primary Antibody concentration

For the determination of GST-Mu protein content by Western-blot analysis, primary antibody (Anti-GST-Mu Rabbit IgG, Abcam, Cambridge, USA) concentrations ranging from 1000 to 10.000 fold were used and according to our results it was observed that, 10.000 fold dilution gave better results for 20 µg protein of control rat liver S1 fractions. The photograph of the membrane is shown in Figure 3.15.

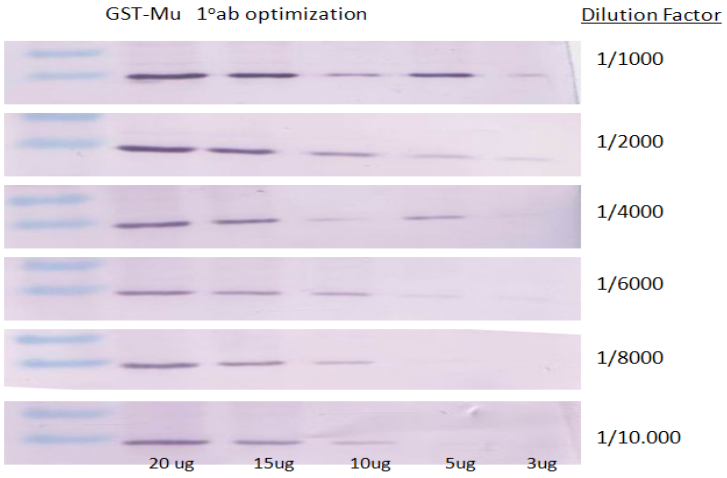


Figure 3.15: Western-blot analysis of control rat liver S1 fractions containing different amounts of protein for determination of GST-Mu by using different dilutions of primary antibody (Anti GST-Mu Rabbit IgG, Abcam: ab-23717).

3.2.1.6 Determination of optimum GAPDH primary antibody concentration

As an internal standard, GAPDH antibody (Anti-GAPDH Rabbit IgG, Santa Cruz, USA) was used for normalization procedures. In order to determine GAPDH protein content in groups, different dilutions; 250, 500, 1000 and 2000 fold were tried and it was found that, 2000 fold diluted antibody solutions was sufficient and gave visible bands at 10 µg of total protein (Figure 3.16). According to our results, we found that increasing the dilutions of the GAPDH antibody decreased the non specific binding of the antibody and as a result more clear bands were obtained. That is, non-specific bands, which were observed when 250 fold diluted antibody was used, were eliminated by using the same antibody at 2000 fold dilution.

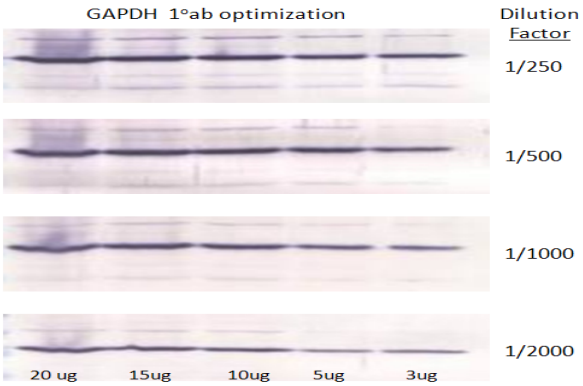


Figure 3.16: Western-blot analysis of control rat liver S1 fractions containing different amount of proteins for the determination of GAPDH by using different dilutions of primary antibodies (Anti GAPDH Rabbit IgG, Santa Cruz: sc25778).

3.2.2 Determination of the optimum Secondary Antibody concentrations for Mn SOD and Cu-Zn SOD Antibodies

Primary antibodies of all CAT, GPx, Mn SOD, GST-Mu and GAPDH proteins were produced in rabbits therefore secondary antibody which will be used in the experiments should react with these rabbit antibodies. For this reason, goat anti rabbit IgG conjugated with alkaline phosphatase (Abcam: Cambridge, USA) was selected and used for these enzymes. On the other hand, for Cu-Zn SOD antibody which was produced from the sheep (Sheap anti Cu-ZnSOD IgG), a distinct secondary antibody (Anti-Sheep IgG alkaline phosphatase conjugated produced in donkey-Sigma, Aldrich) was selected. For each type of secondary antibody, a distinct set of optimization studies were carried out. The results of two distinct sets of experiments are shown in Figure 3.17. Figure 3.17a demonstrates how differentially diluted goat anti rabbit IgG changes the Mn SOD detection at fixed primary antibody and protein concentration. Figure 3.17b displays the results of different dilutions of donkey anti sheep IgG at fixed primary antibody and protein concentrations.

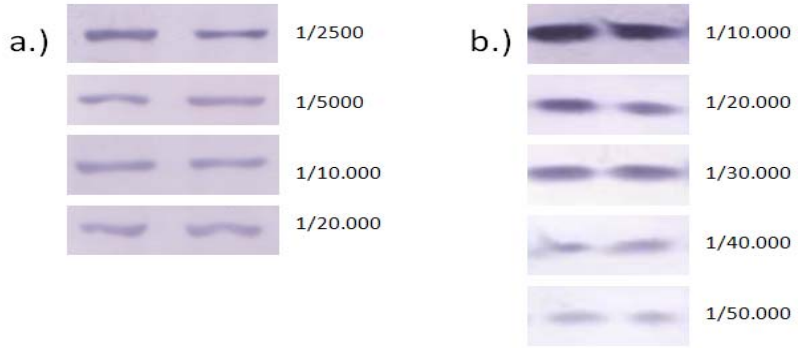


Figure 3.17: The effect of secondary antibody dilutions (a) for goat anti rabbit IgG for the detection of Mn SOD and (b) for donkey anti sheep IgG for the detection of Cu-Zn SOD in 10 µg total protein containing S1 fractions applied to the gels. Nearby bands represents their replicates.

Considering the above results, 1/10.000 dilution was chosen as optimum dilution for secondary antibody (Rabbit IgG) used to detect CAT, Mn SOD, GPx, and GAPDH, and 1/30.000 dilution was selected as optimum for secondary antibody (Sheep IgG) used for Cu-Zn SOD in Western-blot analysis. Table 2.3 (in Materials and Methods) summarizes the overall optimum conditions for primary and secondary antibody dilutions used in this study for the determination of antioxidant enzyme amount through Western-blot analysis.

3.3 Regulation of antioxidant enzyme activities in diabetes with antioxidant treatment

3.3.1 Regulation of CAT activity in diabetes and the effect of antioxidants

Multiplex RT-PCR was performed for the simultaneous amplification of catalase and the internal standard β-actin gene. Agarose gel electrophoresis photographs of multiplex amplification of CAT and β-actin mRNAs in control, diabetic and diabetic groups treated with antioxidants are given in Figure 3.18. As explained in Materials and Methods section, all the gels were scanned by using Image J

software and the intensities of the bands were converted into peaks by using the same software.

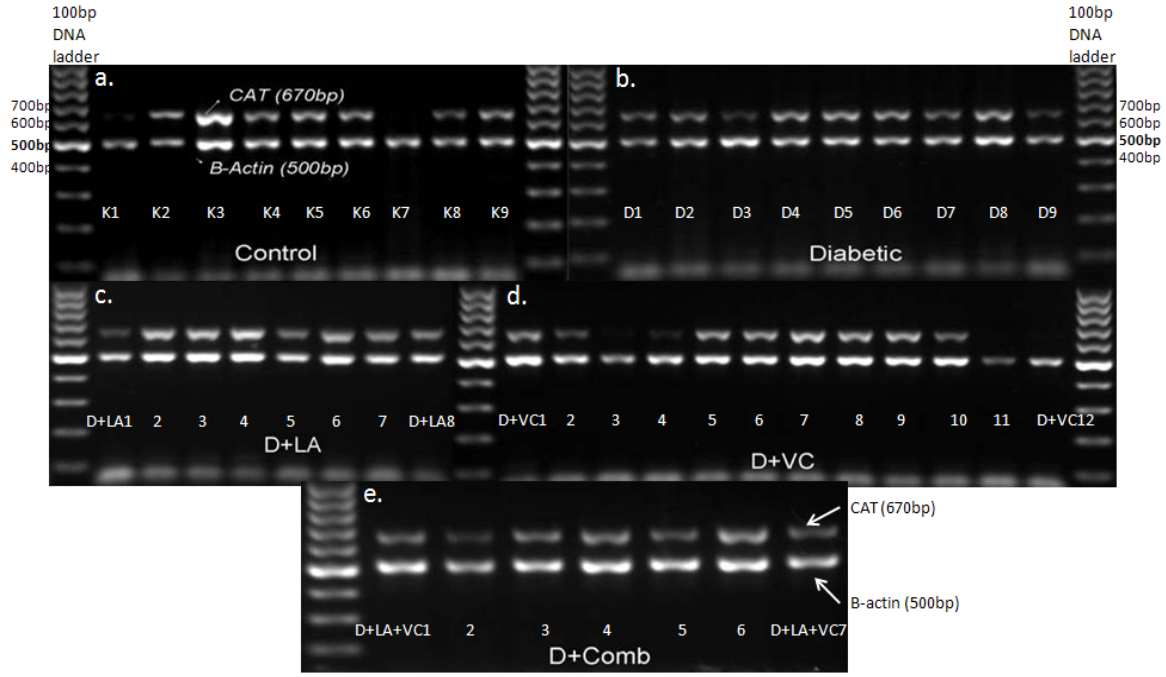


Figure 3.18: Two percent agarose gel electrophoresis of 5 µl of multiplex RT-PCR product of CAT and β-actin genes amplified by using cDNAs synthesized from 1 µg total RNAs isolated from corresponding tissues. 500bp β-actin 670bp CAT bands are clearly observed in individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups.

According to our results, diabetes reduced the relative expression of CAT mRNA around 20% and this reduction was found to be statistically significant ($p<0.005$) as compared to control. α-Lipoic acid (LA), Vitamin C (VC) and combined treatment had no significant effect on diabetic CAT mRNA expressions. Though LA seemed to increase the CAT expression in diabetic rat liver tissues, the results were not statistically significant. The results of densitometric analysis of

bands obtained from multiplex amplification of CAT and β-actin mRNAs in whole groups are summarized in Figure 3.20a, after the Western-blot results.

Protein amounts of CAT were also measured in whole groups with Western-blot analysis by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Co- immunostaining of CAT and internal standard allowed us to compare the relative expression of CAT with respect to GAPDH. Figure 3.19 shows the results of Western-blot analysis of CAT and GAPDH with co-immunostaining.

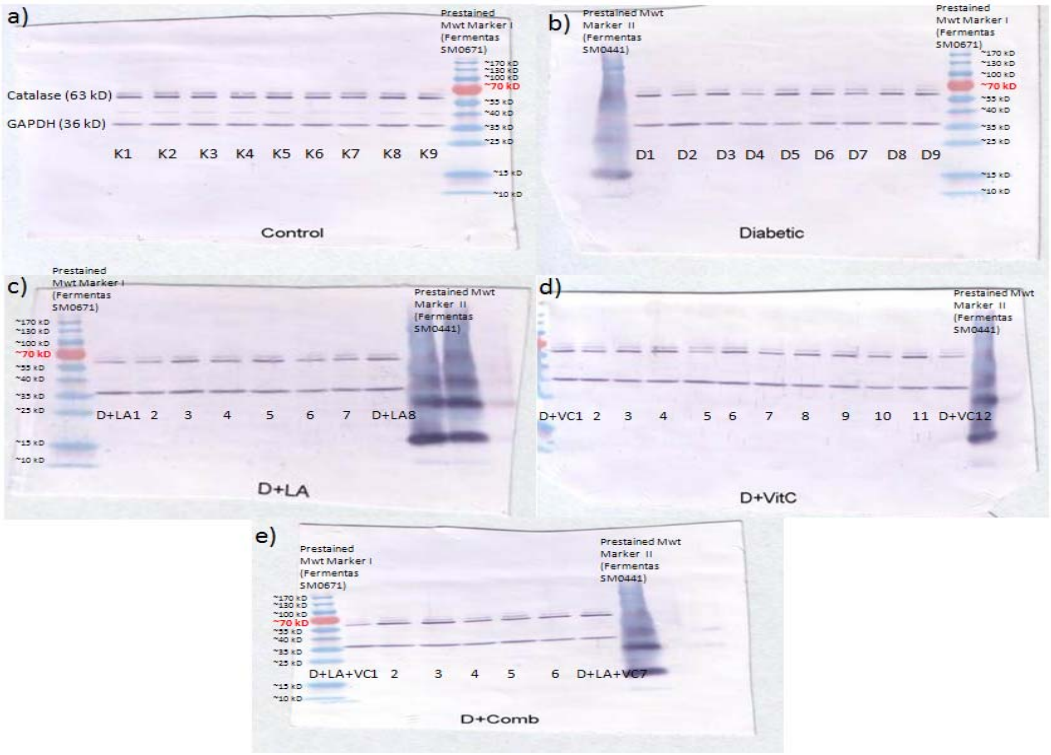


Figure 3.19: Western-blot analysis of CAT and GAPDH proteins from S1 fractions of corresponding tissues of individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups. On the membranes upper band represents 63kD CAT protein and lower band represents 36kD GAPDH protein. 10 µg total protein were loaded in each well and separated by 12% separating gel.

Intensities of the bands were measured with the help of Image J software and relative expressions of CAT with respect to housekeeping GAPDH protein are given in Figure 3.20b. The results of western analysis showed that diabetes caused a decrease in the protein amount of CAT by about 11%. This decrement was found statistically significant ($p<0.005$). Supplementing the animals with VC, LA and combination of them raised the protein amount, hence increased the translation of CAT but, only the effects of LA and combined treatment were found to be statistically significant ($p<0.05$) as compared to untreated diabetics. Application of both antioxidants together was sufficient to normalize the diabetic CAT protein expression value back to the control levels.

Diabetes decreased the CAT activities below the control values. CAT activities were decreased about 54% ($p<0.005$). Application of α-lipoic acid, vitamin C and their combination increased the diabetic CAT activities significantly but, this increment was not sufficient to reach up to the control values in all groups. Effect of diabetes and LA, VC and combined treatment on CAT activities are summarized in Figure 3.20c. All the results of the experiments (raw data) are given in the Appendix G.

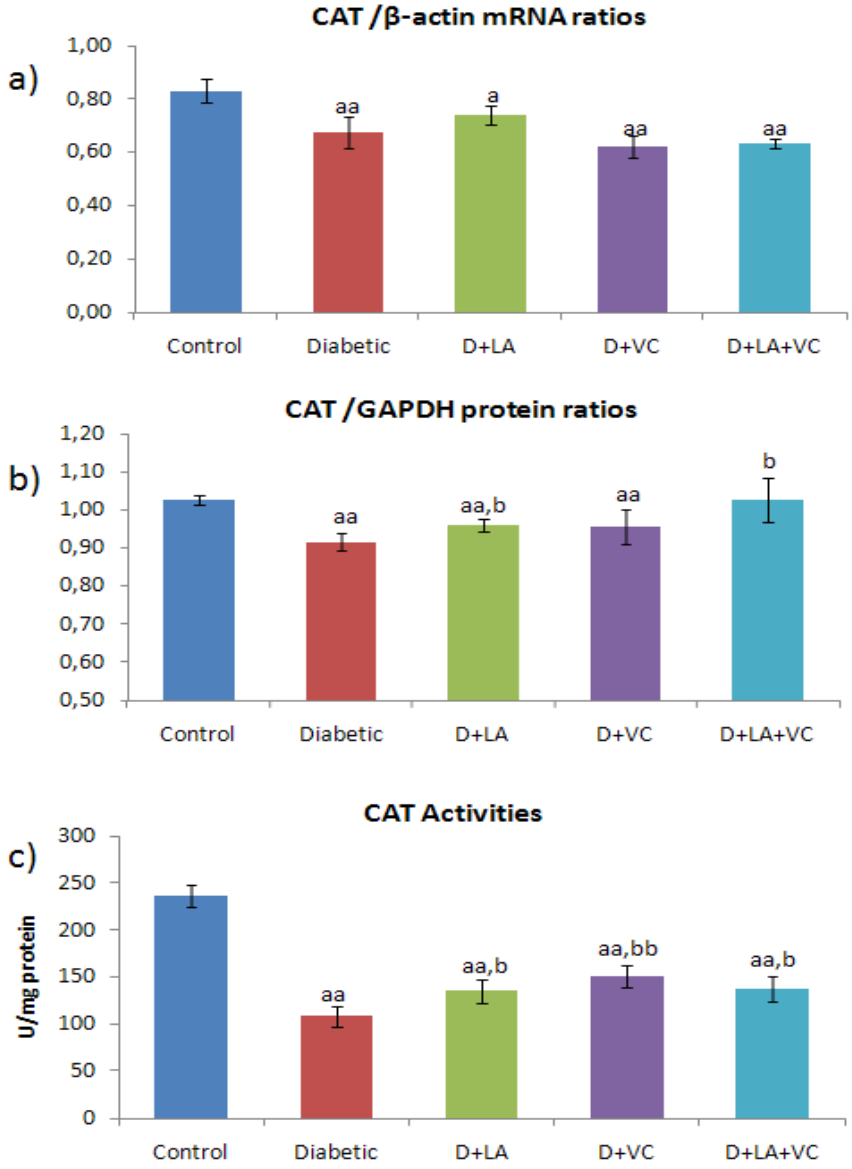


Figure 3.20: Bar diagrams of the results of CAT (a) mRNA expressions, (b) protein expressions, and (c) the activities in control, diabetic, diabetic animals supplemented with α-lipoic acid (D+LA) and diabetic animals supplemented with vitamin C (D+VC), and diabetic animals supplemented with both α-lipoic acid and vitamin C (D+LA+VC).

[a] represents significance at p<0.05 as compared with control groups.
[aa] represents significance at p<0.005 as compared with control groups.
[b] represents significance at p<0.05 as compared with untreated diabetic groups.
[bb] represents significance at p<0.005 as compared with untreated diabetic groups.

3.3.2 Regulation of Cu-Zn SOD activity in diabetes and the effect of antioxidants

mRNA Expressions of Cu-Zn SOD were determined under the optimized conditions and Figure 3.21 shows the agarose gel electrophoresis bands of the RT-PCR products obtained from multiplex amplification of Cu-Zn SOD and β-actin mRNAs in whole groups.

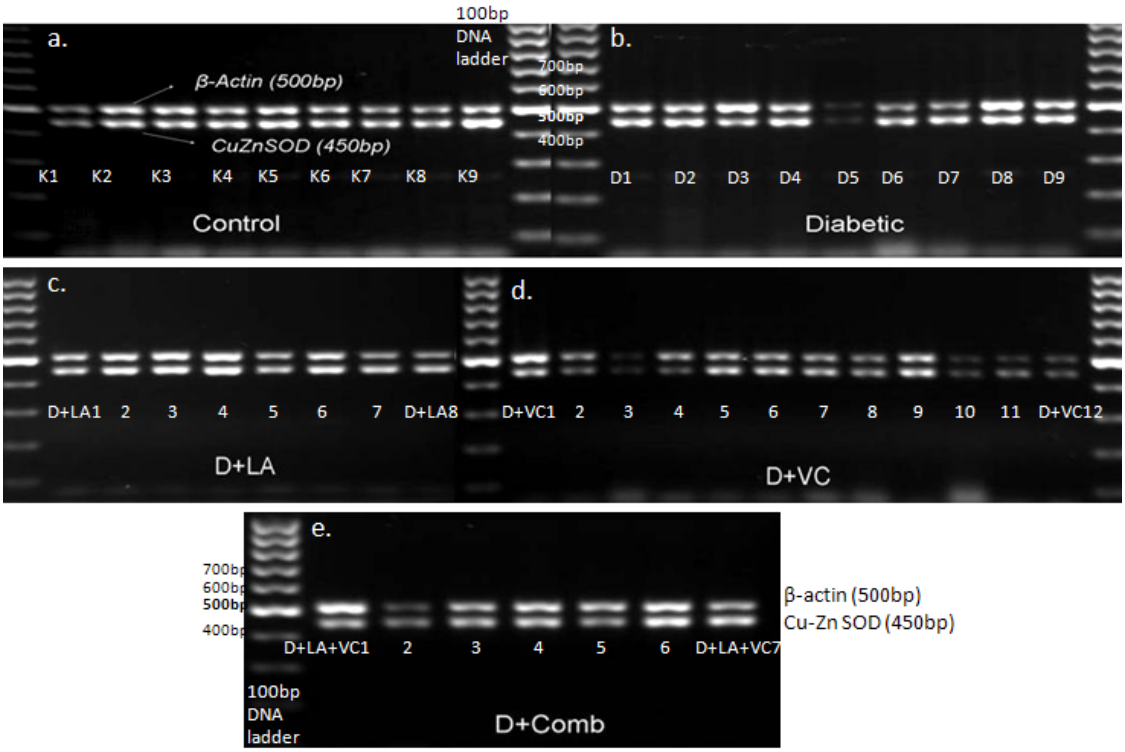


Figure 3.21: Two percent agarose gel electrophoresis of 5 µl of multiplex RT-PCR product of Cu-Zn SOD and β-actin genes amplified by using cDNAs synthesized from 1 µg total RNAs isolated from corresponding tissues. 500bp β-actin 450bp Cu-Zn SOD bands are clearly observed in individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups.

After densitometric analysis with ImageJ, the ratios of the densities of Cu-Zn SOD and β-actin genes are given in Figure 3.23a after the Western-blot results. According to our results, it was clear that, diabetes caused a statistically significant ($p<0.05$) decrease in mRNA levels of Cu-Zn SOD. Supplementing with neither antioxidants alone nor combination, however, changed the mRNA expression of Cu-Zn SOD in diabetic rats.

Protein expressions of Cu-Zn SOD were determined in whole groups with Western-blot analysis which was co-immunostained with GAPDH antibody. Figure 3.22 shows the results of Western-blot analysis of Cu-Zn SOD and GAPDH protein obtained in different groups; control, diabetic, LA, VC and LA+VC treated diabetic rat liver tissues. Intensities of bands were measured with Image J software and relative expressions of Cu-Zn SOD with respect to housekeeping GAPDH protein are given in Figure 3.23b. The results showed that, the relative translation of Cu-Zn SOD were decreased statistically ($p<0.005$) in diabetic animals. Even though, VC alone did not change the translation of Cu-Zn SOD, LA increased the Cu-Zn SOD protein expressions toward the control values. LA supplemented diabetic groups' SOD amounts were increased significantly as compared to diabetics nearly to the control protein amounts. This increase was significant statistically ($p<0.005$). Interestingly, combined application of two antioxidants has almost no effect on protein expressions of Cu-Zn SOD in diabetic animals.

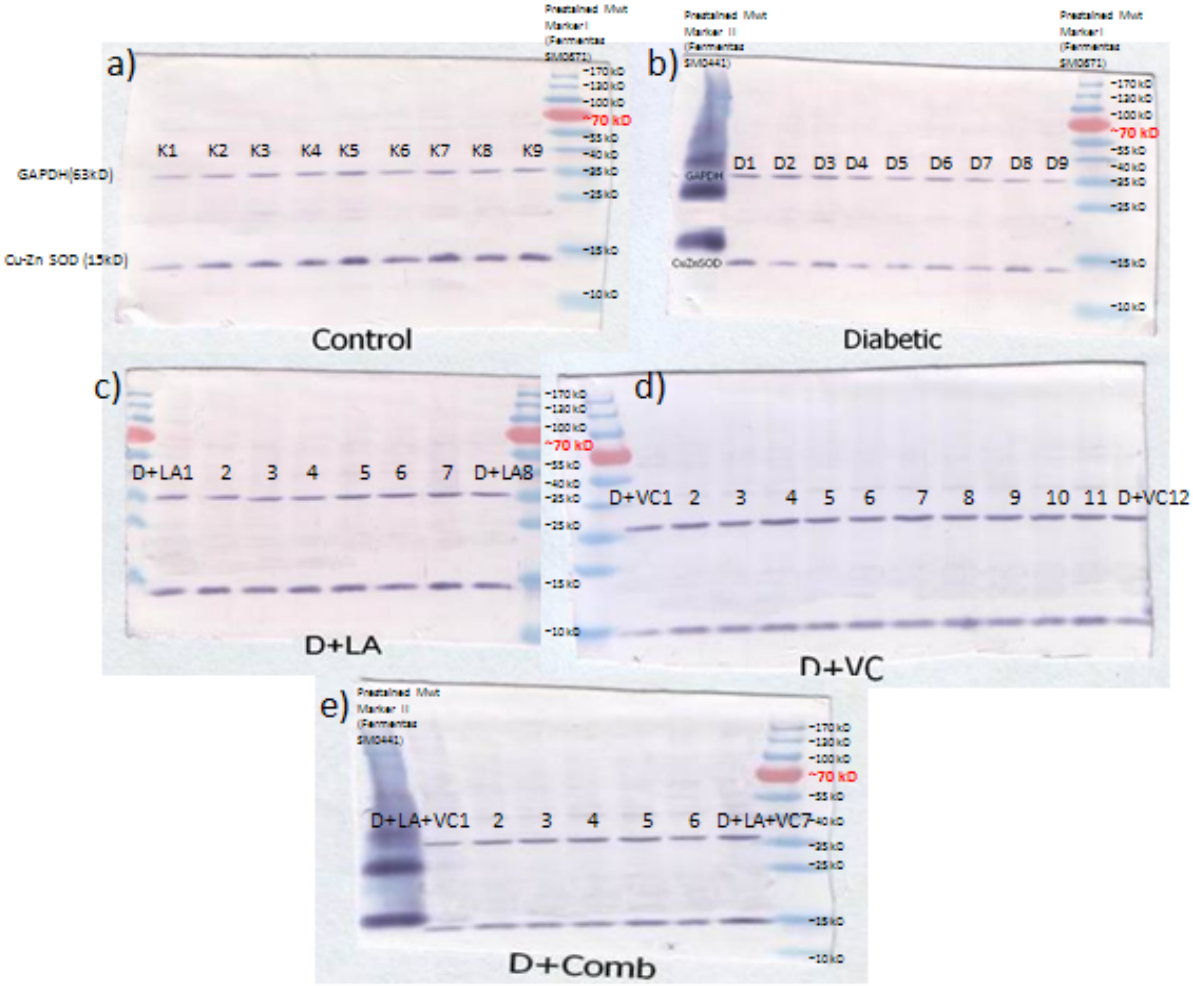


Figure 3.22: Western-blot analysis of Cu-Zn SOD and GAPDH proteins from S1 fractions of corresponding tissues of individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups. On the membranes upper band represents 36kD GAPDH protein and lower band represents 15kD Cu-Zn SOD protein. 10 µg total protein were loaded in each well and separated by 12% separating gel.

Cu-Zn SOD activities were measured in whole groups, and it was observed that diabetes decreased the activities of this enzyme below the control values ($p<0.05$). Cu-Zn SOD activities were decreased about 30% in diabetics. Treatment with LA, VC and combination of them increased diabetic Cu-Zn SOD activities toward control values and, the effect of VC and combined treatment were found to be more powerful then LA treatment alone. That is, the effect of VC and especially the combined treatment normalize the diabetic Cu-Zn SOD activity toward the control values and their effects were statistically significant ($p<0.05$). Effect of diabetes and antioxidant treatment on Cu-Zn SOD activities are summarized in Figure 3.23c.

Table 3.1 summarizes the overall changes in CAT and Cu-Zn SOD on mRNA expression, protein amounts and enzyme activities in control, diabetic and antioxidant supplemented diabetic rat liver tissues.

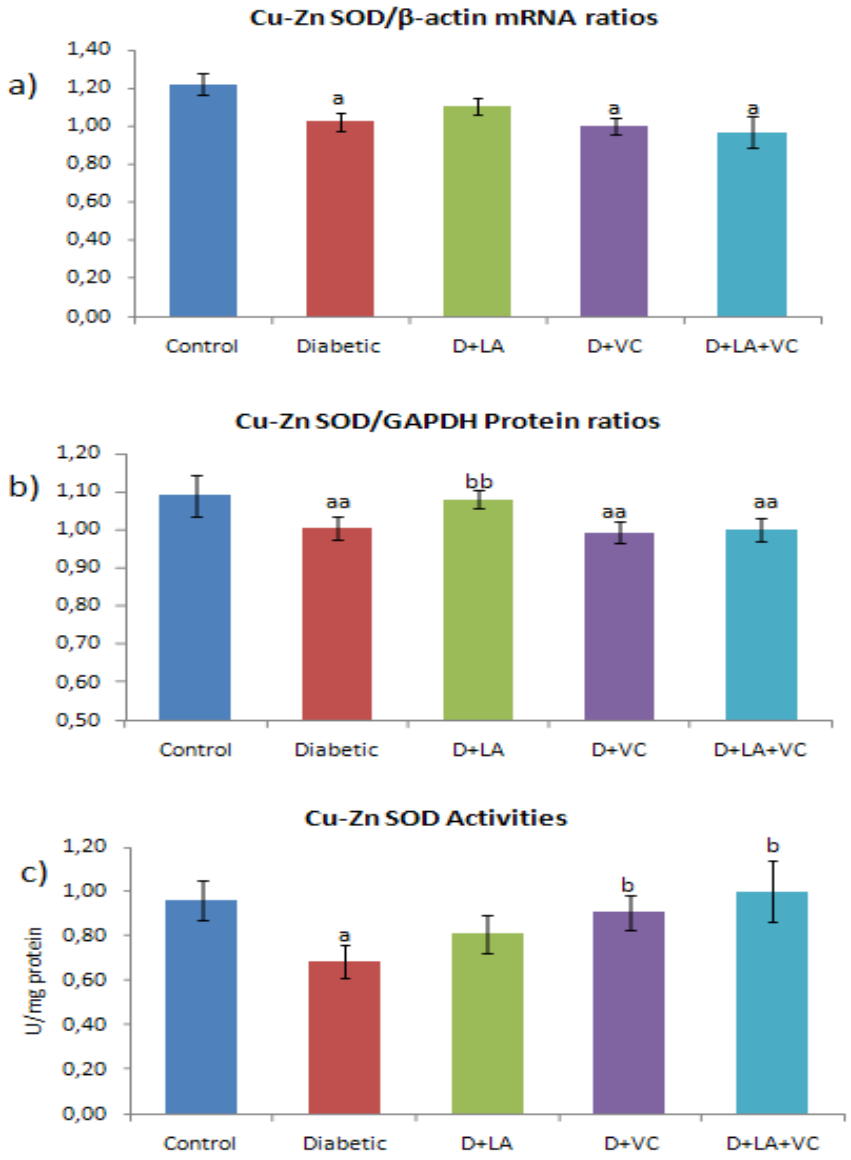


Figure 3.23: Bar diagrams of the results of Cu-Zn SOD (a) mRNA expressions, (b) protein expressions, and (c) the activities in control, diabetic, diabetic animals supplemented with α-lipoic acid (D+LA) and diabetic animals supplemented with vitamin C (D+VC), and diabetic animals supplemented with both α-lipoic acid and vitamin C (D+LA+VC).

[a] represents significance at p<0.05 as compared with control groups.
[aa] represents significance at p<0.005 as compared with control groups.
[b] represents significance at p<0.05 as compared with untreated diabetic groups.
[bb] represents significance at p<0.005 as compared with untreated diabetic groups.

Table 3.1: Summary of overall changes in the mRNA expressions, protein amounts and enzyme activities of antioxidant enzymes (CAT and Cu-Zn SOD) in control, diabetic and antioxidant supplemented diabetic rat liver tissues.

	N	CAT mRNA (CAT/β-actin)	CAT Protein (CAT/GAPDH)	CAT Activity (U/mg protein)	Cu-Zn SOD mRNA (Cu-Zn SOD/β-actin)	Cu-Zn SOD Protein (Cu-ZnSOD/GAPDH)	Cu-Zn SOD Activity (U/mg protein)
Control	*9*	*0,829±0,045*	*1,026±0,013*	*236,2±12,2*	*1,217±0,057*	*1,090±0,054*	*0,962±0,090*
Diabetic	*9*	*0,674±0,057* [aa]	*0,916±0,022* [aa]	*108,5±10,7* [aa]	*1,023±0,048* [a]	*1,004±0,031* [aa]	*0,689±0,075* [a]
Diabetic+ LA	*8*	*0,738±0,037* [a]	*0,960±0,015* [aa,b]	*135,0±12,6* [aa.b]	*1,105±0,045*	*1,079±0,024* [bb]	*0,810±0,085*
Diabetic+ VC	*12*	*0,621±0,040* [aa]	*0,956±0,045* [aa]	*150,8±11,1* [aa,bb]	*1,002±0,043* [a]	*0,993±0,028* [aa]	*0,908±0,079* [b]
Diabetic+LA+VC	*7*	*0,631±0,018* [aa]	*1,027±0,059* [b]	*137,1±13,6* [aa.b]	*0,968±0,083* [a]	*1,002±0,030* [aa]	*1,001±0,136* [b]

Data were expressed as mean ± Standard Error of Mean (S.E.M)

[a]*-represents significance at p<0.05 and* [aa] *represents significance at p<0.005 as compared with control groups.*
[b]*-represents significance at p<0.05 and* [bb] *represents significance at p<0.005 as compared with untreated diabetic groups*

3.3.3 Regulation of Mn SOD activity in diabetes and the effect of antioxidants

We have also performed multiplex RT-PCR for the simultaneous amplification of internal standard β-actin gene and Mn SOD, in order to determine the effect of diabetes and the antioxidants, α-lipoic acid, vitamin C and their combined treatments on MnSOD gene expressions. The results of the multiplex amplification of MnSOD and β-actin mRNAs in whole groups are shown in Figure 3.24.

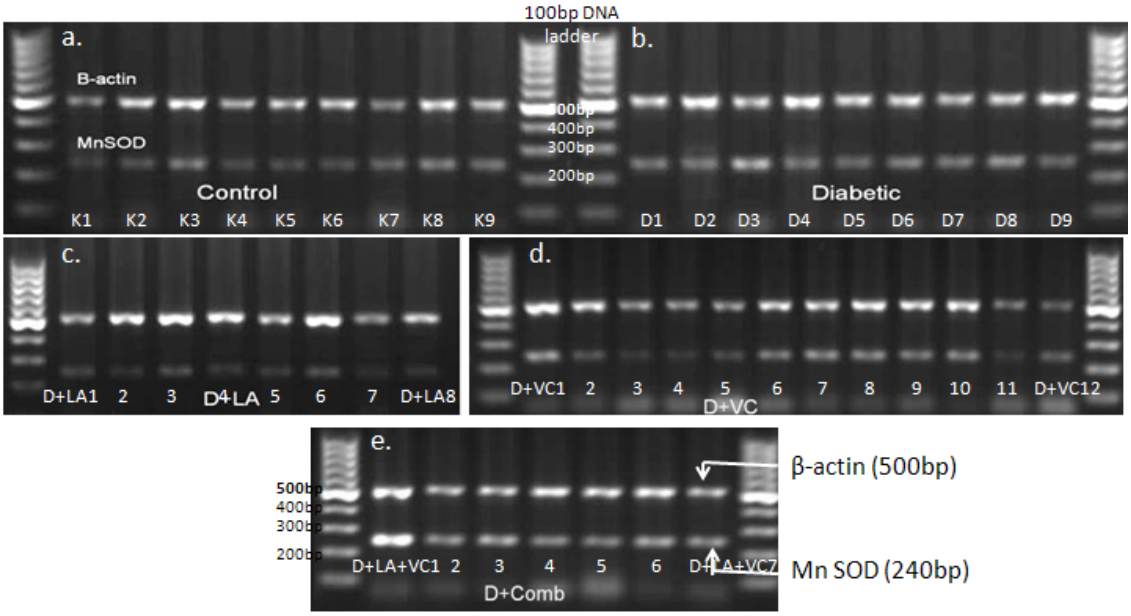


Figure 3.24: Two percent agarose gel electrophoresis of 5 µl of multiplex RT-PCR product of Mn SOD and β-actin genes amplified by using cDNAs synthesized from 1 µg total RNAs isolated from corresponding tissues. 500bp β-actin 240bp Mn SOD bands are clearly observed in individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups.

Figure 3.26a represents the ratios of the densities of Mn SOD and β-actin bands measured by Image J software. As seen from the figure, diabetes

increased the mRNA expressions of Mn SOD only slightly. Supplementing the animals with LA did not change the mRNA expression of Mn SOD whereas, VC treatment alone and also in combination with LA, increased Mn SOD mRNA expressions further, significantly ($p<0.05$). Protein expressions of Mn SOD were also studied with Western-blot analysis and the results are given in Figure 3.25.

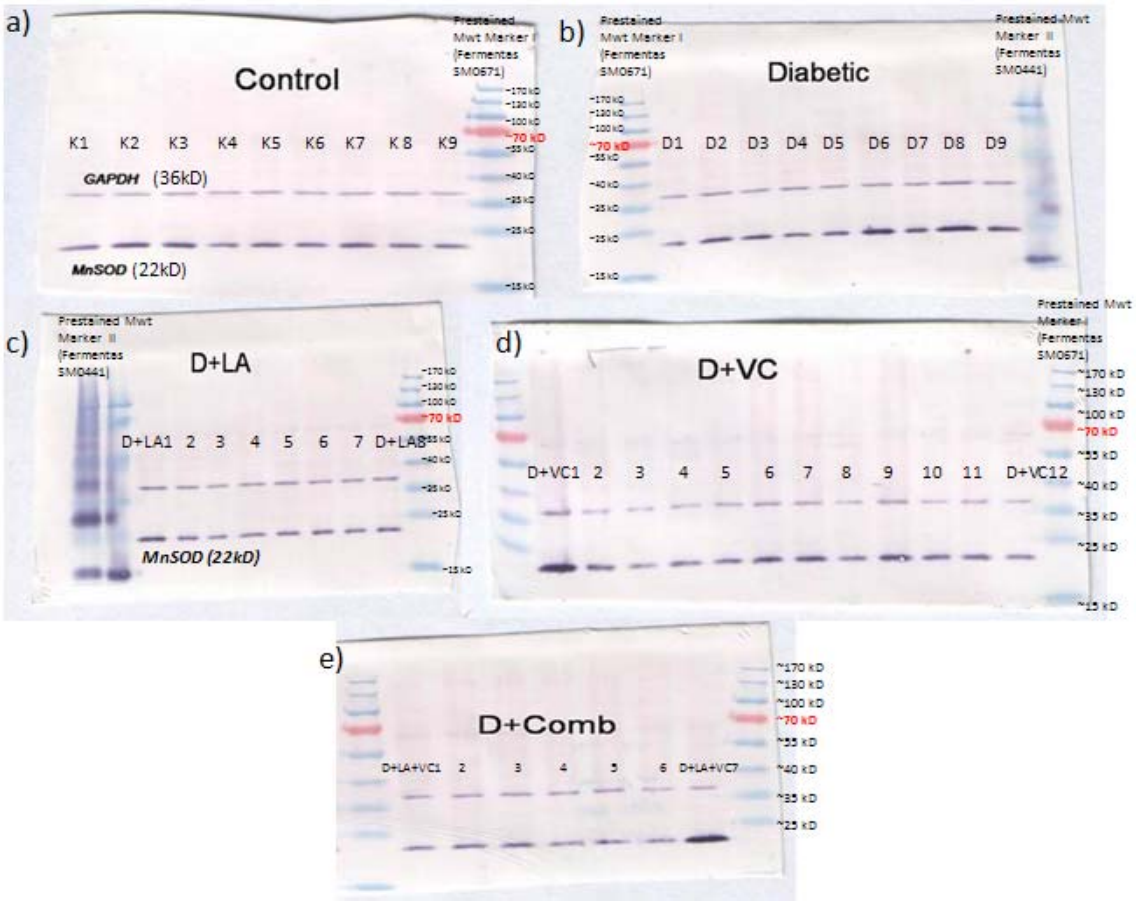


Figure 3.25: Western-blot analysis of MnSOD and GAPDH proteins from S1 fractions of corresponding tissues of individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups. On the membranes upper band represents 36kD GAPDH protein and lower band represents 22kD Mn SOD protein. 10 µg total protein were loaded in each well and separated by 12% separating gel.

Ratios of the intensities of the Mn SOD bands with respect to housekeeping GAPDH protein are given in Figure 3.26b. The results showed that, diabetes did not alter the levels of the protein synthesis of Mn SOD in diabetic animals compared to controls significantly. Furthermore, VC alone or in combination with LA did not change the amount of Mn SOD protein synthesis. However, contrary to our expectations, LA treatment alone decreased the translation of Mn SOD significantly ($p<0.05$).

The effects of diabetes and the antioxidants, α-lipoic acid, vitamin C and their combined treatments on MnSOD activities are given in Figure 3.26c. According to our results, though the activities of MnSOD in diabetic animals seemed to decrease compared to control values, this decrease was not statistically significant. Furthermore, application of LA and VC alone did not alter the diabetic MnSOD activities, but when applied in combinaiton, there was a significant increament in the MnSOD activities compared to diabetics.

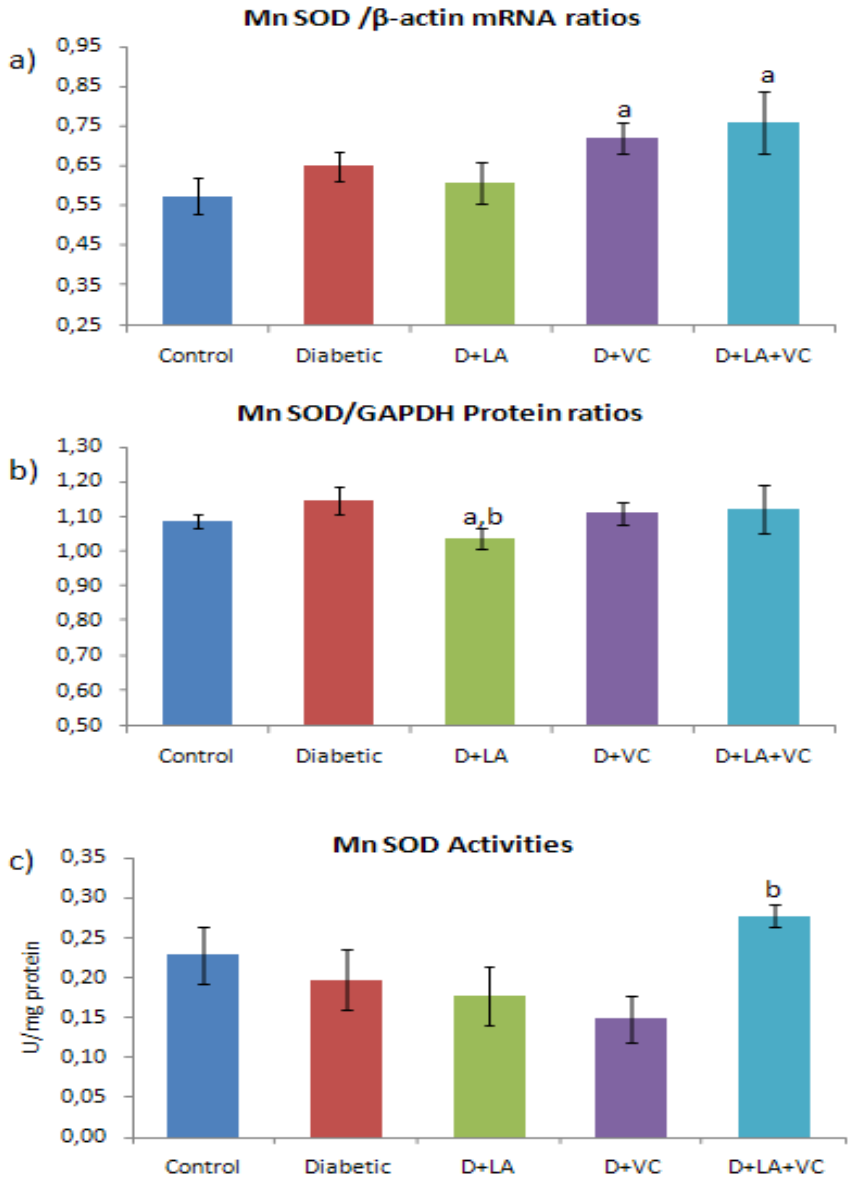


Figure 3.26: Bar diagrams of the results of Mn SOD (a) mRNA expressions, (b) protein expressions, and (c) the activities in control, diabetic, diabetic animals supplemented with α-lipoic acid (D+LA) and diabetic animals supplemented with vitamin C (D+VC), and diabetic animals supplemented with both α-lipoic acid and vitamin C (D+LA+VC).

[a] represents significance at p<0.05 as compared with control groups.
[aa] represents significance at p<0.005 as compared with control groups.
[b] represents significance at p<0.05 as compared with untreated diabetic groups.
[bb] represents significance at p<0.005 as compared with untreated diabetic groups.

3.3.4 Regulation of GPx activity in diabetes and the effect of antioxidants

The ratios of the mRNA expressions of Glutathione Peroxidase and internal standard β-actin were detected in all control, diabetic and antioxidant treated groups, and the multiplex RT-PCR products of the corresponding genes (GPx and β-actin) are shown in Figure 3.27.

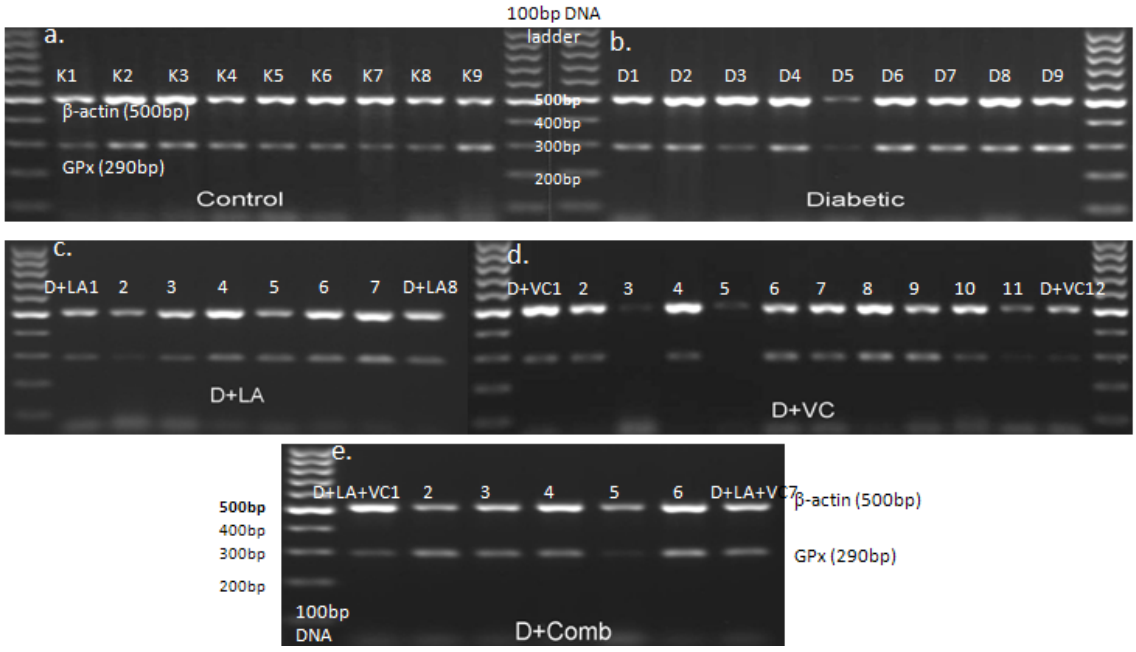


Figure 3.27: Two percent agarose gel electrophoresis of 5 µl of multiplex RT-PCR product of GPx and β-actin genes amplified by using cDNAs synthesized from 1 µg total RNAs isolated from corresponding tissues. 500bp β-actin 290bp GPx bands are clearly observed in individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups.

The ratios of the band intensities are given as bar diagram in Figure 3.29a. According to our results, diabetes increased the relative expression of GPx mRNA compared to controls about 8% but this induction was not statistically significant.

Either LA and VC treatments alone or combination did not show any effects on the expressions of GPx mRNA. Figure 3.28 shows the results of Western-blot analysis of GPx and GAPDH proteins with respect to each other.

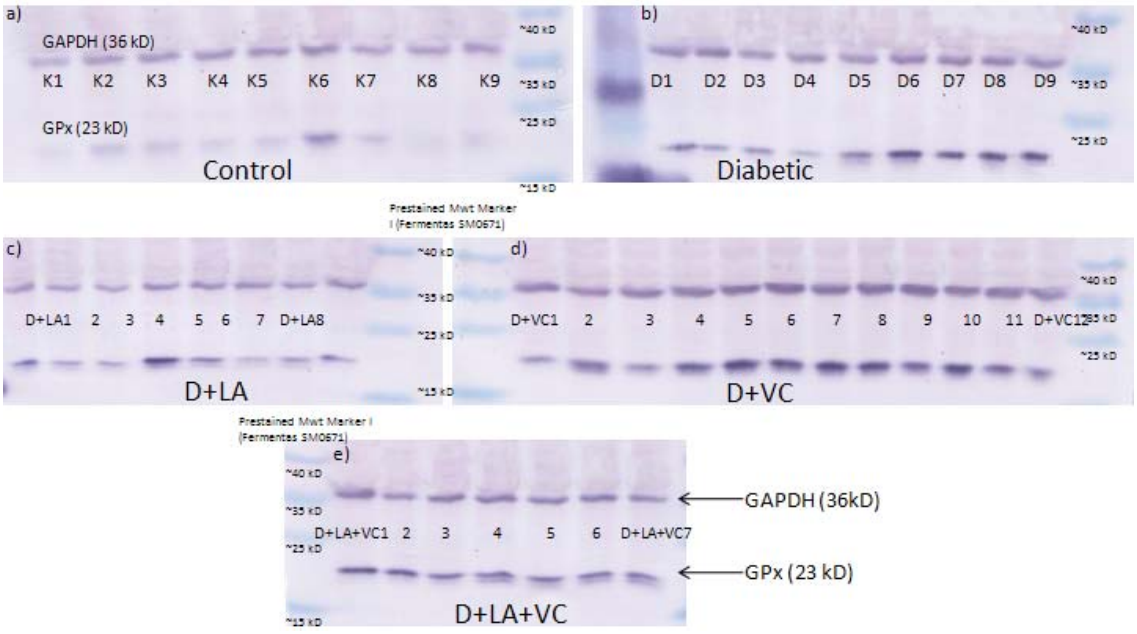


Figure 3.28: Western-blot analysis of GPx and GAPDH proteins from S1 fractions of corresponding tissues of individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups. On the membranes upper band represents 36kD GAPDH protein and lower band represents 23kD GPx protein. 50 µg total protein were loaded in each well and separated by 12% separating gel.

The results of the analysis showed that, diabetes increased the protein synthesis of GPx significantly ($p<0.05$) compared to controls. Supplementing the animals with both antioxidants, LA and Vitamin C (alone or in combination) did not cause any further change in the translation of GPx (Figure 3.29b).

Glutathione peroxidase activities were determined in all control, diabetic and antioxidant treated groups and the results are summarized in Figure 3.29c. As seen from the figure, there was no significant change in GPx activities of diabetic rat livers. On the other hand, when LA, VC or their combination was applied to the diabetic animals, there was a significant induction in GPx activities compared to control values.

Table 3.2 summarizes the overall changes in both mRNA and protein expressions, and enzyme activities of MnSOD and GPx in control, diabetic and antioxidant supplemented diabetic rat liver tissues.

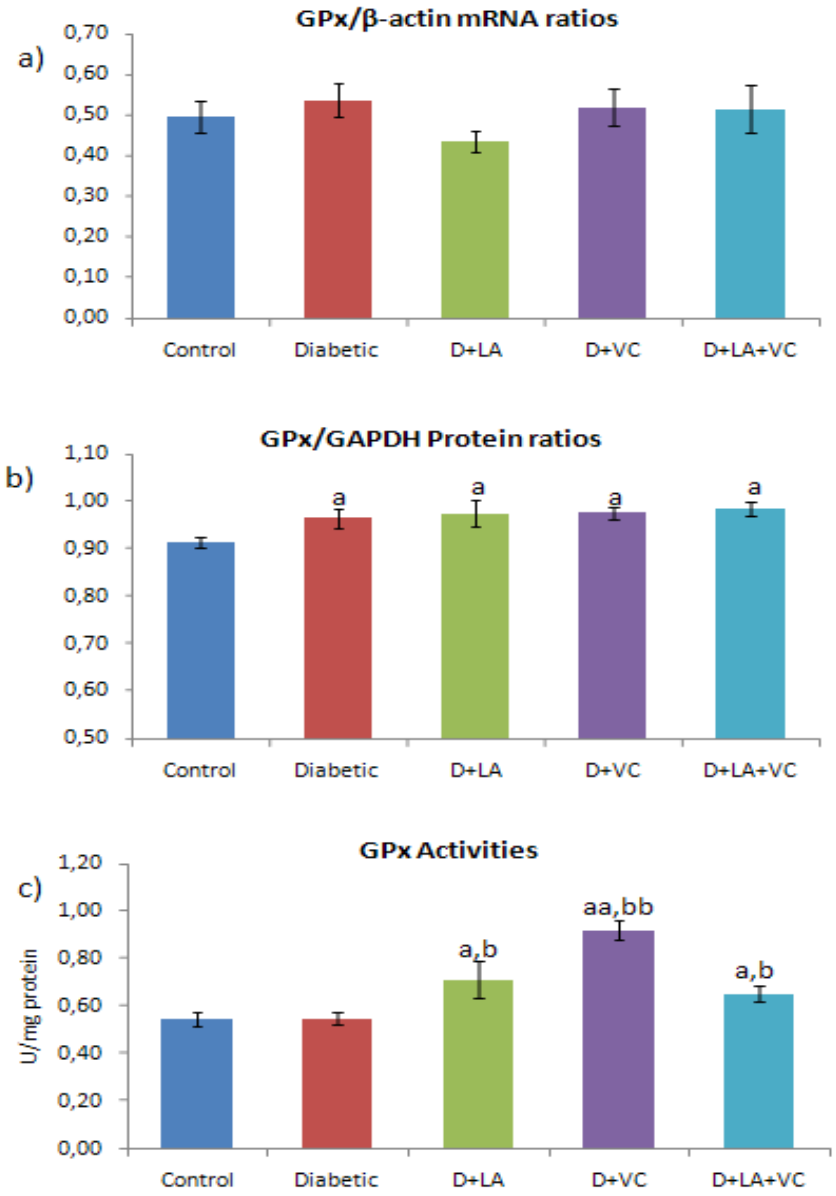


Figure 3.29: Bar diagrams of the results of GPx (a) mRNA expressions, (b) protein expressions, and (c) the activities in control, diabetic, diabetic animals supplemented with α-lipoic acid (D+LA) and diabetic animals supplemented with vitamin C (D+VC), and diabetic animals supplemented with both α-lipoic acid and vitamin C (D+LA+VC).

[a] represents significance at p<0.05 as compared with control groups.
[aa] represents significance at p<0.005 as compared with control groups.
[b] represents significance at p<0.05 as compared with untreated diabetic groups.
[bb] represents significance at p<0.005 as compared with untreated diabetic groups

Table 3.2: Summary of overall changes in the mRNA expressions, protein amounts and enzyme activities of antioxidant enzymes (GPx and Mn SOD) in control, diabetic and antioxidant supplemented diabetic rat liver tissues.

	N	GPx mRNA (GPx /β-actin)	GPx Protein (GPx /GAPDH)	GPx Activity (U/mg protein)	Mn SOD mRNA (Mn SOD /β-actin)	Mn SOD Protein (Mn SOD /GAPDH)	Mn SOD Activity (U/mg protein)
Control	9	0,494±0,039	0,913±0,010	0,546±0,031	0,573±0,045	1,088±0,019	0,228±0,036
Diabetic	9	0,535±0,042	0,964±0,020 [a]	0,547±0,027	0,648±0,037	1,131±0,039	0,197±0,038
Diabetic+ LA	8	0,434±0,027	0,974±0,027 [a]	0,710±0,078 [a,b]	0,608±0,051	1,040±0,030 [a.b]	0,177±0,036
Diabetic+ VC	12	0,518±0,045	0,975±0,014 [a]	0,920±0,042 [aa,bb]	0,719±0,039 [a]	1,136±0,033	0,148±0,029
Diabetic+LA+VC	7	0,513±0,058	0,982±0,009 [a]	0,650±0,034 [a,b]	0,760±0,078 [a]	1,121±0,070	0,277±0,014 [b]

Data were expressed as mean ± Standard Error of Mean (S.E.M)

[a]-represents significance at p<0.05 and [aa] represents significance at p<0.005 as compared with control groups.
[b]-represents significance at p<0.05 and [bb] represents significance at p<0.005 as compared with untreated diabetic groups.

3.3.5 Regulation of GST-Mu activity in diabetes and the effect of antioxidants

As Glutathione S-transferases (GST) defend cells against oxidative stress, regulation of GST expression by induction or inhibition is a crucial factor in determining the sensitivity or resistance of cells to a wide variety of antioxidants in diabetes. For this reason GST Mu isoform was selected as an indicator of defense against oxidative stress. GST-Mu mRNA expressions were determined with multiplex RT-PCR. As an internal standart β-actin was used. Figure 3.30 shows the agarose gel electrophoresis bands of the RT-PCR products obtained from multiplex amplification of GST-Mu and β-actin mRNAs.

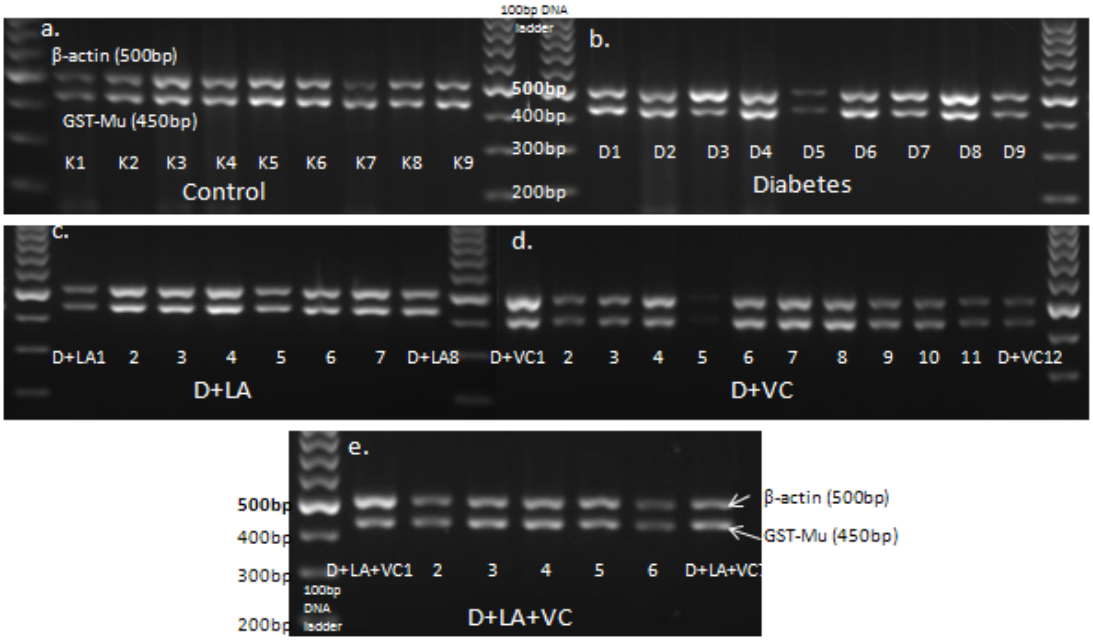


Figure 3.30: Two percent agarose gel electrophoresis of 5 µl of multiplex RT-PCR product of GST-Mu and β-actin genes amplified by using cDNAs synthesized from 1 µg total RNAs isolated from corresponding tissues. 500bp β-actin 450bp GST-Mu bands are clearly observed in individual rats (marked in figure) (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups.

After densitometric analysis of the gels with ImageJ software, the ratios of the densities of GST-Mu and β-actin genes are calculated and the results are given as bar diagrams in Figure 3.32a. According to the results, it was found that, diabetes caused a significant decrease in mRNA expressions of GST-Mu, and this reduction was statistically significant ($p<0.05$). Supplementing the rats with either antioxidant alone or in combination, did not change the mRNA expressions of GST-Mu in diabetic state.

For the determination of GST-Mu protein expression in tissues, Western-blot analysis was carried out by using rabbit anti GST-Mu IgG and the membranes were co-immunostained also by using rabbit anti GAPDH IgG. Figure 3.31 shows the results of multiplex Western-blot analysis of GST-Mu and GAPDH protein in control, diabetic, LA, VC and LA+VC treated diabetic rat liver tissues.

After making the densitometric analysis of protein bands with Image J software, the relative expressions of GST-Mu with respect to housekeeping GAPDH protein were calculated. The results showed that, there was a marked decrease in the relative protein expression of GST-Mu ($p<0.005$) in diabetic animals and application of neither antioxidants showed a restoring effect toward the control values. Instead relative protein expressions were reduced further with antioxidant applications as shown in Figure 3.32b.

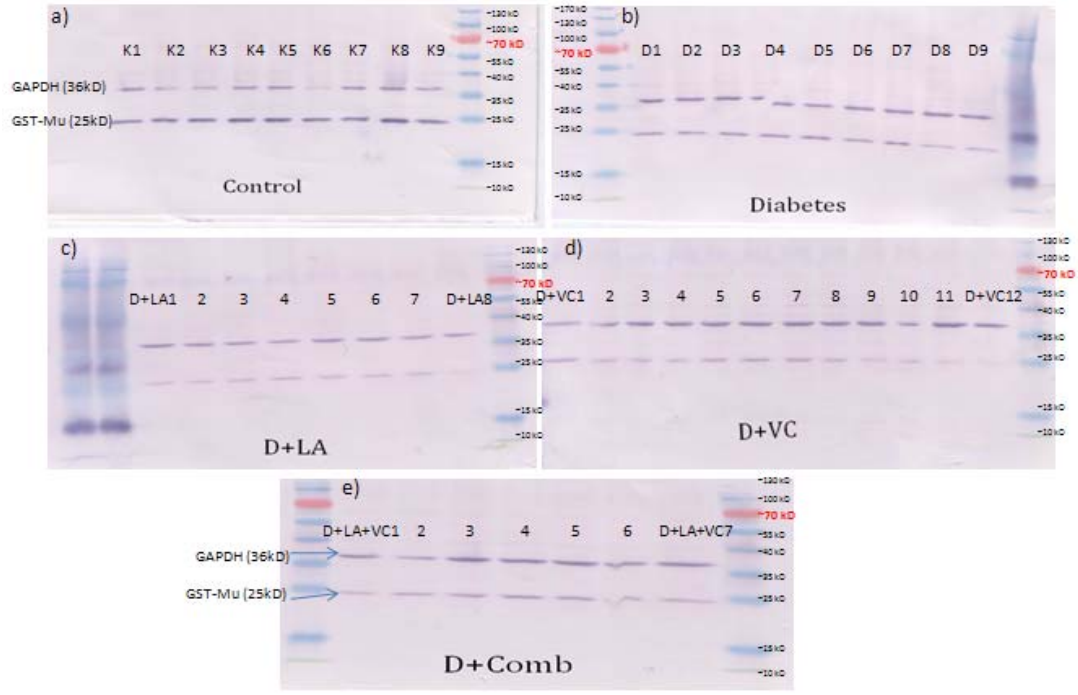


Figure 3.31: Western-blot analysis of GST-Mu and GAPDH proteins from S1 fractions of corresponding tissues of individual rats (marked in figure) belonging (a) control, (b) diabetic, (c) LA treated diabetic (D+LA), (d) VC treated diabetic (D+VC) and (e) LA+VC treated diabetic (D+LA+VC) groups. On the membranes lower band represents 25kD GST-Mu protein and upper band represents 36kD GAPDH protein. 20 µg total protein were loaded in each well and separated by 12% separating gel.

GST-Mu activities were measured in all groups by using DCNB as a specific substrate for that isoform. It was observed that, diabetes leads to a significant reduction in the activities of this enzyme ($p<0.05$) as compared to the control group. Although LA did not change GST-Mu activities in diabetes, VC treatment caused a significant increment in the activities but still below the control values. Interestingly, when two antioxidants were given together, they reduced the diabetic GST-Mu activities further. Effects of diabetes and the antioxidant treatment on GST-Mu activities are summarized in Figure 3.32c and Table 3.3.

Table 3.3: Summary of overall changes in the mRNA expressions, protein amounts and enzyme activities of GST-Mu isoform in control, diabetic and antioxidant supplemented diabetic rat liver tissues.

	N	GST-Mu mRNA (GST-Mu/β-actin)	GST-Mu Protein (GST-Mu/GAPDH)	GST-Mu Activity (U/mg protein)
Control	9	1,188±0,048	1,755±0,200	16,23±1,04
Diabetic	9	0,936±0,083 [a]	0,841±0,066 [aa]	12,96±0,89 [a]
Diabetic+ LA	8	0,953±0,037 [aa]	0,609±0,068 [aa,b]	12,49±1,04 [a]
Diabetic+ VC	12	0,941±0,069 [a]	0,645±0,044 [aa,b]	15,26±0,59 [b]
Diabetic+LA+VC	7	0,999±0,079 [a]	0,653±0,062 [aa,b]	9,59±0,56 [aa,b]

Data were expressed as mean ± Standard Error of Mean (S.E.M)

[a]-represents significance at p<0.05, [aa] represents significance at p<0.005 as compared with control groups.
[b]-represents significance at p<0.05, [bb] represents significance at p<0.005 as compared with untreated diabetic groups.

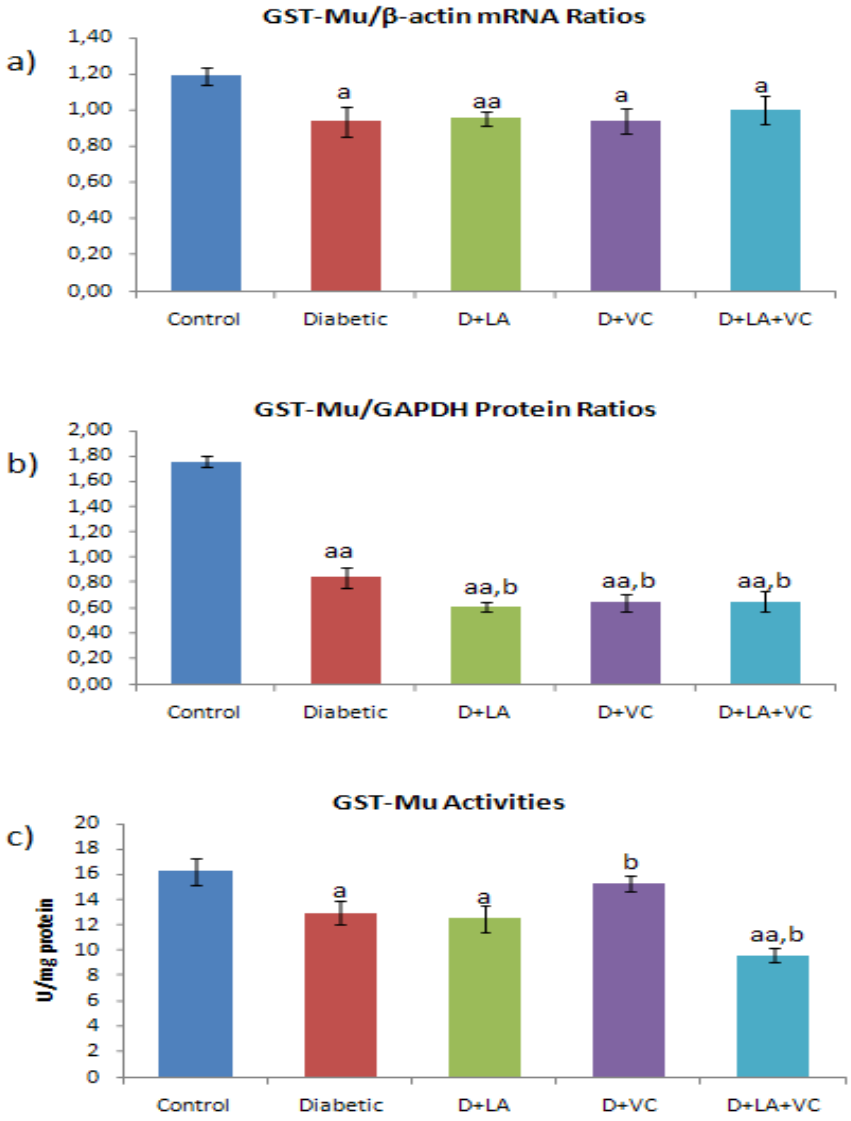


Figure 3.32: Bar diagrams of the results of GST-Mu (a) mRNA expressions, (b) protein expressions, and (c) the activities in control, diabetic, diabetic animals supplemented with α-lipoic acid (D+LA) and diabetic animals supplemented with vitamin C (D+VC), and diabetic animals supplemented with both α-lipoic acid and vitamin C (D+LA+VC).

[a] represents significance at p<0.05 as compared with control groups.
[aa] represents significance at p<0.005 as compared with control groups.
[b] represents significance at p<0.05 as compared with untreated diabetic groups.
[bb] represents significance at p<0.005 as compared with untreated diabetic groups.

3.4 Determination of some biomarkers of oxidative stress: GSH, MDA and protein carbonyl concentrations

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes mellitus. So we studied the oxidative stress in the liver during diabetes and determine the extent of oxidative damage by quantifying the oxidation products of the lipids and proteins by measuring the MDA content and protein carbonylation respectively. Furthermore as an oxidative stress marker, GSH concentrations were also determined in all tissues. Figure 3.33a demonstrates how GSH levels are modified with diabetes and antioxidant treatments. According to the results, diabetes reduced the GSH concentrations below the levels of control groups and this decrement was found statistically significant ($p<0.05$). Even though, individual LA and VC treatments brought the diabetic GSH state toward the control levels, their combination had no significant effect on GSH concentration.

In diabetes free radicals are formed disproportionately by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to increased lipid peroxidation, and damage the cells. Lipid peroxidation is the radical induced process leading to oxidative damage of unsaturated lipids. One of the end products of lipid peroxidation reactions is malonedialdehyde (MDA) and according to our results MDA was found to be elevated significantly ($p<0.05$) in diabetic animals. Supplementing the animals with antioxidants either alone or in combination reduced this effect of diabetes and the MDA values were decreased nearly to the control values as shown in Figure 3.33b. Protein carbonyl contents which are theindicator of radical induced oxidative protein damage were also found to be higher in diabetics compared to controls. About 30% elevation was observed in diabetes which is statistically significant ($p<0.005$). Results are

summarized in Figure 3.33c. Antioxidant LA and VC treatments either alone or in combination, brought the increased protein carbonyl contents observed in diabetes toward the control values. In that respect the antioxidant treatments seem to prevent some of the consequences of oxidative stress which can promote the development of complications of diabetes mellitus.

Table 3.4 summarizes the overall changes in the reduced glutathione concentrations, and the degree of lipid peroxidation and protein carbonylation in control, diabetic, α-lipoic acid supplemented diabetic, vitamin C supplemented diabetic and both α-lipoic acid and vitamin C supplemented diabetic rat liver tissues.

Table 3.4: Summary of overall changes in tissue GSH levels, MDA (final product of lipid peroxidation) levels and protein carbonylation levels in control, diabetic, LA supplemented diabetic, VC supplemented diabetic and LA+VC supplemented diabetic rat liver tissues.

	N	GSH levels (nmol/mg protein)	MDA levels (nmol/mg protein)	Protien Carbonyls (nmol/mg protein)
Control	9	77,04±6,14	0,018±0,002	9,448±0,364
Diabetic	9	58,62±5,11 [a]	0,025±0,003 [a]	12,221±0,733 [aa]
Diabetic+ LA	8	71,85±6,90	0,016±0,002 [b]	5,595±0,452 [aa,bb]
Diabetic+ VC	12	76,95±5,96 [b]	0,021±0,001 [b]	9,018±0,876 [b]
Diabetic+ LA+VC	7	59,17±1,91	0,019±0,001 [b]	7,819±0,548 [a,bb]

Data were expressed as mean ± Standard Error of Mean (S.E.M)

[a]-represents significance at p<0.05, [aa]-represents significance at p<0.005 as compared with control groups.
[b]-represents significance at p<0.05, [bb]-represents significance at p<0.005 as compared with untreated diabetic groups.

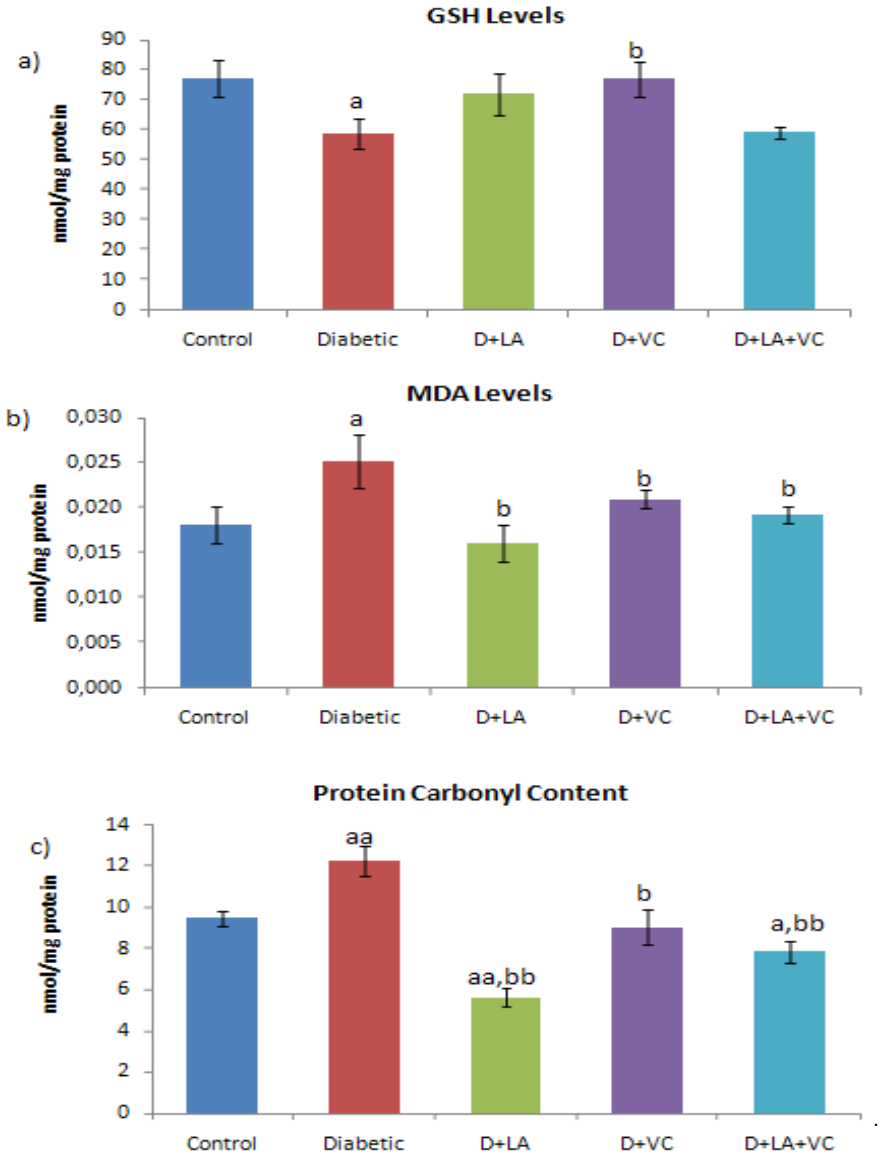


Figure 3.33: Liver tissue (a) GSH concentrations, (b) MDA contents and (c) protein carbony contents of control, diabetic, diabetic animals supplemented with α-lipoic acid (D+LA), diabetic animals supplemented with vitamin C (D+VC), and diabetic animals supplemented with both α-lipoic acid and vitamin C (D+LA+VC).

[a] represents significance at p<0.05 as compared with control groups.
[aa] represents significance at p<0.005 as compared with control groups.
[b] represents significance at p<0.05 as compared with untreated diabetic groups.
[bb] represents significance at p<0.005 as compared with untreated diabetic groups

CHAPTER 4

CONCLUSIONS AND DISCUSSIONS

Diabetes mellitus is a group of metabolic diseases characterized by high blood glucose levels that could result from the defects in insulin secretion, or action, or both. Increased oxidative stress is believed to play an important role in the etiology and pathogenesis of the chronic complications associated with the disease. For this reason, numerous studies proposed that, inhibition of oxidative stress with antioxidants might be an effective strategy for reducing the complications of diabetes. The beneficial effects of antioxidants in the treatment of diabetes have been shown by several investigators (Dinçer *et al*., 2003; Maritim *et al*., 2003; Seven *et al*., 2004). According to the results of these studies, although it is clear that oxidative stress play a major role in diabetic complications, the molecular mechanisms of the effects of antioxidants especially on antioxidant enzymes have not been clearly established. The present study was designed in order to determine the effects of oxidative stress on rat liver tissues during diabetes, as well as the accompanying changes in antioxidant enzyme status of the cell to understand its role in the pathogenesis of the disease. The nature of the molecular mechanisms of the regulation of the primary antioxidant enzymes namely, catalase (CAT), superoxide dismutase (SOD; Cu-Zn and Mn containing), glutathione peroxidase (GPx) and glutathione S-transferase (GST; Mu isoform) were studied at the level of gene and protein expressions, i.e., transcription and translation, respectively. Gene expressions of

these enzymes were studied by measuring their mRNA levels using RT-PCR; protein expressions by studying their protein contents through Western-blot analysis and also by looking at their enzymatic activities. Additionally, the effects of two powerful antioxidants lipid-soluble, α-lipoic acid and water-soluble, vitamin C were investigated simultaneously in the liver tissues of diabetic rats (Sadi *et al*., 2008; Sadi and Guray, 2009). The regulatory effects of these antioxidants on the gene and protein expressions of the antioxidant enzymes were studied at fixed concentrations alone and in combination as previously determined.

At first, we have investigated the presence of oxidative stress during the diabetes in the liver tissues by measuring the extent of oxidative damage, namely by quantifying the oxidation products of the lipids and proteins, by measuring the MDA content and protein carbonylation levels respectively. Furthermore, the concentration of the major cellular antioxidant, reduced glutathione (GSH) was also quantified in diabetes and the effects of antioxidant treatments were evaluated in both control and diabetic rat liver tissues.

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 GSTs. GSH also functions as free radical scavenger and in the repair of radical induced biological damage. Previous studies have shown that, hepatic GSH concentration of STZ induced diabetic rats was significantly lower when compared with the normal rats (Dincer *et al*., 2002; Maritim *et al*., 2003b; Pari and Latha, 2005). According to our results, similarly, diabetes reduced the GSH concentrations below the levels observed in control groups. Even though, individual LA and VC application brought the diabetic GSH state toward the control levels, their combined application yielded no significant changes in GSH concentration. The decrease in GSH in liver during diabetes most probably is the

result of its increased utilization due to oxidative stress and the addition of antioxidants slow down the excess usage of GSH. Interestingly the combined application of both antioxidants did not change the level of the utilization of GSH. Antioxidant treatment may also function in the recycling of oxidized glutathione to the reduced glutathione as demonstrated in antioxidant network which has been given in detail in the Introduction part at Figure 1.7. LA has also the ability to correct deficient thiol status of cells by increasing de novo synthesis of GSH (Han *et al*., 1997), and hence increasing the GSH levels in diabetic animals. The beneficial effects of VC or LA applications were also observed by some other scientists (Cay *et al*., 2001; Dincer *et al*., 2002).

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 leads to peroxidative changes resulting 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 the tissue. Lipid peroxidation also cause 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 (4-HNE) (Halliwell and Gutteridge, 2007). According to our results, lipid peroxidation levels in the liver tissues of diabetic rats were significantly higher compared to controls and these findings are in agreement with the results of other related studies (Kakkar *et al*., 1998; Bhor *et al*., 2004; Seven *et al*., 2004). This marked increase in the lipid peroxidation rates in diabetic tissues suggests an accumulation of oxygen free radicals which can be due to either increased production and/or, decreased elimination. Furthermore, application of antioxidants either alone or in combination reduced the levels towards the control values, most probably by decreasing the cellular redox potential. Any

increase in the recycling of vitamin E radical -which is an important element stopping the radical induced chain lipid peroxidation reactions-will also decrease the amount of the free radicals and hence contribute to the decrease in the redox potential of the cell.

The oxidative modification of proteins has been shown to play an important role in a number of human diseases (Halliwell and Gutteridge, 2007). Protein carbonyl content is actually a general indicator and the most commonly used marker of protein oxidation (Berlett and Stadtman, 1997; Chevion *et al*., 2000). Accumulation of protein carbonyls has been observed in Alzheimer's disease, diabetes, inflammatory bowel disease, and arthritis (Chevion *et al*., 2000; Bhor *et al*., 2004). Our results showed that, the carbonyl content of proteins in diabetic groups were higher compared to control groups, most probably, due to exposure of proteins to reactive oxygen species which could lead to modifications in amino acid side chains resulting in altered structure and/or functions. Other studies also showed that, the increase in the number of carbonyl groups within a protein correlated well with diabetes mellitus (Limaye *et al*., 2003; Bhor *et al*., 2004). Application of LA and VC, alone or in combination brought the increment observed in diabetes toward the control values. Effect of LA on the protein carbonylation was more prominent then the effect of VC alone. It reduced the protein carbonylation even below the control values.

Cytosolic superoxide dismutase (SOD-1 or Cu-Zn SOD) and mitochondrial superoxide dismutase (SOD-2 or Mn SOD) convert intracellular superoxide radicals into hydrogen peroxide which can be removed further by glutathione peroxidase (GPx) and catalase (CAT) by converting it into water. CAT works primarily to catalyze the decomposition of H_2O_2 to water, sharing this function with GPx. Both of these enzymes detoxify H_2O_2 derived from SOD activities. In the presence of low H_2O_2 concentrations, H_2O_2 is the preferred substrate for GPx

since it has low Km value for H_2O_2. At high concentrations, organic peroxides are metabolized by GPx and CAT is responsible for H_2O_2 removal (Yu, 1994).

Another protective mechanism against oxidative stress is Glutathione S-transferases (GSTs) which catalyze the conjugation of glutathione to a wide range of electrophiles. GSTs also exhibit differential response to a wide variety of chemicals and oxidative stress in the normal and pathophysiological conditions such as diabetes mellitus (Raza *et al*., 2000; Raza *et al*., 2004). It has been confirmed that GSTs are also regulated both in vivo and in vitro by reactive oxygen species (ROS) such as superoxides, H_2O_2 and by the products of membrane lipid peroxidation (Awasthi *et al*., 1995; Mari and Cederbaum, 2001). Among the different isoforms of GSTs, the cytosolic Mu isoform is predominantly present in the liver and function in the elimination of diverse array of foreign compounds as well as wide variety of products of oxidative stress. Even though, alpha class GST isoforms have been shown to possess highest activity towards the lipid peroxidation end product which is 4-HNE (4-hydroxynonenal), GST Mu and Pi also display impressive catalytic efficiency toward this end product (Knapen *et al*., 1999). The total pool of GST-Mu, which is quite large in liver cells, might have significant effect on the process of detoxification of the products of the oxidative stress. Therefore GST-Mu family is critical in the protection of cells from reactive oxygen species.

In the present study, when we looked at the gene expressions of several antioxidant enzymes, we found that excessive oxidative stress occurred in the liver due to diabetes mellitus, resulted in the down regulation of the expression of the CAT, Cu-Zn SOD and GST-Mu genes. Our results also showed that, the decrease in the mRNA expressions of these antioxidant enzymes also affected all protein expressions and the activities of these enzymes, indicating a transcriptional regulation of these genes. As the genes are controlled at the level of transcription, the entire pathway through translation was affected and hence

at the end, the activities of all these antioxidant enzymes decreased drastically. The reduction in the mRNA of CAT, Cu-Zn SOD and GST-Mu isoform in diabetes could be due to oxidation of transcriptional factors (e.g. Nrf2) responsible for the initiation machinery of antioxidant enzyme transcription process as in antioxidant response elements (see section 1.3.3.3). Also, the decrease observed in the expression of mRNAs could be due to the decrease in the half lives of mRNAs since increased oxidative stress may lead to destabilization of mRNA (Navarro *et al*., 2008).

On the other hand, according to our results, both the mRNA expressions and the activities of two other antioxidant enzymes, namely Mn SOD and GPx, did not change in diabetic rat liver tissues. Though there was a little decrease in the activities of Mn SOD but the results were not significant statistically. The reason for the unchanged activities of Mn SOD in diabetes might be the result of the overall mechanism of the antioxidant enzyme system. As the gene expressions of Cu-Zn SOD decreased, the unchanged gene expressions of Mn SOD prevent the further decrease in the total SOD activities. As a result, the decrease in the activity of total SOD was due to the decrease in the gene expression of only Cu-Zn SOD, as Mn SOD gene expression and activity did not change at all. Also, the cytosolic Cu-Zn SOD might be more sensitive to oxidative stress caused by diabetes. The diminished Cu-Zn SOD activities reduced H_2O_2 production leading to CAT, to make it deficient of its substrate. Furthermore, decrease in SOD activity might lead to build up high concentrations of superoxide radicals which might inhibit CAT activities as demonstrated by Kono and Fridovich (1982).

The effect of oxidative stress induced by diabetes on antioxidant enzymes reported in literature significantly varied depending on the experimental conditions, such as age of the animals, duration of the diabetes and the type of tissues under examination. There are studies reporting that 2-3 weeks diabetic

state caused the activities of antioxidant enzymes to decline in liver tissues of rats (Anwar and Meki, 2003; Maritim *et al*., 2003a). On the other hand, other researchers (Dincer *et al*., 2002; Yildirim and Buyukbingol, 2002) have reported that CAT and SOD activities were increased in 5-6 week diabetic rats. In a study carried out by using renal cortex of diabetic animals, there was an increase in Cu-Zn SOD and GPx mRNA expression together with an increase in the total SOD and GPx activity, and an increase in CAT gene expression in contrast to a decrease in enzyme activity (Limaye *et al*., 2003). Koya and coworkers (2003) found that mRNA expression of Cu-Zn SOD and CAT were not changed significantly in glomeruli of diabetic rats. Similarly, Bhor and friends (2004) studied the antioxidant enzyme activities in the small intestine of STZ-induced diabetic rats and despite the changes observed in the activities of CAT, SOD and GPx, unaltered mRNA levels for these genes were observed. In another study, total GST (CDNB activity), and GST-alpha (ETA) activities were found to be low in diabetic rat livers as compared to controls (Raza *et al*., 2000). But none of these studies looked for the regulation of the activities of different GST isoforms at the level of gene expression. Sindhu and coworkers (2004) found that, even though there was no significant change in the activities, the protein expression of GPx was decreased in diabetes which is not in agreement with our findings. According to our results similarly, GPx activity was not changed in diabetes but its protein amount on the other hand, was found to be elevated significantly. Several other articles also showed that Mn SOD can be induced to protect against oxidative stress produced by cytokine treatment, ultraviolet light, irradiation, certain tumors, amyotrophic lateral sclerosis, and ischemia/reperfusion (Dobashi *et al*., 2000; Liu *et al*., 2000). However, a decrease in Mn SOD activity was also reported during cancer, aging, asthma, and transplant rejection (Macmillan-Crow and Cruthirds, 2001) but none of these articles studied the role of diabetes on the Mn SOD activity and gene/protein expressions. All the results indicated that

oxidative stress caused by diabetes affected antioxidant defense systems of the different tissues somehow in different ways to protect cellular and tissue injuries.

In this study, we also tried to normalize the changes in gene-protein expressions and hence the activities of antioxidant enzymes, in diabetic rats occurred as a result of oxidative stress, by supplementing the animals with α-Lipoic acid (LA) and vitamin C (VC) either individually or in combination. LA is a naturally occurring compound present as a cofactor in several mitochondrial enzymes that are involved in metabolism and energy production (Packer *et al*., 2001). In its free form, LA is a powerful antioxidant, functioning as a reactive oxygen species scavenger. Antioxidant effects of LA is based on their interactions with peroxyl radicals, which are essential for the initiation of lipid peroxidation; and ascorbyl radicals of vitamin C. Reduced form of lipoic acid, dihydrolipoic acid (DHLA), can recycle ascorbyl radicals and reduce dehydroascorbate generated in the course of ascorbate oxidation by radicals. Therefore, dihydrolipoic acid may act as a strong chain-breaking antioxidant and may enhance the antioxidant potency of other antioxidants like vitamin C and vitamin E in both the aqueous and in hydrophobic membrane phase (Figure 1.7). In literature, there are studies showing that LA reversed the changes in diabetic antioxidant enzyme activities (CAT and SOD) toward the control values (Packer *et al*., 2001; Dincer *et al*., 2002; Moini *et al*., 2002). Limited number of articles has also showed the effect of LA on the Mn SOD and GPx. For instance, Maritim and coworkers (2003b) found that when LA was supplemented, GPx activity was decreased in livers, and increased in kidneys of diabetic rats. According to our results, LA increased the diabetic SOD and CAT activities toward the control values and its effects on CAT activity were more significant than that of SOD. When we look at the CAT mRNA expression and protein amounts, it seems that the increase in activity is not related to the induction of the enzyme synthesis. So

the effect is post-translational. Similarly, the application of LA had almost no effect on mRNA levels but somehow protein expression of Cu-Zn SOD increased. LA increased the rate of translation, without changing the rate of transcription indicated a translational effect of the oxidative damage caused by diabetes. Additionally, LA also decreased the protein expression of Mn SOD with no significant change in the activity. That is, LA exerted its action on Mn SOD mainly at the level of translation. Similar translational control was also observed for GST Mu isoform, and GST-Mu protein expressions were reduced significantly with LA application. The activities were not affected at all. On the other hand, though LA showed no effect on both the mRNA and protein expressions of GPx, it increased the GPx activity significantly, indicating a post-translational modification.

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin and it is a powerful antioxidant, quenching ROS and reactive nitrogen species. Intracellular vitamin C can prevent cell death and inhibit mutations induced by oxidative stress (Lutsenko *et al*., 2002; Halliwell and Gutteridge, 2007). The critical role of VC in eliminating the adverse effects of ROS has been well established. Young and friends (1992) showed that it reduced the diabetes induced lipid peroxidation. Also its protective effect on antioxidant enzyme activities was presented by Garg and Bansal (2000). In our experiments, similar with the literature, application of VC increased both Cu-Zn SOD and CAT activities in diabetic rat livers significantly. It normalized the Cu-Zn SOD activity almost to the control values but not adequate to normalize CAT activity. In the case of Cu-Zn SOD, though there was an increase in the activity, the mRNA and protein amounts of Cu-Zn SOD did not change statistically, indicating a post-translational modification. VC supplementation also did not change the levels of translation but higher enzyme activities of CAT were observed as compared to untreated diabetics. Though VC did not change the activities of the Mn SOD drastically, it affected the mRNA expressions of the enzyme. It enhanced the mRNA expression

of Mn SOD and seemed to induce the transcription without affecting the translation and activity. In the case of GPx, similar to the action of LA, VC also increased the activity of GPx in diabetic groups. That is, supplementing the animals with either antioxidant increased the activities in diabetic groups without any change in both the mRNA and protein expressions. Similarly, effect of VC on GST-Mu was at post-translational level, since it increased the diabetic GST-Mu activity without effecting the mRNA and protein expressions significantly.

Considering α-lipoic acid's ability to recycle other antioxidants such as vitamin C in the cells, the effect of combinational antioxidant treatment (LA+VC) was also studied in diabetic rat liver tissues. It has been proposed that when given in combination, LA might enhance efficacy of VC on the system. Considering Cu-Zn SOD enzyme activities, while LA alone has no significant effect on diabetic Cu-Zn SOD activities, post-translational effect of VC in increasing the activities was further boosted when given in combination with LA. Conversely, post-translational effect of VC in increasing the activities of GST-Mu isoform was reversed when given in combination with LA. Combinational treatment increased the protein expression of CAT significantly, but its effect was not so drastic in terms of activities. Even though individual antioxidant treatments were not sufficient to normalize the CAT protein levels, combined application of antioxidants restored this decline up to control values. This is most probably the result of augmented antioxidant potency of VC on up-regulation of CAT synthesis. But though the protein synthesis was significantly increased with combinational treatment, the overall CAT activities were still below control values after either individual of combined treatment of both antioxidants. The combined treatment of both antioxidants augments all the mRNA, protein and activities of Mn SOD above the control levels significantly. Combined treatment induced gene transcription and protein translation; as a result enzymatic

activities of this mitochondrial antioxidant enzyme were also increased most probably resulting from transcriptional up-regulation. Roles of LA and VC modulating the intracellular signaling through NF-κB pathway have been previously studied by Packer, in 1998 and Carcamo and coworkers in 2004 and this pathway might be considered as potential regulatory mechanism provided by these two antioxidants.

Intracellular redox balance is tightly controlled and its disturbances lead to modifications in the pattern of gene expressions of several enzymes (Schoonbroodt and Piette, 2000). Recent studies have investigated the roles of the oxidants, antioxidants, and other determinants of the intracellular oxidation-reduction (redox) state on the regulation of gene expression (Watai *et al.,* 2007; Surh *et al.,* 2008). Redox sensitive metabolic enzymes can directly modulate cellular metabolism, whereas redox-sensitive signaling proteins execute their function via downstream signaling components, such as kinases, phosphatases, and transcription factors. One of the most important redox regulated proteins in the cell is the protein tyrosine phosphatases (PTPs) and their transient oxidation of thiol groups in active sites leads to their inactivation.

In literature, CAT is found to be regulated by tyrosine phosphorylation at specific tyrosine residues (Cao *et al*., 2003a). At lower levels of ROS, CAT activity may be stimulated by phosphorylation and in the case of very high level of radicals; CAT activity could be decreased by targeting of the phosphorylated CAT for degradation (Cao *et al*., 2003b). This may explain the reduced CAT activity observed in diabetes. Significant increase in CAT activities observed after antioxidant treatment in diabetic rats can be explained by post-translational modifications due to increased phosphorylation state caused by VC and LA as potent activators of tyrosine phosphorylation due to various crosstalks between various kinases and phosphatases. Application of antioxidants may also reduce the cellular oxidant potential that may re-activate several protein tyrosine

phosphatases leading to a situation causing permenant tyrosine phosphorylation on CAT proteins by an unknown mechanism. In order to see the role of phosphorylation on post-translational regulation of CAT we have used different antibodies which recognized the phosphorylated CAT. However, though we have tried different commercially prepared antibodies we could not succeed in showing the phosphorylation. As a future study, we want to use different cell culture systems in order to show the effects of phosphorylation or any other post-translational mechanism following the redox changes induced by diabetes.

According to Cao and coworkers (2003c), the mammalian c-Abl and Arg are the nonreceptor tyrosine kinases and they are activated by the cellular response to oxidative stress and both c-Abl and Arg form a heterodimer and become activated with the H_2O_2 application. This activated complex further mediates the phosphorylation on Tyr-96 of GPx which stimulates the activity providing a protection to cells against oxidative stress. Similarly, the effect of VC and LA on the GPx can be at post-translational level probably through phosphorylation leading to high level of activities. We proposed that reducing the intracellular redox potential by antioxidants might provide a condition which stimulates tyrosine phosphorylation. Knirsch and Clerch (2001) found that a cytoplasmic protein can bind to the 3'untranslated region of Mn SOD mRNA and this binding was regulated by the phosphorylation of Mn SOD binding protein affecting the Mn SOD protein expression. Therefore, expression of Mn SOD is also under the control by phosphorylation/dephosphorylation cascade systems.

As a conclusion, our results showed that, diabetes and the resulting oxidative stress coordinately regulate the activities of the antioxidant enzymes at molecular level. LA and VC, two powerful antioxidants affect all antioxidant enzyme activities at the level of either transcription and/or translation, since, changes in the intracellular redox state appear to regulate several of the critical intracellular pathways. In addition, there appears to be a group of protein targets

within the cell which can alter their function following this redox changes induced by diabetes. These results concerning the antioxidant enzymes and antioxidant treatment in diabetes provide an outline of a regulatory network consisting of specific oxidant generators and targets. In order to understand the molecular mechanism better, future studies should emphasize on the identification of the transcriptional and translational factors, and specific protein targets present in the cell required for the activation of the antioxidant enzymes following the oxidative stress.

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APPENDIX A

BLOOD GLUCOSE CONCENTRATIONS OF ANIMALS (mg/dl)

1. Diabetic Animals

D-1	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	92	421	227	165	436
R(1)	109	438	231	131	185
R(2)	110	372	480	370	194
R(3)					

D-2	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	130	360	460	232	562
R(1)	120	360	441	178	530
R(2)	120	362	200	92	583
R(3)	120	379	469	213	490

D-3	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	115	432	272	365	467
R(1)	85	421	361	244	ex
R(2)	110	367	156	163	ex
R(3)	117	412	149	352	280

2. Vitamin C Supplemented Diabetic Animals

D+VC-1	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	90	454	504	440	450
R(1)	104	409	403	427	434
R(2)	109	432	527	480	491
R(3)	101	512	532	444	496

D+VC-2	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	102	421	435	497	478
R(1)	106	432	427	423	441
R(2)	96	421	427	420	491
R(3)	106	430	477	485	482

D+VC-3	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	95	463	485	489	499
R(1)	91	375	482	456	434
R(2)	102	470	469	468	509
R(3)	107	436	498	496	461

3. α-Lipoic Acid Supplemented Diabetic Animals

D+LA-1	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	95	377	432	162	470
R(1)	97	392	376	350	482
R(2)	105	371	424	368	460
R(3)	95	390	102	143	ex

D+LA-2	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	96	367	464	326	428
R(1)	110	360	Ex	Ex	Ex
R(2)	99	289	166	112	100
R(3)	106	344	Ex	Ex	Ex

D+LA-3	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	107	382	Ex	ex	ex
R(1)	106	378	329	314	540
R(2)	102	360	252	297	478
R(3)	110	349	184	181	315

4. α-Lipoic Acid and Vitamin C Supplemented Diabetic Animals

D+VC+LA-1	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	102	335	238	126	117
R(1)	102	345	371	364	428
R(2)	98	335	519	370	525
R(3)	92	348	364	171	ex

D+VC+LA-2	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	102	330	334	453	402
R(1)	110	315	429	379	174
R(2)	101	374	KUYRUĞU	KESİK	EX
R(3)	111	337	327	425	ex

D+VC+LA-3	*02.11.2006*	*08.11.2006*	*21.11.2006*	*30.11.2006*	*13.12.2006*
R(0)	100	354	89	Ex	
R(1)	95	340	126	Ex	
R(2)	96	435	127	144	327
R(3)	107	321	197	384	405

APPENDIX B

WEIGHTS OF ANIMALS (gr)

1. Diabetic Animals

D-1	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	243	214	203	198	198	207
R(1)	241	238	237	255	238	235
R(2)	236	215	213	220	215	234
R(3)	-----	-----	-----	-----	-----	

D-2	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	280	277	245	251	265	243
R(1)	285	240	217	204	218	207
R(2)	290	238	209	184	184	179
R(3)	207	192	174	188	178	172

D-3	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	163	157	132	127	129	125
R(1)	205	194	163	149	ex	
R(2)	216	196	176	152	ex	
R(3)	168	183	186	185	214	210

2. Vitamin C Supplemented Diabetic Animals

D+VC-1	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	180	175	165	159	164	154
R(1)	178	165	157	148	157	161
R(2)	180	174	176	165	176	179
R(3)	184	159	150	146	149	155

D+VC-2	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	189	176	154	149	158	134
R(1)	190	185	160	177	174	158
R(2)	194	176	159	154	160	155
R(3)	195	183	167	165	168	166

D+VC-3	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	195	178	169	160	168	162
R(1)	196	175	182	170	168	169
R(2)	186	173	167	164	163	157
R(3)	184	162	161	141	148	136

3. α-lipoic acid Supplemented Diabetic Animals

D+LA-1	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	243	225	195	184	196	185
R(1)	241	217	209	208	210	210
R(2)	245	208	189	183	183	175
R(3)	233	213	194	175	148	ex

D+LA-2	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	245	229	224	230	222	220
R(1)	245	205	EX	ex	ex	
R(2)	242	266	280	305	306	309
R(3)	246	EX	ex	ex	ex	

D+LA -3	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	225	EX		ex		
R(1)	233	226	211	214	213	219
R(2)	232	196	177	176	186	185
R(3)	224	198	179	177	179	175

4. α-lipoic acid and Vitamin C Supplemented Diabetic Animals

D+VC+LA-1	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	264	241	223	238	225	167
R(1)	266	238	216	217	218	225
R(2)	258	228	203	197	206	192
R(3)	269	245	218	215	199	ex

D+VC+LA-2	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	254	226	202	197	209	205
R(1)	257	227	184	195	197	208
R(2)	248	223	195	182	173	ex
R(3)	251	218	193	201	177	ex

D+VC+LA-3	*02.11.2006*	*14.11.2006*	*21.11.2006*	*28.11.2006*	*4.12.2006*	*11.12.2006*
R(0)	247	227	186	ex		
R(1)	243	213	187	ex		
R(2)	241	228	214	218	243	239
R(3)	247	219	185	188	185	193

APPENDIX C

THE PROTEIN CONTENTS

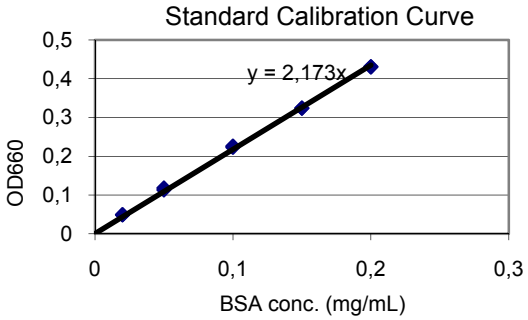


Figure A1: Standard calibration curve for lowry protein determination methods

Table A1: The protein contents (mg/ml) of the cellular cytoplasmic fractions measured by Lowry (1951) method.

	SAMPLE	1.000g	16.000g		SAMPLE	1.000g	16.000g
K1	K1R0	*58,08*	*52,71*	D+VC1	VC1R0	*60,33*	*40,17*
K2	K1R2	*55,15*	*50,18*	D+VC2	VC1R1	*47,31*	*36,95*
K3	K2R0	*50,78*	*44,72*	D+VC3	VC1R2	*68,27*	*46,37*
K4	K2R2	*49,45*	*45,02*	D+VC4	VC1R3	*48,36*	*35,71*
K5	K2R3	*61,13*	*57,27*	D+VC5	VC2R0	*53,49*	*41,41*
K6	K3R0	*59,92*	*52,41*	D+VC6	VC2R1	*49,04*	*36,95*
K7	K3R1	*61,36*	*54,03*	D+VC7	VC2R2	*42,31*	*32,98*
K8	K3R2	*60,15*	*74,5*	D+VC8	VC2R3	*51,82*	*41,9*
K9	K3R3	*74,12*	*70,29*	D+VC9	VC3R0	*57,54*	*45,87*
D1	D1R0	*72,4*	*60,63*	D+VC10	VC3R1	*73,71*	*36,95*
D2	D1R1	*64*	*59,13*	D+VC11	VC3R2	*60,46*	*51,82*
D3	D1R2	*63,66*	*43,1*	D+VC12	VC3R3	*33,66*	*26,78*
D4	D2R0	*76,59*	*57,87*	D+LA+VC1	C1R0	*37,54*	*49,65*
D5	D2R1	*76,31*	*58,77*	D+LA+VC2	C1R1	*81,83*	*78,8*
D6	D2R2	*68,95*	*52,53*	D+LA+VC3	C1R2	*64,69*	*62,24*
D7	D2R3	*75,39*	*64,17*	D+LA+VC4	C2R0	*66,5*	*57,93*
D8	D3R0	*49,34*	*43,88*	D+LA+VC5	C2R1	*79,36*	*72,23*
D9	D3R3	*65,67*	*65,55*	D+LA+VC6	C3R2	*66,7*	*60,37*
D+LA1	LA1R0	*63*	*61,64*	D+LA+VC7	C3R3	*85,4*	*59,3*
D+LA2	LA1R1	*92,35*	*45,27*				
D+LA3	LA1R2	*66,05*	*61,7*				
D+LA4	LA2R0	*66,24*	*49,84*				
D+LA5	LA2R2	*58,19*	*34,67*				
D+LA6	LA3R1	*105,8*	*65,63*				
D+LA7	LA3R2	*90,4*	*64,78*				
D+LA8	LA3R3	*62,74*	*62,78*				

APPENDIX D

RNA CONCENTRATIONS

Table A2: Spectrophotometric determination of total RNA concentration *(µg/ml)* and OD_{260}/OD_{280} rations of all samples.

	OD260	OD280	OD260/280	RNA(µg/ml)	Average RNA	
K1R0	0,251	0,12	2,081	1.002,93	1.014,40	K1
K1R0'	0,256	0,124	2,072	1.025,88		
K1R2	0,465	0,232	2,007	1.861,33	1.854,25	K2
K1R2'	0,462	0,225	2,049	1.847,17		
K2R0'	0,396	0,191	2,077	1.584,96	1.567,87	K3
K2R0''	0,388	0,186	2,089	1.550,78		
K2R2*	0,157	0,079	1,992	629,395	617,43	K4
K2R2**	0,151	0,078	1,947	605,469		
K2R3*	0,289	0,15	1,924	1.155,76	1.100,10	K5
K2R3**	0,27	0,138	1,959	1.080,08		
K2R3***	0,266	0,135	1,968	1.064,45		
K3R0	0,485	0,25	1,944	1.940,43	1.934,08	K6
K3R0'	0,482	0,245	1,964	1.927,73		
K3R1	0,406	0,206	1,974	1.623,54	1.645,26	K7
K3R1'	0,417	0,211	1,971	1.666,99		
K3R2	0,081	0,04	2,024	325,195	323,24	K8
K3R2'	0,08	0,039	2,037	321,289		
K3R3*	0,195	0,1	1,948	781,738	783,2	K9
K3R3**	0,196	0,1	1,967	784,668		
D1R0	0,17	0,078	2,187	679,199	655,52	D1
D1R0'	0,158	0,072	2,201	631,836		
D1R1	0,412	0,204	2,017	1.649,41	1.636,47	D2
D1R1'	0,406	0,198	2,045	1.623,54		
D1R2	0,346	0,168	2,056	1.382,32	1.396,48	D3
D1R2'	0,353	0,173	2,042	1.410,65		
D2R0	0,427	0,212	2,015	1.706,06	1.700,68	D4
D2R0'	0,424	0,211	2,007	1.695,31		
D2R1	0,195	0,092	2,125	779,297	813,97	D5
D2R1'	0,212	0,109	1,953	848,633		
D2R2	0,337	0,172	1,96	1.346,68	1.346,68	D6
D2R2	0,337	0,172	1,96	1.346,68		
D2R3	0,196	0,1	1,962	784,668	780,76	D7
D2R3'	0,194	0,1	1,947	776,855		
D3R0	0,154	0,078	1,969	614,258	617,92	D8
D3R0'	0,155	0,08	1,952	621,582		

D3R3	0,267	0,139	1,916	1.066,41	1.072,75	D9
D3R3'	0,27	0,143	1,891	1.079,10		
LA1R0	0,197	0,095	2,073	787,598	794,43	D+LA1
LA1R0'	0,2	0,097	2,069	801,27		
LA1R1	0,194	0,095	2,049	775,391	758,06	D+LA2
LA1R1'	0,185	0,091	2,036	740,723		
LA1R2	0,191	0,091	2,097	762,695	742,68	D+LA3
LA1R2'	0,181	0,086	2,102	722,656		
LA2R0	0,199	0,097	2,044	794,434	744,79	D+LA4
LA2R0'	0,159	0,089	1,788	637,207		
LA2R0''	0,201	0,098	2,042	802,734		
LA2R2	0,064	0,027	2,395	257,324	338,7	D+LA5
LA2R2'	0,128	0,058	2,216	511,719		
LA2R2''	0,062	0,026	2,398	247,07		
LA3R1	0,26	0,129	2,013	1.039,06	891,28	D+LA6
LA3R1'	0,164	0,088	1,86	657,715		
LA3R1''	0,244	0,138	1,774	977,051		
LA3R2	0,19	0,099	1,911	758,789	769,29	D+LA7
LA3R2'	0,195	0,102	1,917	779,785		
LA3R3	0,181	0,083	2,186	723,633	739,58	D+LA8
LA3R3'	0,179	0,083	2,148	715,332		
LA3R3**	0,195	0,102	1,917	779,785		
VC1R0	0,246	0,124	1,981	985,84	979,49	D+VC1
VC1R0''	0,243	0,124	1,962	973,145		
VC1R1'	0,229	0,117	1,956	914,063	902,83	D+VC2
VC1R1''	0,223	0,115	1,938	891,602		
VC1R2	0,272	0,137	1,983	1.086,43	1.103,27	D+VC3
VC1R2'	0,28	0,143	1,961	1.120,12		
VC1R3	0,291	0,148	1,97	1.163,09	1.199,22	D+VC4
VC1R3'	0,309	0,157	1,964	1.235,35		
VC2R0	0,251	0,127	1,981	1.005,86	977,05	D+VC5
VC2R0'	0,237	0,119	1,986	948,242		
VC2R1	0,251	0,127	1,97	1.003,42	1.034,42	D+VC6
VC2R1'	0,266	0,135	1,969	1.065,43		
VC2R2	0,169	0,085	1,994	675,781	716,55	D+VC7
VC2R2'	0,189	0,096	1,971	757,324		
VC2R3	0,274	0,141	1,951	1.097,17	1.061,85	D+VC8
VC2R3'	0,256	0,13	1,969	1.025,88		
VC2R3''	0,266	0,136	1,946	1.062,50		
VC3R0	0,087	0,044	1,98	346,191	335,69	D+VC9
VC3R0'	0,081	0,04	2,037	325,195		
VC3R1	0,223	0,111	1,999	891,113	888,43	D+VC10
VC3R1'	0,221	0,112	1,972	885,742		
VC3R2	0,234	0,117	1,999	937,988	911,62	D+VC11
VC3R2'	0,224	0,113	1,978	896,484		
VC3R2''	0,225	0,115	1,962	900,391		
VC3R3	0,07	0,035	1,993	278,32	289,55	D+VC12

VC3R3*	0,075	0,04	1,867	300,781		
C1R0	0,267	0,134	1,988	1.066,90	1.057,13	D+LA+VC1
C1R0'	0,262	0,131	2,001	1.047,36		
C1R1	0,198	0,104	1,908	790,039	798,99	D+LA+VC2
C1R1'	0,216	0,11	1,954	863,281		
C1R1''	0,186	0,093	2,009	743,652		
C1R2	0,084	0,042	2,024	335,938	329,1	D+LA+VC3
C1R2'	0,081	0,04	2,025	322,266		
C2R0	0,238	0,12	1,992	952,148	945,56	D+LA+VC4
C2R0'	0,235	0,118	1,991	938,965		
C2R1	0,206	0,104	1,985	824,707	811,77	D+LA+VC5
C2R1'	0,2	0,101	1,978	798,828		
C3R2	0,22	0,113	1,936	878,418	862,3	D+LA+VC6
C3R2'	0,212	0,109	1,943	846,191		
C3R3	0,2	0,101	1,977	798,34	819,34	D+LA+VC7
C3R3'	0,21	0,105	1,999	840,332		

APPENDIX E

RESULTS OF NUCLEIC ACID SEQUENCING AND DATA CROSS-CHECK WITH NCBI-BLAST2

Obtained B-Actin Forward Sequence

TTTTGACGGGCGTGAGGCCAGGATAGAGCCACCAATCCACACAGAGTACTTGCGCTCAGGAGGAGCAAT
GATCTTGATCTTCATGGTGCTAGGAGCCAGGGCAGTAATCTCCTTCTGCATCCTGTCAGCAATGCCTGGGT
ACATGGTGGTGCCACCAGACAGCACTGTGTTGGCATAGAGGTCTTTACGGATGTCAACGTCACACTTCATG
ATGGAATTGAATGTAGTTTCATGGATGCCACAGGATTCCATACCCAGGAAGGAAGGCTGGAAGAGAGCCT
CGGGGCATCGGAACCGCTCATTGCCGATAGTGATGACCTGACCGTCAGGCAGCTCATAGCTCTTCTCCAG
GGAGGAAGAGGATGCGGCAGTGGCCATCTCTTGCTCGAAGTCTAGGGCAACATAGCACAGCTTCTCTTTA
ATGTCACGCACGATTTCCCTCTCAGCTGTGGTGGTGAAGCTGAGCCCCCCCCCCGGTACGGAAAAAAAAA
AAAGGGAGCAATAAGGGGTTCCAATGCCCGGAGGTTTTCCCCCCCCCTCCGGGTAGGAACCGGGACCAAC
ACCTATTTCTAGGGGGGAGGTAACCAAAAACCCCCCCAGGTCCGGGCCCCGCCCACCGTAGGAAAGAAAA
AAAACACCCCCCCGGAAAATAAATATCCAACAACATTTTAAAAAAAAAAGAACAAAAA

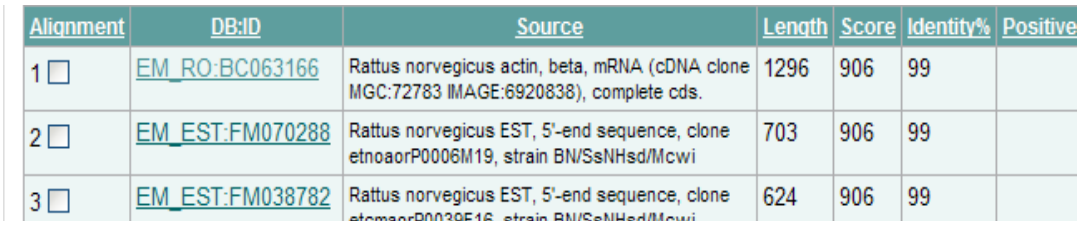
NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:EF156276	Rattus norvegicus beta-actin mRNA, partial cds.	849	904	100		0.0
2 ☐	EM_RO:BC063166	Rattus norvegicus actin, beta, mRNA (cDNA clone MGC:72783 IMAGE:6920838), complete cds.	1296	904	100		0.0
3 ☐	EM_EST:FM070288	Rattus norvegicus EST, 5'-end sequence, clone	703	904	100		0.0

Obtained B-Actin Reverse Sequence

TGCCCCCAGCTGAGAGGGAATCGTGCGTGACATTAAAGAGAAGCTGTGCTATGTTGCCCTAGACTTCGAG
CAAGAGATGGCCACTGCCGCATCCTCTTCCTCCCTGGAGAAGAGCTATGAGCTGCCTGACGGTCAGGTCA
TCACTATCGGCAATGAGCGGTTCCGATGCCCCGAGGCTCTCTTCCAGCCTTCCTTCCTGGGTATGGAATCCT
GTGGCATCCATGAAACTACATTCAATTCCATCATGAAGTGTGACGTTGACATCCGTAAAGACCTCTATGCC
AACACAGTGCTGTCTGGTGGCACCACCATGTACCCAGGCATTGCTGACAGGATGCAGAAGGAGATTACTG
CCCTGGCTCCTAGCACCATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCTGTGTGGATTGGT
GGCTCTATCCTGGCCTCACTGTCCACCTTCCAGCAGATTACCCCCCAAAAAAGCAGGGAACAAGCCCATCC
GATTATGGGCTGGGAAGAGAGTGATTTTTATCTTCTGGTGCTGGGAACGGGGCGGGATCCTCGCGCGTCC
AGCGGGTTATGGTGGGTCACATCTAGATTGCTAAGCTTTTCGAGGCACCTTCATGAAGTAGCCTTCGGAG
AAGAACTCGAAGAAGCGTAGAAGCGGGGTGCAATTTTAACGAA

NCBI-BLAST2 Results

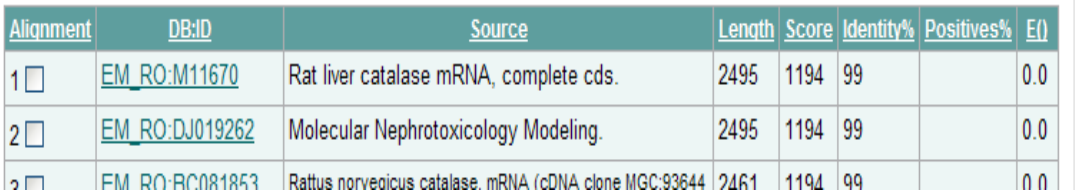


Alignment	DB:ID	Source	Length	Score	Identity%	Positive
1 ☐	EM_RO:BC063166	Rattus norvegicus actin, beta, mRNA (cDNA clone MGC:72783 IMAGE:6920838), complete cds.	1296	906	99	
2 ☐	EM_EST:FM070288	Rattus norvegicus EST, 5'-end sequence, clone etnoaorP0006M19, strain BN/SsNHsd/Mcwi	703	906	99	
3 ☐	EM_EST:FM038782	Rattus norvegicus EST, 5'-end sequence, clone	624	906	99	

CAT Forward Sequence

GTGCTGTCGTTAATGACTGACCAGCGCATCACACCTTGCCTGTTGAAGAGGCAGGAAGACTTGCACAGGA
AGACCCGGATTATGGCCTCCGAGATCTTTTCAATGCCATCGCCAGTGGCAATTACCCATCCTGGACTTTTTA
CATCCAGGTCATGACTTTCAAGGAGGCAGAAACCTTCCCATTTAATCCATTTGACCTGACCAAGGTTTGGC
CTCACAAGGACTACCCTCTTATACCAGTTGGCAAACTGGTCTTAAACAGAAATCCTGCTAATTATTTTGCTG
AAGTTGAACAGATGGCTTTTGACCCAAGCAACATGCCCCCTGGCATTGAGCCCAGCCCGGACAAGATGCT
CCAGGGCCGCCTTTTTTGCTTACCCAGACACTCACCGCCACCGCCTGGGACCAAACTATCTGCAGATACCTG
TGAACTGTCCCTACCGTGCTCGCGTGGCCAACTACCAGCGCGATGGCCCCATGTGCATGCATGACAACCA
GGGTGGTGCTCCCAACTACTACCCCAACAGCTTCAGCGCACCAGAGCAGCAGGGCTCGGCCCTGGAGCAC
CATAGCCAGTGCTCTGCAGATGTGAAGCGCTTCAACAGTGCTATAAACACAACCGTCCATCCA

NCBI-BLAST2 Results



Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:M11670	Rat liver catalase mRNA, complete cds.	2495	1194	99		0.0
2 ☐	EM_RO:DJ019262	Molecular Nephrotoxicology Modeling.	2495	1194	99		0.0
3 ☐	EM_RO:BC081853	Rattus norvegicus catalase, mRNA (cDNA clone MGC:93644	2461	1194	99		0.0

CAT Reverse Sequence

TTTGGAAGCTCTTCAATCTTGCAGAGCACTGGCTATGGTGCTCCAGGGCCGAGCCCTGCTGCTCTGGTGCG
CTGAAGCTGTTGGGGTAGTAGTTGGGAGCACCACCCTGGTTGTCATGCATGCACATGGGGCCATCGCGCT
GGTAGTTGGCCACGCGAGCACGGTAGGGACAGTTCACAGGTATCTGCAGATAGTTTGGTCCCAGGCGGT
GGCGGTGAGTGTCTGGGTAAGCAAAAAGGCGGCCCTGGAGCATCTTGTCCGGGCTGGGCTCAATGCCAG
GGGGCATGTTGCTTGGGTCAAAAGCCATCTGTTCAACTTCAGCAAAATAATTAGCAGGATTTCTGTTTAAG
ACCAGTTTGCCAACTGGTATAAGAGGGTAGTCCTTGTGAGGCCAAACCTTGGTCAGGTCAAATGGATTAA
ATGGGAAGGTTTCTGCCTCCTTGAAAGTCATGACCTGGATGTAAAAAGTCCAGGATGGGTAATTGCCACT
GGCGATGGCATTGAAAAGATCTCGGAGGCCATAATCCGGGTCTTCCTGTGCAAGTCTTCCTGCCTCTTCAA
CAGGCAAGTTTTTGATGCCCTGGTCAGTCTTGTAATGGAACTTGCAGTACATGTTTTTTTTTTATTTAAAAA
AAAAAAAAAAAGATTAAAAAACTTGAATGGCGAGGAGGCGGGAAGACTTGAACAGAAAGACCGGATTAT
TGAGCTCCAAAGATCATATTCAATGCCATCGCAGGTGGCAATTAACCATCATGACTTTATACATGAGGTCA
TTAACTTTAAAGGAAGAAAAAAACATAGCAATGATAATATAGCAAAGCA

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:M11670	Rat liver catalase mRNA, complete cds.	2495	1170	98		0.0
2 ☐	EM_RO:DJ019262	Molecular Nephrotoxicology Modeling.	2495	1170	98		0.0
3 ☐	EM_RO:BC081853	Rattus norvegicus catalase, mRNA (cDNA clone MGC:93644 IMAGE:7109889), complete cds.	2461	1170	98		0.0

SOD1 Forward Sequence

TTGTGGTGTATGTCGATTAAGGATTACTGAGGGCGAGCATCGGGTTCCATCGTCCATCAATATGGGGACA
ATACACAAGGCTGTACCACTGCAGGACCTCATTTTAATCCTCACTCTAAGAAACATGGCGGTCCAGCGGAT
GAAGAGAGGCATGTTGGAGACCTGGGCAATGTGGCTGCTGGAAAGGACGGTGTGGCCAATGTGTCCATT
GAAGATCGTGTGATCTCACTCTCAGGAGAGCATTCCATCATTGGCCGTACTATGGTGGTCCACGAGAAAC
AAGATGACTTGGGCAAAGGTGGAAATGAAGAAAGTACAAAGACTGGAAATGCTGGAAGCCGCTTGGCTT
GTGGTGTGATTGGGATTGCCCAATAAACATTCCCTATGTGGTCTGAGTCTCAGACTCATTCGGGGTCCGGC
TACCACGCATGAGTGGGTAAGGGGTAATGCAAATATAAACAATAAAAGATTGGGGGGGAAAAAAAAAAG
GAGAAAAAAAAAAAAAAAAAAAAAAAAAGGAAAAGAGGGGGGAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAACAAAAGAAAAATAAAAAAAAAGATAAAAAAAAAAAAAAGAAGAAAGAAAAAAAAAGA
AAAAAAAAAAAAACAAAAAGAATAATAAGAAAAGAGAAATACATAGATACAAACACAATAAAAACAATA
AAATAAAGAAAAAAAAACAAAAGGAAAATAAAAAAACAAAAAAAAAAACAATAAACAAAAAAAAAAAAA
AAAAAAAAAAAAAATAAAAAAAAAAAA

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:Y00404	Rat mRNA for copper-zinc-containing superoxide dismutase	650	758	96		0.0
2 ☐	EM_RO:X05634	Rat mRNA for CuZn supteroxide dismutase (EC 1.15.1.1)	547	758	96		0.0
3 ☐	EM_RO:DJ007501	Primary rat hepatocyte toxicity modeling.	650	758	96		0.0
4 ☐	EM_RO:BC082800	Rattus norvegicus superoxide dismutase 1, soluble, mRNA (cDNA clone MGC:105454 IMAGE:7304983), complete cds.	665	758	96		0.0

SOD1 Reverse Sequence

CTGACTCGACCACTAGGGATGTTTATTGGGCATCCCAATCACACCACAAGCCAAGCGGCTTCCAGCATTTC
CAGTCTTTGTACTTTCTTCATTTCCACCTTTGCCCAAGTCATCTTGTTTCTCGTGGACCACCATAGTACGGCC
AATGATGGAATGCTCTCCTGAGAGTGAGATCACACGATCTTCAATGGACACATTGGCCACACCGTCCTTTC
CAGCAGCCACATTGCCCAGGTCTCCAACATGCCTCTCTTCATCCGCTGGACCGCCATGTTTCTTAGAGTGAG
GATTAAAATGAGGTCCTGCAGTGGTACAGCCTTGTGTATTGTCCCCATATTGATGGACATGGAACCCATGC
TCGCCTTCAGTTAATCCTGTAATCTGTCCTGACACCACAACTGGTTCACCGTTTTGCCTTCTGCAAAAATTTT
TTTATTAAAAAGAAGGGGGGGGGGAGGAGAAAAAAAAAAACAAAAAAAAAAAAAAAATAAGAAAAGGG
AGGCGGGGGGAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATAAAAAAAAAAAAAAAAAAAAATAAAAAAAAAAA
AAAACAAAAAAAAAAAAAATAAATAAAAACAAAAAAAAACAAAAAAAAAAAAAATAAAAAAAAAAAAAA
A

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_EST:AI410612	EST238905 Normalized rat heart, Bento Soares Rattus sp. cDNA clone RHECW01 3' end, mRNA sequence.	520	802	98		0.0
2 ☐	EM_RO:Y00404	Rat mRNA for copper-zinc-containing superoxide dismutase	650	798	98		0.0
3 ☐	EM_RO:X05634	Rat mRNA for CuZn supteroxide dismutase (EC 1.15.1.1)	547	798	98		0.0

SOD2 Forward Sequence

TGATGCCCTGCAGCACCACGCGACCTACGTGACATCTGAACGTCACCGAGGAGAAGTACCACGAGGCGCT
GGCCAAGGGAGATGTTACAACTCAGGTTGCTCTTCAGCCTGCACTGAAGTTCAATGGCGGGGGCCATATC
AATCACAGCATTTTCTGGACAAACCTGAGCCCTAAGGGTGGTGGAGAACCCAAAGGAGATTTGCTGGAGG
CTTAGAGGGTGGGAGAAAGCTAAGTTAACAAAAAAAAAAATTAAGGAAACTAAAAAAAAAAAAAAAAAA
AAGGGCAAAAAGTATA

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:Y00497	Rat mRNA for manganese-containing superoxide dismutase (MnSoD)	1438	382	98		2e-91
2 ☐	EM_RO:DJ007908	Primary rat hepatocyte toxicity modeling.	1438	382	98		2e-91
3 ☐	EM_RO:BC070913	Rattus norvegicus superoxide dismutase 2, mitochondrial, mRNA (cDNA clone MGC:91729 IMAGE:7104698), complete cds.	2090	382	98		2e-91
4 ☐	EM_PAT:DL486416	Molecular Toxicology Modeling.	1492	382	98		2e-91

SOD2 Reverse Sequence

TTGGTTCCCCCCTTAGGGCTCAGGTTTGTCCAGAAATGCTGTGATTGATATGGCCCCCGCCATTGAACTTCA
GTGCAGGCTGAAGAGCAACCTGAGTTGTAACATCTCCCTTGGCCAGCGCCTCGTGGTACTTCTCCTCGGTG
ACGTTCAGATTGTTCACGTAGGTCGCGTGGTGCTTGCTGTGGTGCAGCTGCATGATCTGGCCGTTAATGTG
CCAACGGAGGACTTTCGCGTATTTTTTTTAATTCAAAAAAAAGTCCGGGGCTGCAGGAAAATTCACTGGCC
TATCCCGAGAAATAAGGGAGGACAACCCATTTTAAACAAAACAAGAAAAAGTAAAACAACGTTATAAAAA
AAAAAAAAAAACAATAAAAAAAATAAAAAAGAAAAA

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:Y00497	Rat mRNA for manganese-containing superoxide dismutase (MnSoD)	1438	396	98		5e-95
2 ☐	EM_RO:DJ007908	Primary rat hepatocyte toxicity modeling.	1438	396	98		5e-95
3 ☐	EM_RO:BC070913	Rattus norvegicus superoxide dismutase 2, mitochondrial, mRNA (cDNA clone MGC:91729 IMAGE:7104698), complete cds.	2090	396	98		5e-95
4 ☐	EM_PAT:DL486416	Molecular Toxicology Modeling.	1492	396	98		5e-95

GPx Forward Sequence

TGCGTATGCTTCTCCGCGCGCCCGCTGGCGGGCGGGGAGCCCGTGAGCCTGGGCTCCCTGCGGGGCAAG
GTGCTGCTCATTGAGAATGTCGCGTCCCTCTGAGGCACCACGACCCGGGACTACACCGAAATGAATGATC
TGCAGAAGCGTCTGGGGCCTCGTGGCCTGGTGGTGCTCGGTTTCCCGTGCAATCAGTTCGGACATCAGGA
GAATGGCAAGAATGAAGAGATTCTGAATTCCCTCAAGTATGTCCACACCCGGTGGTGGATTTCCCCACCCC
GTGGCGGCTCTGATACCAATGTGCCCCGAACAAACCCCGCCAAGCCCAACTTTCAATATAATTCTTTAACG
AACGGGCTCAAGGGACGATTAAAAACCCCCTAAAAACCAAGAAAAACCCCCCCCCCCAAAAGGAAATAAC
TTTCCCTCCCAAAACCAAAAAAAAAAAAAAAATATAAAATTCCCTCAAAAATGTCCAAACGGGGTGGTGG
GGGGGAAAAAAAAAAAAAAAAAAAAAAAATAAAATACAAAAAAGAGCGAGCAGACAAATCATAAAAAA
ATAAAAAAAAAAAATACTACAACACAAAAAAAAAAAAAAAACAAATAAATGAAAGAGAAAAAAAATAA
CACCCCATAAAACTAAAAAAAAAAAAAAAAAAACACAAAAAAACAAAATAAAAAAAAAAA

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:X12367	R.norvegicus mRNA for glutathione peroxidase I	1539	508	98		1e-125
2 ☐	EM_RO:X07365	Rat mRNA for glutathione peroxidase	1161	508	98		1e-125
3 ☐	EM_RO:DJ019559	Molecular Nephrotoxicology Modeling.	1539	508	98		1e-125
4 ☐	EM_RO:BC058438	Rattus norvegicus glutathione peroxidase 1, mRNA (cDNA clone MGC:72654 IMAGE:6918849), complete cds.	880	508	98		1e-125

GPx Reverse Sequence

TGGAGGATCGATCTCTTCTTCTTGCCTTCTCCTGATGTCCGAACTATTGCACGGGAAACCGAGCACCACCA
GGCCACGAGGCCCCAGACGCTTCTGCAGATCATTCATTTCGGTGTAGTCCCGGGTCGTGGTGCCTCAGAG
GGACGCGACATTCTCAATGAGCAGCACCTTGCCCCGCAGGGAGCCCAGGCTCACGGGCTCCCCGCCCGCC
AGCGGGCGCGCGGAGAAGGCATACACGGTGGACAGCCCTCCGCGGAGTGACTCTCCCCCCCCCCCAGA
CCGCCGGCCGCCCGTGGGCCTGGGCTTCTTGAGATGATTGGTTTTGCTGTTTTCCAATGAAATGTTGTTCA
GAAAAAAACAACTTTCTCAACGAAAAAAAATATGACAAAAAAAAAAAAAAAAAAACAAAAAAAAAACGC
CAAAAAAAGGGCGCAAAAGAGAAAAAAAAAAAAATGAAAAAAAAAAAACAAAAAAAGAGAAAAAAAAG
GGAAAAAAAAAAATAATAAAAAAACAAAGCAAAGAGAGATAGATAAAAAAAAAAAAAAAAAATAGAA
AAATAAAGTAAAATATATATAAATAAATATATATAAATAAAAAAAAAACAATAAAAAAAAAAAAAAAAA
AAAAAAAAAAACAAAAA

NCBI-BLAST2 Results

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1 ☐	EM_RO:X12367	R.norvegicus mRNA for glutathione peroxidase I	1539	452	97		1e-110
2 ☐	EM_RO:X07365	Rat mRNA for glutathione peroxidase	1161	452	97		1e-110
3 ☐	EM_RO:DJ019559	Molecular Nephrotoxicology Modeling.	1539	452	97		1e-110
4 ☐	EM_RO:BC058438	Rattus norvegicus glutathione peroxidase 1, mRNA (cDNA clone MGC:72654 IMAGE:6918849), complete cds.	880	452	97		1e-110

APPENDIX F

APPROVALS OBTAINED FROM ETHICAL COMMITEE

T.C.
FIRAT ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURULU BAŞKANLIĞI

Sayı : 56 02.08.2008
Konu : Prof. Dr. Tülin GÜRAY'ın Araştırma Projesi hk.

Sayın: Prof. Dr. Tülin GÜRAY

"Lipoik Asit ve C Vitaminin'in STZ ile indüklenmiş Diyabetli Sıçanlarda Glutatyon Peroksidaz, Mangan Süperoksit Dismutaz ve Glutatyon S-Transferaz Gen Ekspresyonlarına Etkilerinin İncelenmesi" başlıklı çalışmanız Etik Kurulumuzca görüşülmüş olup Etik Kurulu Kararı örneği ekte gönderilmiştir.

Bilgileriniz ile gereğini rica ederim.

Prof. Dr. İbrahim H. ÖZERCAN
Etik Kurulu Başkanı

EK: Etik Kurulu Kararı 1 (bir) sayfa

T.C.
FIRAT ÜNİVERSİTESİ
HAYVAN DENEYLERİ ETİK KURULU
ELAZIĞ

ETİK KURULU KARARI

TOPLANTI TARİHİ	TOPLANTI SAYISI	KARAR NO	ÖZÜ
02.08.2008	12	56	Prof. Dr. Tülin GÜRAY

KARAR

"Lipoik Asit ve C Vitaminin'in STZ ile indüklenmiş Diyabetli Sıçanlarda Glutatyon Peroksidaz, Mangan Süperoksit Dismutaz ve Glutatyon S-Transferaz Gen Ekspresyonlarına Etkilerinin İncelenmesi" başlıklı çalışmanız Etik Kurulumuzda görüşülmüş ve etik yönden uygun bulunduğuna oybirliği ile karar verilmiştir.

.

GÖREVİ	ADI SOYADI	BÖLÜMÜ	İMZA
Başkan	Prof. Dr. İbrahim H. ÖZERCAN	Tıp Fakültesi Patoloji A.D.	
Raportör	Yrd. Doç. Dr. Mehmet TUZCU	Fen Edebiyat Fakültesi Moleküler Biyoloji A.D.	
Üye	Doç. Dr. Ramazan BAL	Tıp Fakültesi Biyofizik A.D.	Bulunmadı
Üye	Doç. Dr. Abdullah ÖZEN	Veteriner Fakültesi Deontoloji A.D.	Bulunmadı
Üye	Doç. Dr. Cihan GÜNAY	Veteriner Hekim	
Üye	Tahsin AVCI	Sivil üye	
Üye	Hıdır DOĞAN	Sivil üye	

T.C.
FIRAT ÜNİVERSİTESİ
TIP FAKÜLTESİ ETİK KURUL BAŞKANLIĞI
ELAZIĞ

Sayı : **28.04.2006**
Konu : Çalışma

Sayın: Doç.Dr.Ökkeş YILMAZ

" **İnsülin Uygulanmayan Tip-I Diyabetik Albino Sıçanların Karaciğer Dokusundaki Antioksidan Enzimlerin Ekspresyonları Üzerine α-Lipoik Asit ile Vitamin C'nin Etkilerinin Araştırılması"** adlı çalışması, etik kurulumuzda uygun görülmüş olup; Etik Kurul Karar Örneği ekte gönderilmiştir.

Bilgilerinizi ve gereğini rica ederim.

Prof. Dr. Emir DÖNDER
Etik Kurul Başkanı

T.C.
FIRAT ÜNİVERSİTESİ
TIP FAKÜLTESİ
ETİK KURULU
ELAZIĞ

ETİK KURUL YÖNETİM KURULU KARARI

TOPLANTI TARİHİ	TOPLANTI SAYISI	KARAR NO	ÖZÜ
17.04.2006	5	1	Doç.Dr.Ökkeş YILMAZ

KARAR

"İnsülin Uygulanmayan Tip-I Diyabetik Albino Sıçanların Karaciğer Dokusundaki Antioksidan Enzimlerin Ekspresyonları Üzerine α-Lipoik Asit ile Vitamin C'nin Etkilerinin Araştırılması " adlı araştırma teklifi; Etik Kurul toplantısında görüşülmüş olup; çalışmanın Tıbbı Deontolojik ve Etik açısından mevcut ilke ve kurallara uygun olduğuna oy birliği ile karar verilmiştir.

GÖREVİ	ADI SOYADI	BÖLÜMÜ	İMZA
Başkan	Prof.Dr.Emir DÖNDER	İç.Hastalıkları A.B.D	İmza
Başkan Yardımcısı	Prof.Dr.Adnan SEYREK	Mik.ve Kli.Mik.A.B.D	İmza
Raportör	Doç.Dr.Doç.Dr.Ahmet AYAR	Fizyoloji A.B.D	İmza
Üye	Doç.Dr.Bayram YILMAZ	Fizyoloji A.B.D	İmza
Üye	Doç.Dr.Ziya ÇETİNKAYA	Genel Cerrahi A.B.D	İmza
Üye	Doç.Dr.İ.Hanifi ÖZERCAN	Patoloji A.B.D	İmza
Üye	Doç.Dr.Engin ŞAHNA	Farmakoloji A.B.D	İmza

APPENDIX G

RAW DATA

Table A3: GSH, MDA and Protien Carbonyl Levels in individual rats

Individual Animals	*GSH (nmol/mg protein)*	*MDA Levels (nmol/mg protein)*	*Protein Carbonyls (nmol/mg protein)*
K1	71,46	0,0219	9,00
K2	86,92	0,0173	10,54
K3	94,98	0,0208	9,88
K4	97,52	0,0192	8,97
K5	62,18	0,0167	10,57
K6	70,32	0,0181	11,03
K7	55,88	0,0166	8,78
K8	77,65	0,0188	8,27
K9	77,15	0,0143	7,99
D1	68,33	0,0170	9,90
D2	51,68	0,0187	14,02
D3	75,66	0,0306	14,93
D4	57,84	0,0209	11,97
D5	69,61	0,0252	13,18
D6	36,77	0,0204	14,12
D7	50,47	0,0293	12,49
D8	59,01	0,2437	11,31
D9	58,68	0,0395	8,07
D+LA1	75,49	0,0153	7,43
D+LA2	84,77	0,0245	4,94
D+LA3	55,71	0,0104	6,37
D+LA4	82,84	0,0172	6,46
D+LA5	81,96	0,0197	5,98
D+LA6	68,64	0,0124	4,99
D+LA7	60,5	0,0132	3,20
D+LA8	64,85	0,0157	5,39
D+VC1	79,52	0,0149	9,91
D+VC2	86,58	0,0174	9,89
D+VC3	93,05	0,0184	12,23
D+VC4	55,14	0,0227	13,14
D+VC5	66,43	0,0186	13,17
D+VC6	62,71	0,0289	5,02
D+VC7	99,6	0,0259	7,94
D+VC8	55,29	0,0224	11,72
D+VC9	72,75	0,0186	6,42
D+VC10	85,29	0,0150	5,76
D+VC11	49,03	0,0181	5,89
D+VC12	118,03	0,0303	7,13
D+LA+VC 1	60,02	0,0224	9,67
D+LA+VC 2	59,59	0,0141	8,10
D+LA+VC 3	61,74	0,0227	8,85
D+LA+VC 4	57,27	0,0155	8,10
D+LA+VC 5	50,63	0,0178	5,06
D+LA+VC 6	67,37	0,0191	7,59
D+LA+VC 7	57,56	0,0231	7,36

Table A4: Activitis of antioxidant enzymes in individual rats

Individual Animals	*CAT Activities (U/mg protein)*	*Cu-Zn SOD Activities (U/mg protein)*	*Mn SOD Activities (U/mg protein)*	*GPx Activities (U/mg protein)*	*GST-Mu Activities (U/mg protien)*
K1	276,37	1,392	0,139	0,533	17,02
K2	272,01	1,357	0,199	0,601	20,74
K3	211,24	0,659	0,195	0,678	12,06
K4	236,50	0,659	0,508	0,671	14,94
K5	293,23	0,874	0,208	0,489	17,96
K6	217,73	1,042	0,164	0,556	19,79
K7	213,95	0,804	0,208	0,537	17,41
K8	223,39	1,027	0,250	0,412	12,67
K9	181,28	0,843	0,184	0,440	13,51
D1	107,35	0,950	0,107	0,490	14,51
D2	127,77	0,777	0,159	0,521	10,82
D3	55,02	0,486	0,245	0,736	15,74
D4	105,64	0,605	0,046	0,558	15,20
D5	126,13	0,812	0,086	0,542	16,63
D6	65,53	0,575	0,358	0,489	10,29
D7	133,86	0,604	0,187	0,476	13,19
D8	99,80	0,343	0,378	0,598	9,45
D9	155,29	1,046	0,205	0,516	10,83
D+LA1	151,81	0,951	0,266	0,573	12,97
D+LA2	92,45	0,719	0,065	0,870	15,81
D+LA3	172,15	1,035	0,189	0,563	10,06
D+LA4	136,55	0,779	0,059	0,761	11,03
D+LA5	181,18	1,089	0,085	1,178	17,67
D+LA6	91,64	0,479	0,179	0,588	10,60
D+LA7	101,69	0,468	0,231	0,571	12,48
D+LA8	152,28	0,958	0,338	0,577	9,27
D+VC1	141,68	0,876	0,069	0,912	14,53
D+VC2	136,70	0,973	0,102	0,939	14,68
D+VC3	168,42	1,092	0,185	0,761	15,60
D+VC4	101,71	0,663	0,244	0,983	11,46
D+VC5	185,54	0,983	0,080	0,860	14,19
D+VC6	204,23	1,167	0,080	1,033	18,12
D+VC7	161,26	1,166	0,119	1,008	15,98
D+VC8	194,75	1,044	0,081	0,843	15,86
D+VC9	163,55	0,694	0,193	0,775	13,67
D+VC10	119,07	0,693	0,098	0,964	19,30
D+VC11	159,51	1,237	0,116	0,711	13,97
D+VC12	73,74	0,306	0,413	1,248	15,79
D+LA+VC 1	105,07	1,022	0,257	0,785	11,50
D+LA+VC 2	144,04	1,158	0,261	0,535	8,94
D+LA+VC 3	194,15	1,463	0,333	0,576	11,68
D+LA+VC 4	128,31	1,115	0,317	0,719	9,38
D+LA+VC 5	84,49	0,277	0,238	0,617	8,36
D+LA+VC 6	161,42	1,007	0,243	0,613	9,35
D+LA+VC 7	141,88	0,968	0,293	0,706	7,89

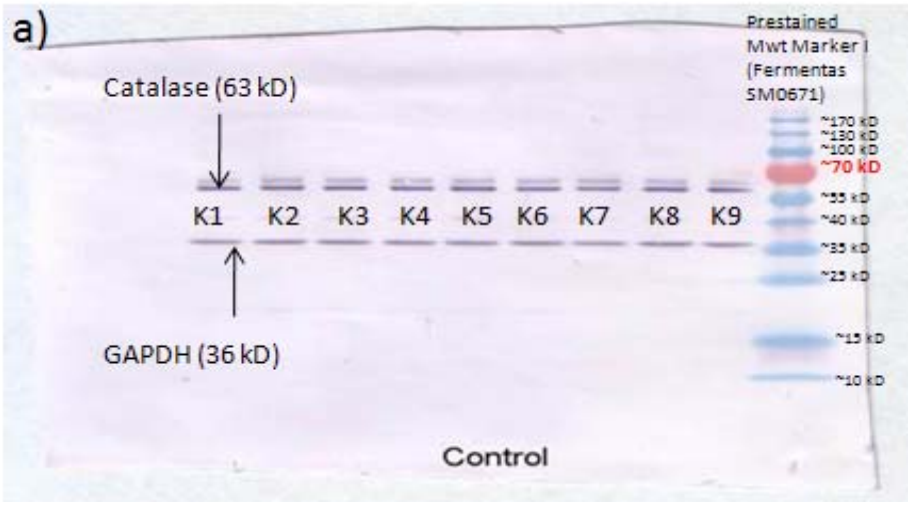


Figure A2: Western-blot results of Control rat liver S1 fractions immunostained by using CAT (above band) and GAPDH (below band) antibodies. Lane 1-9 contains individual control (K1-K9) samples (10 µg total protein/well) and Lane 10 contains 2.5 µl of Type I prestained protein marker.

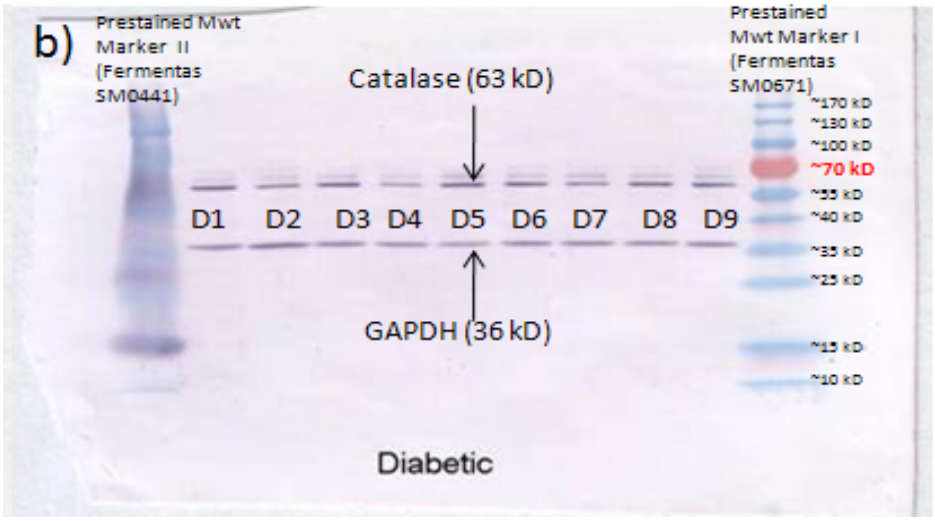


Figure A3: Western-blot results of Diabetic rat liver S1 fractions immunostained by using CAT (above band) and GAPDH (below band) antibodies. Lane 1 contains 2.5 µl Type II prestained protein marker. Lane 2-10 contains individual diabetic (D1-D9) samples (10 µg total protein/well) and Lane 11 contains 2.5 µl of type I prestained protein marker.

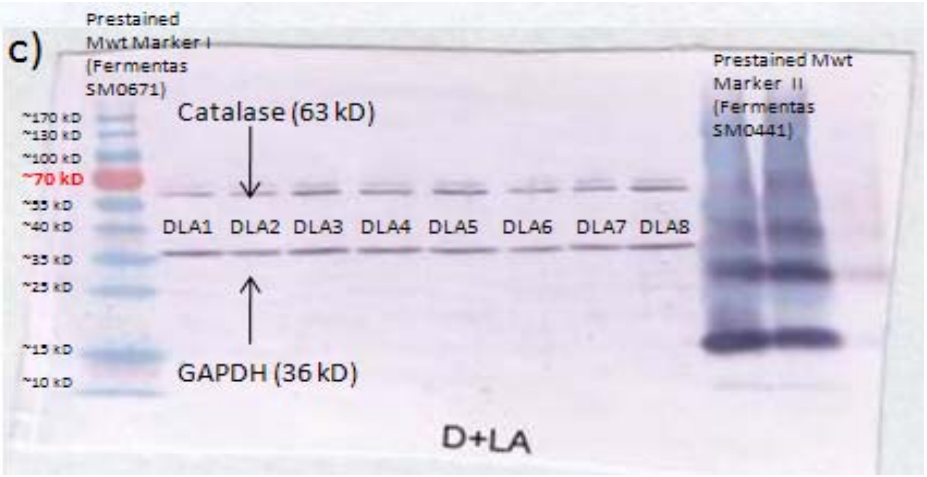


Figure A4: Western-blot results of LA treated Diabetic (D+LA) rat liver S1 fractions immunostained by using CAT (above band) and GAPDH (below band) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-9 contains individual LA treated diabetic (D+LA1-D+LA8) samples (10 µg total protein/well) and Lane 10-11 contains 2.5 µl of type II prestained protein marker.

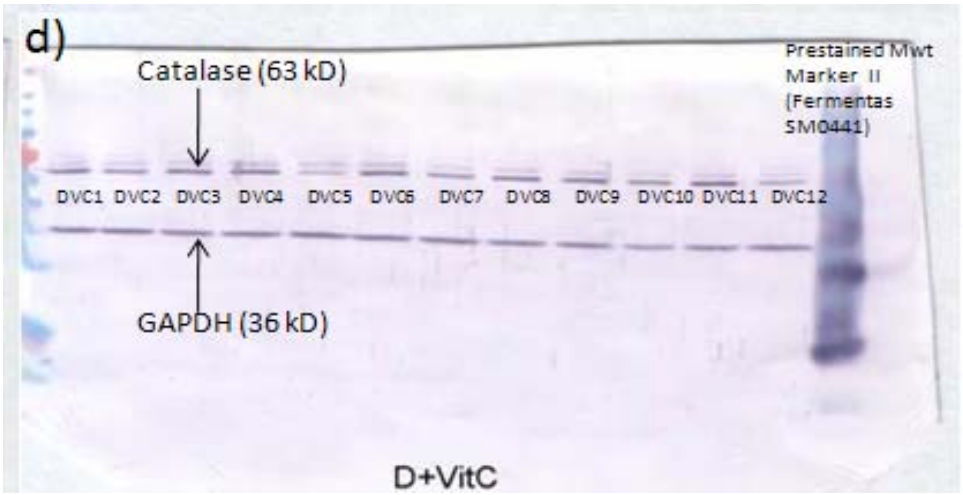


Figure A5: Western-blot results of VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using CAT (above band) and GAPDH (below band) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-13 contains individual VC treated diabetic (D+VC1-D+VC12) samples (10 µg total protein/well) and Lane 13 contains 2.5 µl of type II prestained protein marker.

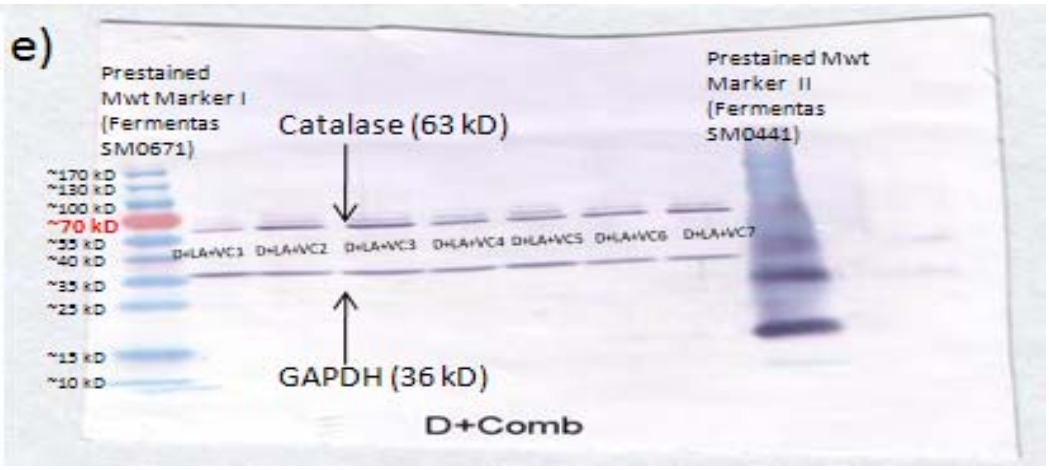


Figure A6: Western-blot results of LA and VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using CAT (above band) and GAPDH (below band) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-8 contains individual LA+VC treated diabetic (D+LA+VC1-D+LA+VC7) samples (10 µg total protein/well) and Lane 9 contains 2.5 µl of type II prestained protein marker.

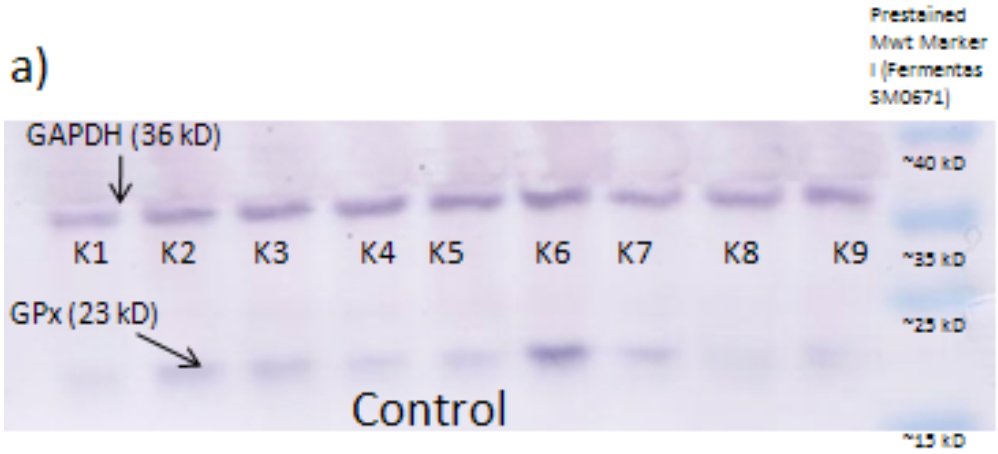


Figure A7: Western-blot results of Control rat liver S1 fractions immunostained by using GPx (below bands) and GAPDH (above bands) antibodies. Lane 1-9 contains individual control (K1-K9) samples (50 µg total protein/well) and Lane 10 contains 2.5 µl of Type I prestained protein marker.

Figure A8: Western-blot results of Diabetic rat liver S1 fractions immunostained by using GAPDH (above band) and GPx (below band) antibodies. Lane 1 contains 2.5 µl Type II prestained protein marker, lane 2-10 contains individual diabetic (D1-D9) samples (50 µg total protein/well) and Lane 11 contains 2.5 µl of type I prestained protein marker.

Figure A9: Western-blot results of LA treated Diabetic (D+LA) rat liver S1 fractions immunostained by using GAPDH (above band) and GPx (below band) antibodies. Lane 9 contains 2.5 µl Type I prestained protein marker, Lane 2-9 contains individual LA treated diabetic (D+LA1-D+LA8) samples (50 µg total protein/well).

Figure A10: Western-blot results of VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above band) and GPx (below band) antibodies. Lane 1 and 14 contains 2.5 ul Type I prestained protein marker, Lane 2-13 contains individual VC treated diabetic (D+VC1-D+VC12) samples (50 µg total protein/well).

Figure A11: Western-blot results of LA and VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and GPx (below bands) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-8 contains individual LA+VC treated diabetic (D+LA+VC1-D+LA+VC7) samples (50 µg total protein/well).

Figure A12: Western-blot results of Control rat liver S1 fractions immunostained by using GAPDH (above bands) and Cu-Zn SOD (below bands) antibodies. Lane 1-9 contains individual control (K1-K9) samples (10 µg total protein/well) and Lane 10 contains 2.5 µl of Type I prestained protein marker.

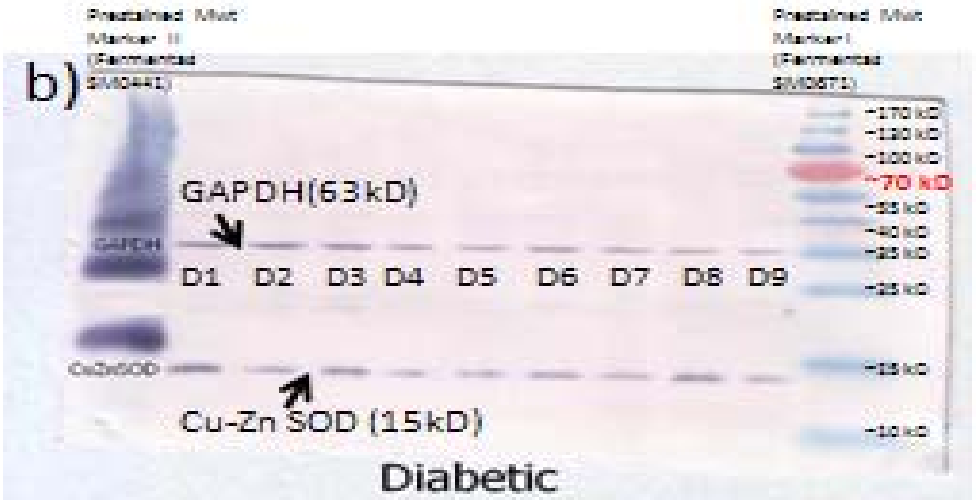


Figure A13: Western-blot results of Diabetic rat liver S1 fractions immunostained by using GAPDH (above bands) and Cu-Zn SOD (below bands) antibodies. Lane 1 contains 2.5 µl Type II prestained protein marker, Lane 2-10 contains individual diabetic (D1-D9) samples (10 µg total protein/well) and Lane 11 contains 2.5 µl of type I prestained protein marker.

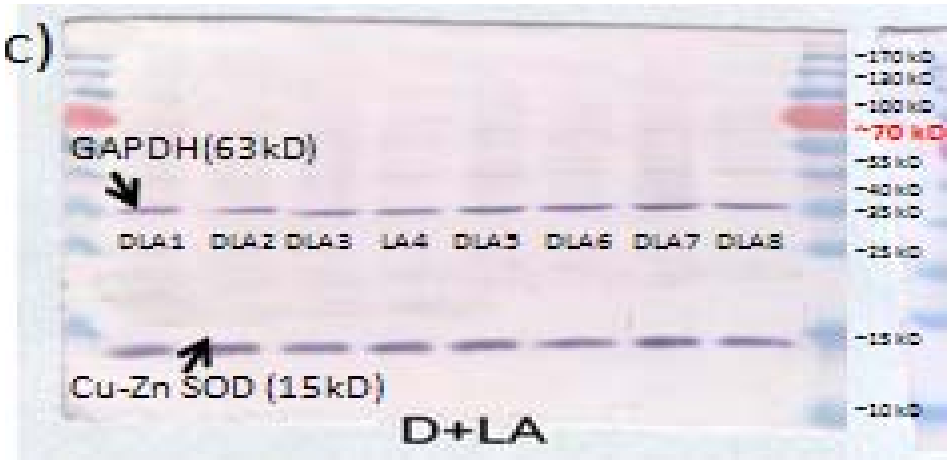


Figure A14: Western-blot results of LA treated Diabetic (D+LA) rat liver S1 fractions immunostained by using GAPDH (above bands) and Cu-Zn SOD (below bands) antibodies. Lane 1 and 10 contains 2.5 µl Type I prestained protein marker, Lane 2-9 contains individual LA treated diabetic (D+LA1-D+LA8) samples (10 µg total protein/well).

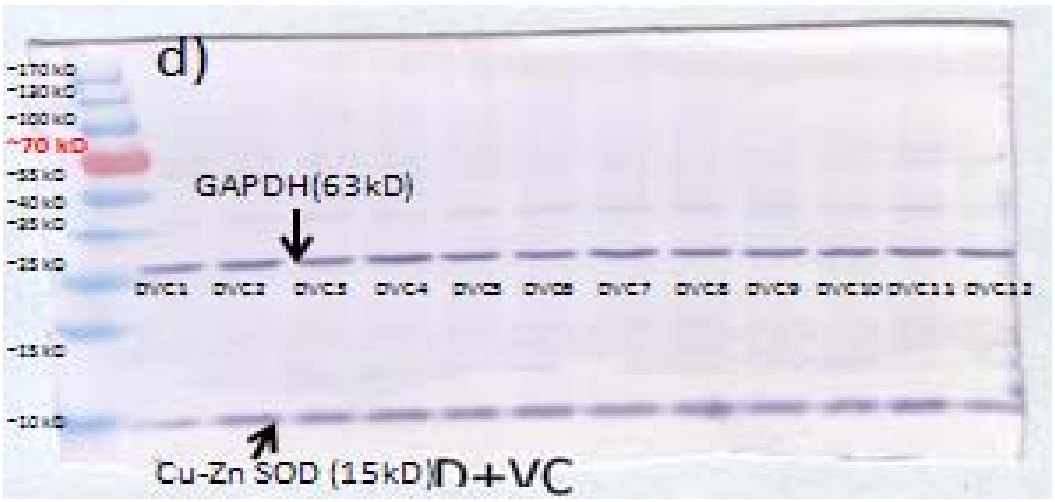


Figure A15: Western-blot results of VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and Cu-Zn SOD (below bands) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-13 contains individual VC treated diabetic (D+VC1-D+VC12) samples (10 µg total protein/well).

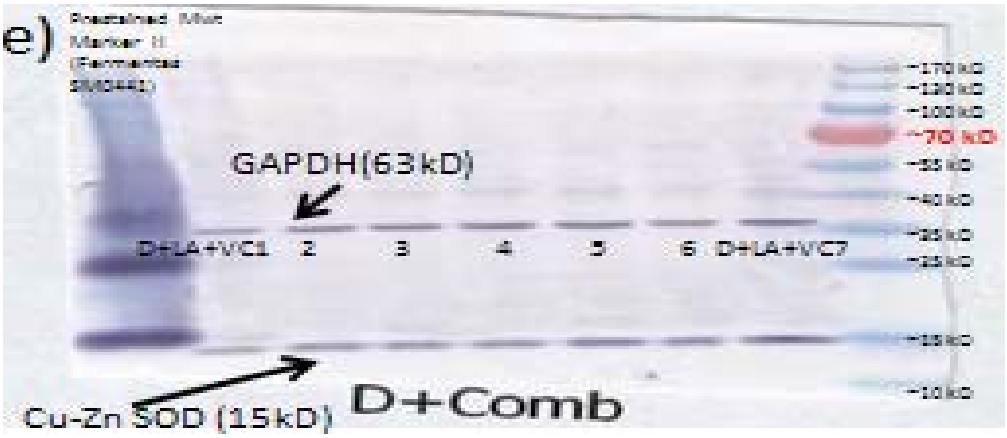


Figure A16: Western-blot results of LA and VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and Cu-Zn SOD (below bands) antibodies. Lane 1 contains 2.5 µl Type II prestained protein marker, Lane 2-8 contains individual LA+VC treated diabetic (D+LA+VC1-D+LA+VC7) samples (10 µg total protein/well) and Lane 9 contains 2.5 µl of type I prestained protein marker.

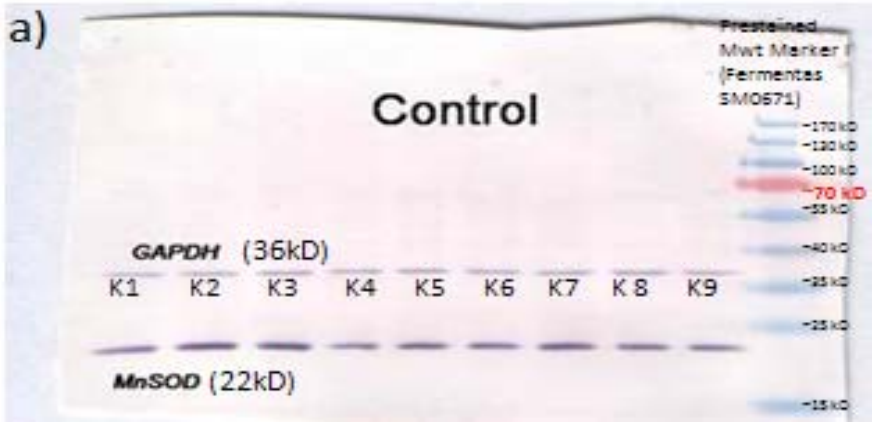


Figure A17: Western-blot results of Control rat liver S1 fractions immunostained by using GAPDH (above bands) and Mn SOD (below bands) antibodies. Lane 1-9 contains individual control (K1-K9) samples (10 µg total protein/well) and Lane 10 contains 2.5 µl of Type I prestained protein marker.

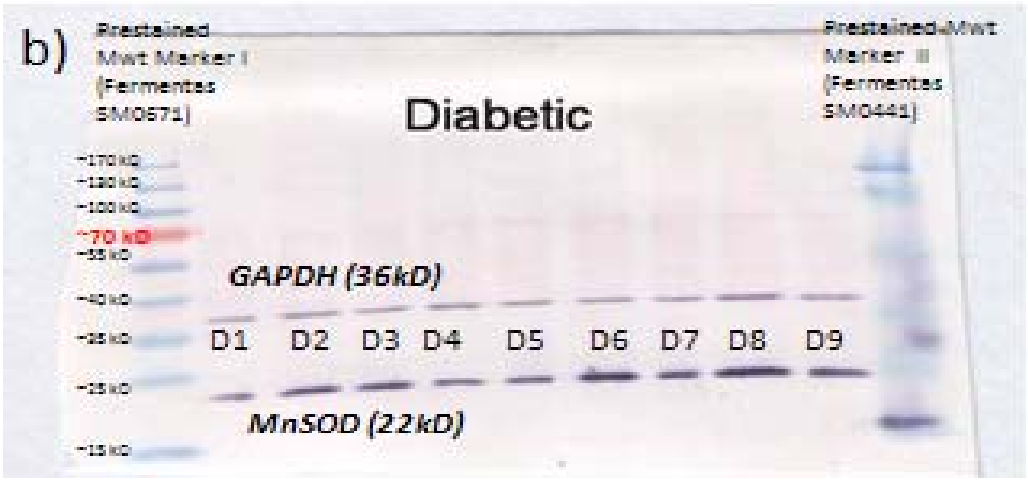


Figure A18: Western-blot results of Diabetic rat liver S1 fractions immunostained by using GAPDH (above bands) and Mn SOD (below bands) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-10 contains individual diabetic (D1-D9) samples (10 µg total protein/well) and Lane 11 contains 2.5 µl of type II prestained protein marker.

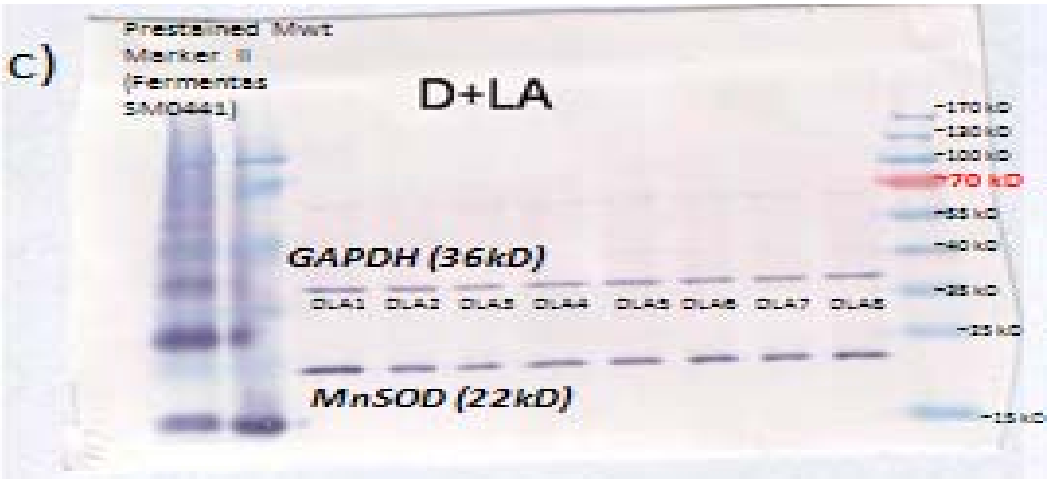


Figure A19: Western-blot results of LA treated Diabetic (D+LA) rat liver S1 fractions immunostained by using GAPDH (above bands) and Mn SOD (below bands) antibodies. Lane 1-2 contains 2.5 µl Type II prestained protein marker, Lane 2-9 contains individual LA treated diabetic (D+LA1-D+LA8) samples (10 µg total protein/well), Lane 11 contains 2.5 µl Type I prestained protein marker.

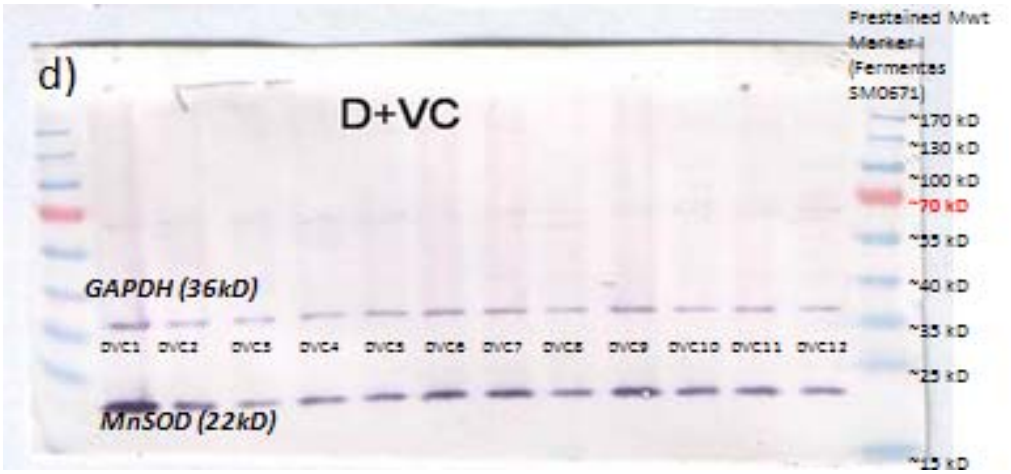


Figure A20: Western-blot results of VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and Mn SOD (below bands) antibodies. Lane 1 and 14 contains 2.5 µl Type I prestained protein marker, Lane 2-13 contains individual VC treated diabetic (D+VC1-D+VC12) samples (10 µg total protein/well).

Figure A21: Western-blot results of LA and VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and Mn SOD (below bands) antibodies. Lane 1 contains 2.5 µl Type II prestained protein marker, Lane 2-8 contains individual LA+VC treated diabetic (D+LA+VC1-D+LA+VC7) samples (10 µg total protein/well) and Lane 9 contains 2.5 µl of type I prestained protein marker.

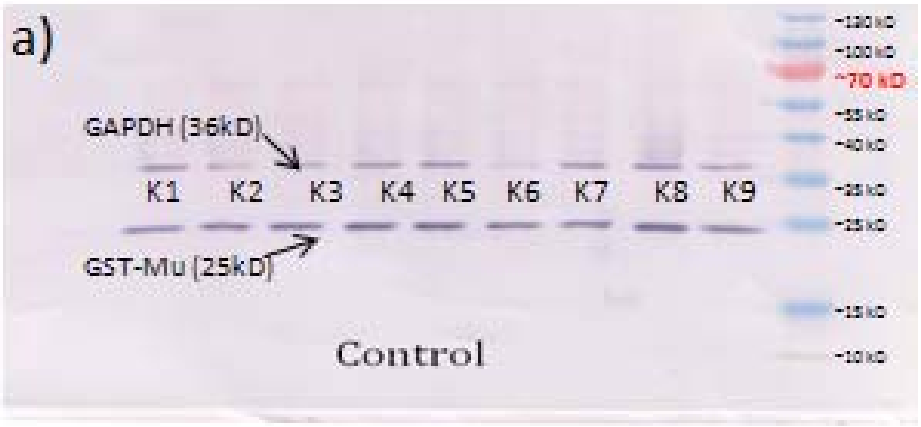


Figure A22: Western-blot results of Control rat liver S1 fractions immunostained by using GAPDH (above bands) and GST-Mu (below bands) antibodies. Lane 1-9 contains individual control (K1-K9) samples (20 µg total protein/well) and Lane 10 contains 2.5 µl of Type I prestained protein marker.

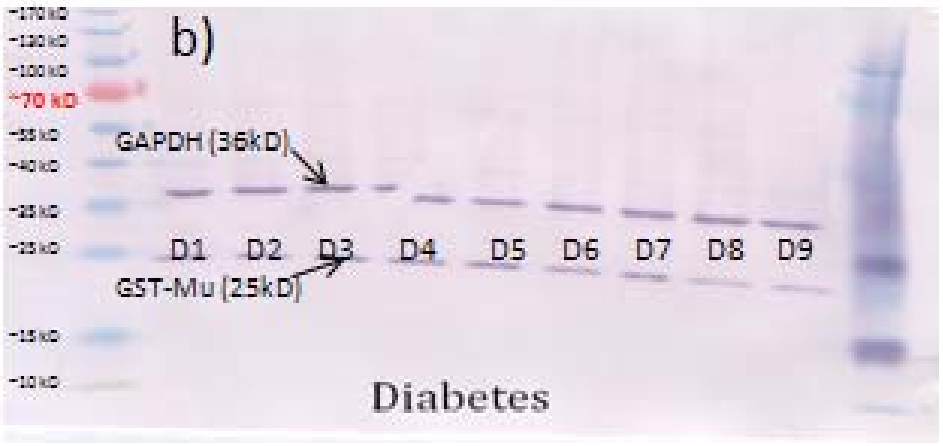


Figure A23: Western-blot results of Diabetic rat liver S1 fractions immunostained by using GAPDH (above bands) and GST-Mu (below bands) antibodies. Lane 1 contains 2.5 µl Type I prestained protein marker, Lane 2-10 contains individual diabetic (D1-D9) samples (20 µg total protein/well) and Lane 11 contains 2.5 µl of type II prestained protein marker.

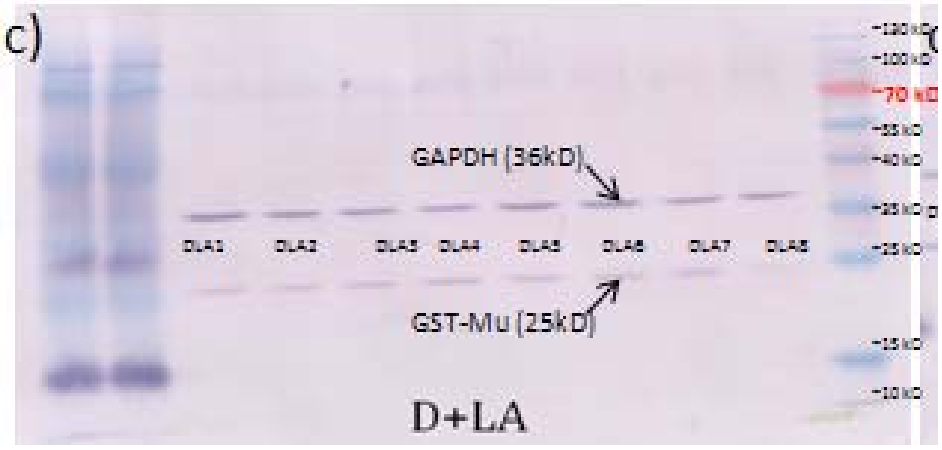


Figure A24: Western-blot results of LA treated Diabetic (D+LA) rat liver S1 fractions immunostained by using GAPDH (above bands) and GST-Mu (below bands) antibodies. Lane 1-2 contains 2.5 µl Type II prestained protein marker, Lane 2-9 contains individual LA treated diabetic (D+LA1-D+LA8) samples (20 µg total protein/well), Lane 11 contains 2.5 µl Type I prestained protein marker.

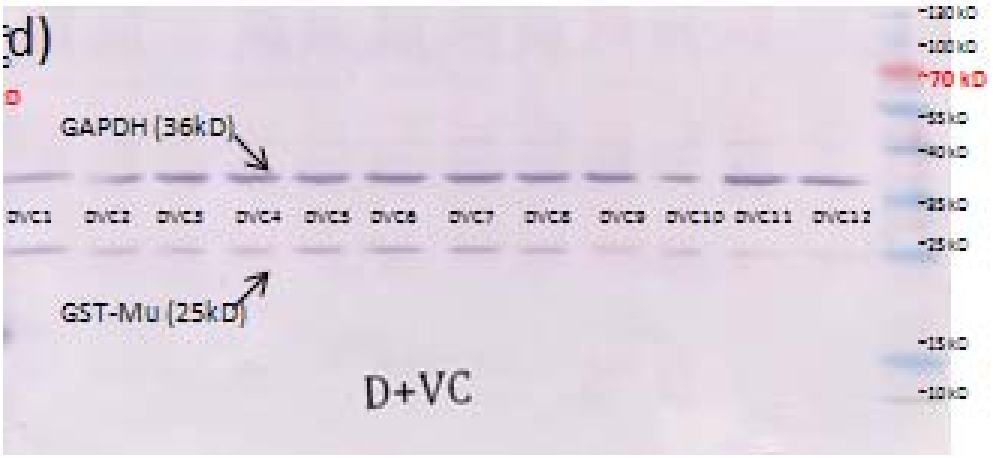


Figure A25: Western-blot results of VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and GST-Mu (below bands) antibodies. Lane 13 contains 2.5 µl Type I prestained protein marker, Lane 1-12 contains individual VC treated diabetic (D+VC1-D+VC12) samples (20 µg total protein/well).

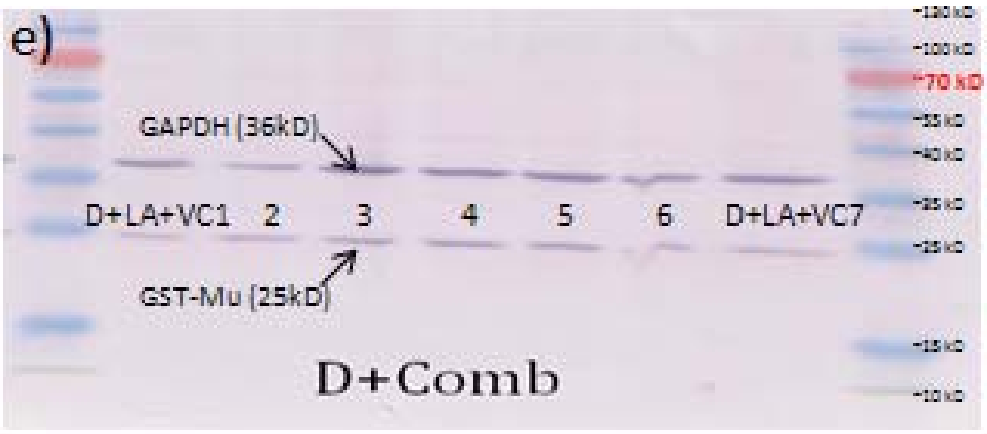


Figure A26: Western-blot results of LA and VC treated Diabetic (D+VC) rat liver S1 fractions immunostained by using GAPDH (above bands) and GST-Mu (below bands) antibodies. Lane 1 and 9 contains 2.5 µl Type I prestained protein marker, Lane 2-8 contains individual LA+VC treated diabetic (D+LA+VC1-D+LA+VC7) samples (20 µg total protein/well).

Table A5: Overall protein expressions results obtained from Western-blot analysis of antioxidant enzymes normalized to internal standart GAPDH protein.

Individual Animals	*CAT/GAPDH Protein ratios*	*Cu-Zn SOD/GAPDH Protein Ratios*	*Mn SOD/GAPDH Protein Ratios*	*GPx/GAPDH Protein Ratios*	*GST-Mu/GAPDH Protein Ratios*
K1	1,027	0,980	1,060	0,916	1,326
K2	1,029	1,044	1,108	0,913	2,064
K3	1,009	1,071	1,116	0,908	1,929
K4	1,010	1,084	1,058	0,887	1,709
K5	1,042	1,131	1,094	0,881	1,794
K6	1,010	1,098	1,080	0,978	1,794
K7	1,028	1,158	1,125	0,934	1,713
K8	1,036	1,111	1,089	0,898	1,749
K9	1,040	1,130	1,064	0,899	1,749
D1	0,905	1,029	1,016	0,935	0,804
D2	0,918	0,950	1,109	0,869	0,769
D3	0,908	0,994	1,132	0,941	0,855
D4	0,926	0,978	1,049	0,890	0,880
D5	0,923	1,002	1,085	0,982	1,215
D6	0,882	1,001	1,280	1,061	0,964
D7	0,936	1,000	1,111	0,982	0,915
D8	0,892	1,058	1,258	1,009	0,659
D9	0,953	1,018	1,139	1,008	0,506
D+LA1	0,933	1,072	1,097	0,986	0,444
D+LA2	0,962	1,111	1,033	0,959	0,555
D+LA3	0,958	1,080	1,022	0,966	0,680
D+LA4	0,957	1,103	1,026	1,149	0,988
D+LA5	0,986	1,092	1,028	0,965	0,441
D+LA6	0,973	1,042	1,043	0,895	0,516
D+LA7	0,955	1,083	1,025	0,958	0,765
D+LA8	0,952	1,052	1,046	0,910	0,480
D+VC1	0,969	0,942	1,468	0,904	0,836
D+VC2	0,917	0,972	1,149	1,020	0,806
D+VC3	0,976	1,007	1,059	0,922	0,623
D+VC4	0,998	0,967	1,068	0,988	0,545
D+VC5	0,873	0,992	1,052	1,042	0,617
D+VC6	0,977	1,011	1,135	0,983	0,760
D+VC7	0,905	0,974	1,153	1,035	0,657
D+VC8	0,970	1,040	1,085	0,996	0,622
D+VC9	0,981	0,997	1,164	0,926	0,570
D+VC10	0,993	0,993	1,133	1,013	0,880
D+VC11	1,008	1,035	1,100	0,944	0,381
D+VC12	0,900	0,984	1,066	0,926	0,442
D+LA+VC 1	0,912	0,974	1,009	0,963	0,545
D+LA+VC 2	1,063	1,003	1,064	1,021	1,003
D+LA+VC 3	1,088	0,983	1,083	0,954	0,500
D+LA+VC 4	1,013	0,968	1,051	0,973	0,667
D+LA+VC 5	1,001	1,013	1,048	0,986	0,623
D+LA+VC 6	1,042	1,016	1,057	0,975	0,647
D+LA+VC 7	1,068	1,055	1,535	1,004	0,590

Table A6: Overall mRNA expressions results obtained from RT-PCR analysis of antioxidant enzymes normalized to internal standart β-actin gene.

Individual Animals	*CAT/β-actin mRNA ratios*	*Cu-Zn SOD/β-actin mRNA Ratios*	*Mn SOD/β-actin mRNA Ratios*	*GPx/β-actin mRNA Ratios*	*GST-Mu/β-actin mRNA Ratios*
K1	0,568	1,035	0,521	0,451	1,176
K2	0,963	1,030	0,321	0,445	1,193
K3	1,049	1,113	0,492	0,424	0,926
K4	0,789	1,285	0,440	0,585	1,121
K5	0,850	1,244	0,346	0,457	1,179
K6	0,796	1,189	0,337	0,414	1,224
K7	0,864	1,228	0,825	0,400	1,280
K8	0,735	1,228	0,510	0,500	1,403
K9	0,847	1,604	0,551	0,769	1,224
D1	0,785	1,061	0,434	0,534	1,074
D2	0,680	1,100	0,449	0,457	1,101
D3	0,392	0,754	0,812	0,315	0,529
D4	0,839	1,083	0,421	0,475	1,246
D5	0,450	1,017	0,388	0,712	1,064
D6	0,891	1,277	0,445	0,578	1,170
D7	0,662	1,027	0,513	0,558	0,688
D8	0,768	0,878	0,603	0,480	0,813
D9	0,599	1,008	0,278	0,707	0,740
D+LA1	0,540	1,007	0,434	0,442	0,837
D+LA2	0,831	1,047	0,341	0,440	0,854
D+LA3	0,788	0,989	0,378	0,360	0,881
D+LA4	0,878	1,024	0,353	0,346	0,982
D+LA5	0,668	1,089	0,358	0,596	1,047
D+LA6	0,774	1,079	0,383	0,409	0,944
D+LA7	0,733	1,303	0,390	0,464	0,931
D+LA8	0,691	1,303	0,314	0,411	1,147
D+VC1	0,595	0,746	0,512	0,322	0,720
D+VC2	0,532	0,830	0,318	0,416	0,783
D+VC3	0,470	1,007	0,337	0,782	0,751
D+VC4	0,407	0,748	0,362	0,312	0,660
D+VC5	0,739	1,069	0,498	0,731	0,594
D+VC6	0,737	1,073	0,539	0,573	1,061
D+VC7	0,807	1,083	0,677	0,432	1,059
D+VC8	0,738	1,100	0,541	0,445	1,289
D+VC9	0,778	1,036	0,515	0,659	1,221
D+VC10	0,566	1,065	0,540	0,403	1,220
D+VC11	0,626	1,222	0,503	0,645	1,020
D+VC12	0,459	1,045	1,089	0,492	0,916
D+LA+VC 1	0,618	0,636	1,096	0,304	0,595
D+LA+VC 2	0,595	1,266	0,822	0,802	1,013
D+LA+VC 3	0,647	1,011	0,896	0,565	1,050
D+LA+VC 4	0,634	0,933	0,529	0,451	1,060
D+LA+VC 5	0,570	0,799	0,537	0,423	0,885
D+LA+VC 6	0,723	0,923	0,646	0,515	1,179
D+LA+VC 7	0,632	1,210	0,794	0,532	1,208

CURRICULUM VITAE

PERSONAL INFORMATION

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Nationality : Republic of Turkey
Date of birth : 01.01.1980
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WORK EXPERIENCE

Research Assistant (2002-2009) Middle East Technical University, Department of Biological Sciences

EDUCATION AND TRAINING

PhD (2004-2009): Middle East Technical University, Joint Biochemistry Graduate Programme,
M.S (2002-2004): Middle East Technical University, Joint Biochemistry Graduate Programme
B.S (2000-2002): Middle East Technical University, Department of Chemistry
(Double Minor Program)
B.S (1997-2002): Middle East Technical University, Educational Faculty, Biology Education

IN SERVICE TRAINING

- International Symposium on Biotechnology: Developments and Trends (27-30 September 2009, METU, Ankara, Turkey)
- 2nd Microarray Training and Data Analysis Course, METU Central Laboratory, Molecular Biology and Biotechnology R&D Center (29 June-1 July 2009, Ankara, Turkey)
- 9th FEBS Young Scientist Forum (2-4 July 2009, Prague, Czech Republic)

- EMBO Young Scientists Forum (20-22 February 2008, Bogazici University, İstanbul, Turkey)
- 2nd National HPLC and Other Separation Techniques (7-9 October, 2004, GATA, Ankara, Turkey)

RESEARCH TOPICS

Regulation of gene expression of antioxidant and detoxification enzymes in diabetes, Effect of lipoic acid, vitamin C and Resveratrol on antioxidant enzyme systems and Phase I and II enzymes (GSTs and Cytochrome P450s)

MEMBERSHIPS

Turkish Biochemical Society
Turkish Biotechnology Society
International Society for the Study of Xenobiotics (ISSX)

PUBLICATIONS

Full paper published in a peer reviewed journal covered by SCI, SSCI or AHCI core list

1. **Sadi G**, Güray T (2009) Gene Expressions of Mn-SOD and GPx-1 in Streptozotocin Induced Diabetes: Effect of Antioxidants. Mol Cell Biochem 327:127-134 (SCI Core)

2. **Sadi G**, Yilmaz O, Güray T (2008) Effect of vitamin C and lipoic acid on streptozotocin-induced diabetes gene expression: mRNA and protein expressions of Cu-Zn SOD and catalase. Mol Cell Biochem 309(1-2):109-316 (SCI Core)

Abstract of a paper presented at and published in the proceedings of a refereed conference regularly held by an international organization

1. **Sadi G,** Güray T (2009) Effect of Antioxidants on Hepatic Cytochrome P450 Gene Expressions in Streptozotocin Induced Diabetes. 34th FEBS Congress (4-9 July, 2009, Prague, Czech Republic) The FEBS Journal 273(1): 387

2. **Sadi G**, Kartal DI, Guray T (2009) Regulation of GST-Mu and CYP 2E1 Gene Expressions in Streptozotocin Induced Diabetes: Effect of Vitamin C. 11th European Regional ISSX Meeting (17-20 May 2009, Lisbon, Portugal) Drug Metabolism Reviews 41(1) PP: 215

3. Irtem D, **Sadi G**, Guray T (2007) Effects of lipoic acid and vitamin C administration on diabetic rat liver total GST activities. 15th Meeting of the Balkan Clinical Laboratory Federation (4-7 Sept 2007, Antalya, Turkey) Balkan Journal of Clinical Laboratory, XIV, 07(1): 167

4. **Sadi G**, Guray T (2007) Effects of lipoic acid and vitamin C on streptozotocin induced diabetic rat liver catalase and superoxide dismutase activities. 15th Meeting of the Balkan Clinical Laboratory Federation (4-7 Sept 2007, Antalya, Turkey) Balkan Journal of Clinical Laboratory, XIV, 07(1): 114

5. **Sadi G**, Görgülü G, Güray T (2006) The effects of streptozotocin induced diabetes on some oxidative biomarkers of rat liver. 31th FEBS Congress (24-29 June 2006, İstanbul, Turkey) The FEBS Journal 273(1): 350

6. Görgülü G, **Sadi G**, Guray T (2005) The Effects of Antioxidant Supplementation on Diabetes-Induced Oxidative Stress in Rat Liver Tissues. 13th Meeting of the Balkan Clinical Laboratory Federation. Balkan Journal of Clinical Laboratory PP-T-049

7. Ozhan HG, Kızıl C, Gorgulu G, **Sadi G**, Severcan F, Guray T (2003) The Effects of Melatonin on lipid peroxidation and antioxidant enzyme activities of rat liver. 13th Balkan Biochemical and Biophysical Days (12-15 Oct 2003, Kuşadası, Turkey) Turkish Journal of Biochemistry, 28(3): 139

Unpublished presentation in a refereed conference regularly held by an international organization

1. **Sadi G,** Güray T (2009) Reduction of Free Radical Induced Protein Carbonylation by Antioxidant Treatment in Diabetic Rat Tissues. FEBS Advance Lecture Course.(15-20 April 2009, Antalya, Turkey)

2. **Sadi G**, Güray T (2007) Effects of Lipoic Acid and Vitamin C Administration on Streptozotocin Induced Diabetic Rat Liver Catalase Activities. International Symposium on Drug Research and Development "From Chemistry to Medicine. 17-20 May 2007, Antalya, Turkey

Abstract of a paper presented at and published in the proceedings of a refereed, regularly held on national conference

1. **Sadi G**, Eryılmaz N, Tütüncüoğlu E et al (2009) Differential Expressions of CAT and SOD In STZ Induced Diabetic Rats In Response to Antioxidants. 21st National Biochemistry Congress (28-31 Oct 2009, İstanbul, Turkey) Turkish Journal of Biochemistry 34: 100-101 (oral presentation)

2. Eryılmaz N, **Sadi G**, Yılmaz Ö et al (2009) Regulation of GPx mRNA Expression and GSH Levels by Antioxidant Treatment in Diabetic Rat Kidney Tissues. 21st National Biochemistry Congress (28-31 Oct 2009, İstanbul, Turkey) Turkish Journal of Biochemistry 34: 101 (oral presentation)

3. **Sadi G,** Güray T (2009) Resveratrol Uygulamasının Diyabetik Sıçan Karaciğer CYP1A2 Gen Expresyonuna Etkilerinin Real-Time PCR Tekniği İle İncelenmesi. 7.Uluslararası Katılımlı Türk Toksikoloji Derneği kongresi (30 Mayıs-1 Haziran, 2009, Ankara, Turkiye) P:023

4. **Sadi G,** Guray T (2008) Effects of Resveratrol on Streptozotocin Induced Diabetic Rat Liver CuZn-SOD Expression. 20th National Biochemistry Congress (29-Oct-1 Nov 2008, Nevşehir, Turkey) Turkish Journal of Biochemistry 33(S1): 100 (oral presentation)

5. Kartal DI, **Sadi G**, Güray T (2008) Diabetic Rat Liver GST Alpha and Mu Gene Expressions and Activities. 20th National Biochemistry Congress (29-Oct-1 Nov 2008, Nevşehir, Turkey) Turkish Journal of Biochemistry 33(S1): 142-143

6. **Sadi G**, Guray T (2004) Antioxidants and oxidative modifications in diabetic rat liver tissues. 18th National Biochemistry Congress (15-19 May 2004, Trabzon, Turkey) Turkish Journal of Biochemistry, 29(1): 69-70

International and National Scientific Grants

1. Travel Grant for the 11th European Regional ISSX Meeting (from ISSX)
2. Travel Grant for 9th YSF Conference July 2 - 4, 2009 and during 34th FEBS Congress July 4 - 9, 2009 (from FEBS)
3. TUBİTAK PhD Scholarship