T.R
VAN YUZUNCU YIL UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCE
DEPARTMENT OF CHEMISTRY

DETERMINATION OF SOME ANTIOXIDANT ACTIVITIES (SUPEROXIDE DISMUTASE, CATALASE, AND REDUCING GLUTATHIONE) AND OXIDATIVE STRESS LEVEL (MALONDIALDEHYDE ACID) IN CIRRHOTIC LIVER PATIENTS

M. SC. THESIS

PREPARED BY: Hamam HAMAM
SUPERVISOR I: Prof. Dr. Halit DEMİR
SUPERVISOR II: Dr. Öğr. Üyesi. Mesut AYDIN

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ACCEPTANCE AND APPROVAL PAGE

This thesis entitled "**DETERMINATION OF SOME ANTIOXIDANT ACTIVITIES (SUPEROXIDE DISMUTASE, CATALASE, AND REDUCING GLUTATHIONE) AND OXIDATIVE STRESS LEVEL (MALONDIALDEHYDE ACID) IN CIRRHOTIC LIVER PATIENTS**" and prepared by Hamam HAMAM under consultation of Prof. Dr. Halit DEMIR and Dr. Öğr. Mesut AYDIN in Department of Chemistry, on date of 30/09/2019 it has been successful with a unanimous vote by the following jury and it has been recognized as a Master's Thesis, in accordance with Postgraduate Education and training regulation with the relevant provisions.

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The Board of Directors of the Institute of Science: 04./10./2019..... date and is approved by decision No: 2019/154-I

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Director of the Institute

THESIS STATEMENT

All information presented in the thesis that ethical behavior and academic rules were obtained in the frame, as well as all kinds of work that does not belong to me in this statement prepared in accordance with the rules of writing theses and reports that I referred to the complete information source.

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Hamam HAMAM

ABSTRACT

DETERMINATION OF SOME ANTIOXIDANT ACTIVITIES (SUPEROXIDE DISMUTASE, CATALASE, AND REDUCING GLUTATHIONE) AND OXIDATIVE STRESS LEVEL (MALONDIALDEHYDE ACID) IN CIRRHOTIC LIVER PATIENTS

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Cirrhosis is a condition in which the liver does not function properly due to long-term damage. This damage is characterized by the replacement of normal liver tissue by scar tissue. Cirrhosis is most commonly caused by alcohol, hepatitis B, and hepatitis C as the most common etiological factors. In this study, blood serum samples were collected from patients with liver cirrhosis from the Gastroenterology Clinic of Batman Regional Hospital. The aim of this study was to determine the levels of lipid peroxidation (MDA) and antioxidants such as reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) in the blood serum of liver cirrhosis patients. SOD, CAT and GSH activities were significantly decreased in the patient groups compared to the healthy control group ($p<0.05$). MDA levels were significantly higher in the patient group compared to the healthy control group ($p <0.05$). In conclusion, this study, oxidative stress may play an important role in the development of liver cirrhosis.

Keywords: Liver Cirrhosis, SOD, GSH, CAT, MDA

ÖZET

SİROTİK KARACİĞER HASTALARINDA OKSİDATİF STRES DÜZEYİ (MALONDIALDEHIT ASIT) VE BAZI ANTİOKSİDANT AKTİVİTELERİN (SÜPEROKSİT DİSMUTAZ, KATALAZ, VE REDÜKTE GLUTATYON) BELİRLENMESİ

HAMAM Hamam
Yüksek Lisans Tezi, Kimya Anabilim Dalı
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Danışman II: Dr. Öğr. Üyesi. Mesut AYDIN
Ekim 2019, 69 Sayfa

Siroz, karaciğerde uzun süreli hasar nedeniyle işlevini yapamaduğı bir durumdur. Bu hasar normal karaciğer dokusunun skar dokusu ile değiştirilmesi ile karakterize olan bir durumdur. Siroz en sık olarak alkol, hepatit B ve en yaygın etiyolojik faktörler olarakta hepatit C'den kaynaklanır. Bu çalışmada, Batman Bölge Hastanesi Gastroenteroloji Kliniği karaciğer sirozu olan hastalardan kan serum örnekleri alındı. Bu çalışmanın amacı, karaciğer siroz hastalarının kan serumunda lipid peroksidasyon düzeyi (MDA) ve redukte glutatyon (GSH), katalaz (CAT) ve süperoksit dismutaz (SOD) gibi antioksidanların aktivitelerini belirlemektir. Hasta gruplarında SOD, CAT ve GSH aktiviteleri sağlıklı kontrol grubuna göre anlamlı olarak azaldı ($p<0.05$). MDA düzeyi, sağlıklı kontrol grubuyla karşılaştırıldığında hasta grubunda anlamlı olarak yükseldiği görüldü ($p<0.05$). Sonuç olarak, bu çalışmada, oksidatif stres karaciğer sirozu gelişiminde önemli bir rol oynayabilir.

Anahtar kelimeler: Karaciğer Sirozu, SOD, GSH, CAT, MDA

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04/10/2019

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SYMBOLS AND ABBREVIATIONS

Some symbols and abbreviations used in this study are presented below, along with descriptions.

Symbols	Description
Cu	Cupper
Fe	Iron
Zn	Zinc
O_2	Oxygen
O_3	Ozone
O_2^-	Superoxide
OH^-	Hydroxyl
°C	Centigrade temperature
min	Minute
g	Gram
h	Hour
L	Liter
Ml	Milliliter
µl	Microliter
mg	Milligram
mM	Mill molar
M	Molar
N	Normal
rpm	Round/Minute
µg	Microgram
µm	Micrometer
β	Beta
α	Alfa
U/L	Unit/liter

Abbreviations	Explanation
Apo	Apolipoprotein
ATP	Adenine triphosphate
CAT	Catalase
DNA	Deoxyribo nucleic acid
RNA	Ribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide
Kcal	kilocalorie
LDL	Low density lipoprotein
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
NO_2	Nitrogen dioxide
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RSS	Reactive sulphur species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
UV	Ultraviolet
GSH	Reduced glutathione
Mn	Manganese
GSSG	Oxidized glutathione
KOH	Potassium hydroxide
Na_2CO_3	Sodium carbonate
BSA	Bovine Serum Albumin
NH_4 (SO_4)	Ammonium sulphate

Abbreviations	Explanation
$CuCI_2$	Copper chloride
NaOH	Sodium hydroxide
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
BHT	Butylhydroxytoluene solution
TBA	Thiobarbituric acid solution
TCA	Trichloro acetic acid solution
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2, 2'-azino-bis (3-ethylbenzothiazoline-6-Sulphonic acid)
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HCC	Hepatocellular Carcinoma
HE	Hepatic encephalopathy
NASH	Non-Alcoholic Steatohepatitis
NAFLD	Non-alcoholic fatty liver disease
IBD	Inflammatory bowel disease
BPD	Borderline personality disorder
ROP	Retinopathy of prematurity

1. INTRODUCTION

A free radical is basically an element with an atom or molecule that has an unpaired electron in its outer orbits. Due to the unpaired electron it contains, the atom, ion, or molecule of the free radical is very reactive .This free electron is harmful to various biological systems as it is capable of “stealing” an electron off of a stable molecule, and causes it to become a free radical as well. He tissue may lose some of its functionality (Tiwari, 2004).

There are three primary sources of free radicals that make the majority of all free radicals. These are oxygen, sulfur, and reactive nitrogen species. Reactive oxygen and nitrogen species (ROS and RNS) are naturally produced in the body as a result of normal physiological reactions. ROS and RNS partake in both beneficial and harmful reactions, and a healthy human is believed to be subject to 10 to 20 thousand free radicals of these types every day (Valko et al., 2006).

ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (Valko et al., 2004). Beneficial effects of ROS involve physiological roles in cellular responses to anoxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids, membranes, proteins and nucleic acids (termed oxidative stress) (Poli et al., 2004).

Reactive oxygen species (ROS) are produced by living organisms as a result of normal cellular metabolism. Oxidative stress is thought to contribute to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease (Wood-Kaczmar et al., 2006), the pathologies caused by diabetes, rheumatoid arthritis, and neurodegeneration in motor neuron diseases (Giugliano et al., 1996).

In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease and from general tissue damage; One case in which this link is particularly well-understood is the role of oxidative stress in cardiovascular disease. Here, low density lipoprotein (LDL) oxidation appears to trigger

the process of atherogenesis, which results in atherosclerosis, and finally cardiovascular disease (Giugliano et al., 1996).

Oxidative damage in DNA can cause cancer. Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S- transferase etc. protect DNA from oxidative stress. It has been proposed that polymorphisms in these enzymes are associated with DNA damage and subsequently the individual's risk of cancer susceptibility (Khan et al., 2010).

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The harmful effects of ROS and RNS are balanced by the antioxidant action of nonenzymatic antioxidants in addition to antioxidant enzymes (Halliwell, 1996). Several antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S- transferase protect DNA from oxidative stress. It has been proposed that polymorphisms in these enzymes are associated with DNA damage and subsequently the individual's risk of cancer susceptibility (Khan et al., 2010).

Liver cirrhosis is defined as the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury, and is associated with the development of liver failure and portal hypertension (Sivanathan et al., 2014). Infection with Hepatitis B (HBV) or C (HCV), alcohol abuse and nonalcoholic fatty liver disease (NAFLD) are the main etiologic factors of liver cirrhosis worldwide (Sivanathan et al., 2014). However, certain genetic polymorphisms may influence the progression of liver fibrosis (Bataller et al., 2003).

Cirrhosis is an advanced stage of liver fibrosis that is accompanied by distortion of the hepatic vasculature. It leads to shunting of the portal and arterial blood supply directly

into the hepatic outflow (central veins), compromising exchange between hepatic sinusoids and the adjacent liver parenchyma, i.e., hepatocytes (Schaffner et al., 1963).

Cirrhosis is characterized by vascularized fibrotic septa that link portal tracts with each other and with central veins, leading to hepatocyte islands that are surrounded by fibrotic septa and which are devoid of a central vein. The major clinical consequences of cirrhosis are impaired hepatocyte (liver) function, an increased intrahepatic resistance (portal hypertension) and the development of hepatocellular carcinoma (HCC). Cirrhosis and its associated vascular distortion are traditionally considered to be irreversible but recent data suggest that cirrhosis regression or even reversal is possible (Wanless et al., 2000; Desmet et a.,l 2004).

The aim of this study was to determine the level of oxidative stress products like malondialdehyde (MDA), and antioxidant enzymes by measuring the levels of GSH, CAT and SOD enzymes in cirrhotic patients.

2. LITERATURE REVIEW

2.1. Free radicals

The events of World War II (1939-1945) led directly to the birth of free radical biochemistry. The two atom bombs (6th August 1945, Hiroshima and 9th August 1945, Nagasaki) led to massive deaths to entire population, and the survivors had shortened life-span. In 1954, Gershman and Gilbert speculated that the lethal effects of ionizing radiation might be ascribed to formation of reactive oxygen species (ROS). Since then free radicals (atoms with an unpaired electron) such as ROS and reactive nitrogen species (RNS) have gained notoriety (Gilbert, 1981).

In popular scientific/biomedical literature the term 'free radical' is used in a broad sense and also includes related reactive species such as 'excited states' that lead to free radical generation or those species that results from free radical reactions. In general, free radicals are very short lived, with half-lives in milli-, micro- or nanoseconds. Details about some of the biologically important reactive species are presented . Free radicals have been implicated in the etiology of several human diseases as well as ageing (Harraan, 1955; Halliwell et al., 1985).

Free radicals as molecules or molecular fragments containing one or more unpaired electrons.The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from oxygen represent the most important class of such species generated in living systems (Valko et al,. 2004).

Free radicals include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxyl ($ROO^{\bullet}$) and lipid peroxyl ($LOO^{\bullet}$). Also, hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH), are not free radicals and generally called oxidants, but can easily lead to free radical reactions in living organisms (Genestra, 2007).

Elevated of free radicals level, breaks the balance between the oxidation and reduction reactions in biological systems that in favor of reactive oxidants, potentially leading to oxidative stress (OS). Free radicals are chemical reactive molecules containing

oxygen and nitrogen that known as reactive oxygen/nitrogen species (ROS/RNS). Free radical is atomic or molecular species with single or more unpaired electrons in their structure. ROS/RNS easily react with other molecules that cause damage to cellular components, including proteins, lipids, carbohydrates, and nucleic acids. Aging, chronic inflammatory diseases, tobacco (smoking), alcohol consumption, diabetes neurodegenerative diseases, cancer, etc., lead to generation of oxidative stress (Valko et al., 2007).

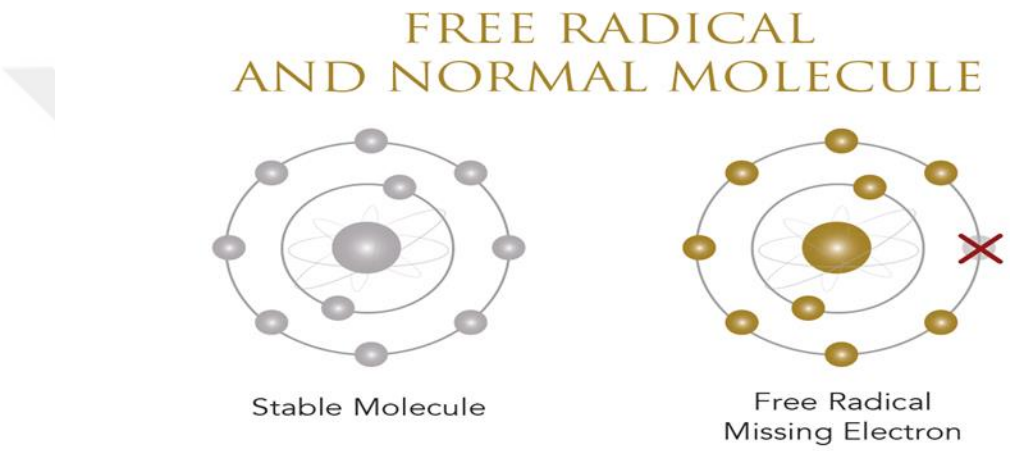

Figure 2.1. Free radical and normal molecule.

2.1.1. Reactive oxygen/nitrogen species (ROS/RNS)

ROS and RNS are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants. Radicals are less stable than non-radical species, although their reactivity is generally stronger (Halliwell et al ., 2015). Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron, by cleavage of a radical to give another radical, and also via redox reactions (Bahorun et al ., 2006; Halliwell et al ., 2015).

ROS are generated during cellular metabolism and functional activities, and have vital roles in cell signalling, apoptosis, gene expression and ion transportation (Vajragupta et al., 2004) The aggregation of ROS is providentially controlled in vivo by a broad range of spectrum of non-enzymatic antioxidant systems, such as bilirubin; glutathione (GSH);

vitamins A, C, and E; and defense-related enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Guemouri et al., 1991).

The high amounts of reactive oxygen species can influence harming of molecules presenting lipid, protein, DNA and RNA, due to they have high chemical reactivity and tiny. Bases pair inside nucleic acids, on amino acid side chain in protein and double bonds in unsaturated fatty acids may be attacked via (ROS) during ($OH^{\bullet}$) . It has a high oxidant of the free radical (Lu et al., 2010).

Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalyzed processes (Fridovich, 1986).

$$O_2^{\bullet-} + 2H^+ - SOD \rightarrow H_2O_2 + O_2 \ldots\ldots\ldots\ldots (2.1)$$

SOD enzymes accelerate this reaction in biological systems by about four orders of magnitude. It should be noted that SOD enzymes work in conjunction with $H_2O_2^-$ removing enzymes, such as catalases and glutathione peroxidases (Michiels et al ., 1994). The generation of various free radicals is closely linked with the participation of redox-active metals (Valko et al., 2005).

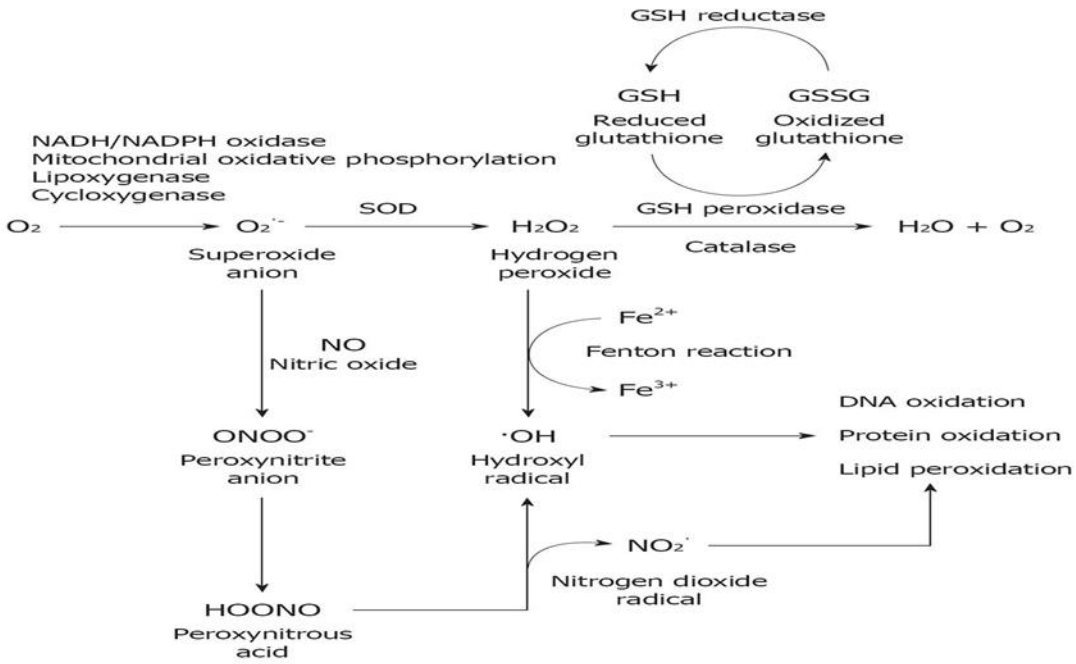

Figure 2.2. Mechanism of oxidative cellular damage (Wong et al., 2013).

Nitric oxide ($NO^{\bullet}$) is an abundant reactive radical that acts as an important oxidative biological signalling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Archer, 1993; Förstermann et al ., 1998). This small molecule contains one unpaired electron on the antibonding 2π *y orbital and is, therefore, a radical. A connection between RNS and septic shocks, asthma, and atherosclerosis was drawn. Nitric oxide and nitrogen dioxide are the primary examples of RNS. Nitric oxide is formed by the nitric oxide synthase (NOS) enzyme and is a very reactive free radical species that can alter carbohydrates, fats, proteins, and the cell nucleus. The damage can be to such an extent that the body starts an inflammatory reaction, which may lead to tissue damage and various adhesions. Nitric oxide has other effects like suppressing platelet aggregation and relaxing the arterial and venous muscles (Agarwal et al., 2005).

$NO^{\bullet}$ is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline with the formation of $NO^{\bullet}$ via a five-electron oxidative reaction (Ghafourifar et al ., 2005). Overproduction of reactive nitrogen species is called nitrosative stress (Klatt et al ., 2000). This may occur when the generation of reactive species in a system exceeds the system's ability to neutralize and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. $NO^{\bullet}$ has a half-life of only a few seconds it an aqueous environment (Chiueh, 1999).

2.1.2. Scavenging of free radicals

Organisms are equipped with an armamentarium of defense systems, termed antioxidants in order to safeguard them against the onslaught of ROS. The main methods comprise superoxide radicals scavenging ($O_2^{\bullet-}$); hydrogen peroxide scavenging (H2O2); hypochlorous acid scavenging (HOCl); hydroxyl radical scavenging ($HO^{\bullet}$); peroxyl radical scavenging ($ROO^{\bullet}$), among them are the methods that use azo-compounds to generate peroxyl radicals, such as the ``TRAP'' method (Total Radical-Trapping Antioxidant Parameter) and the ``ORAC'' method (Oxygen-Radical Absorbance Capacity); the scavenging of radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6-

sulphonate) or the ABTS or the ``TEAC" method (Trolox Equivalent Antioxidant Capacity); the scavenging of stable radical 2,2-diphenyl-1-picrylhydrazyl or DPPH . method and the scavenging of radical cation N,N-dimethyl-p-phenylenediamine or DMPD method (Sánchez-Moreno, 2002). The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening of many samples for radical scavenging activity (Marxen et al., 2007). The method DPPH is widely used for measurement of free radical scavenging ability of antioxidants (Perez et al., 2008).

At present, in spite of the diversity of methods, there is a great need to standardize measurements of antioxidant activity. Therefore the researchers usually meditate the radical scavenging analyses for antioxidant studies in the cell-free systems in advance of further studies in cellular lines and/or animal models (Lü et al., 2010).

O_2^{-}• is also generated in riboflavin/methionine /illuminate and assayed by the reduction of Nitro blue tetrazolium (NBT) to form blue formazan. Briefly, the reaction mixture is illuminated at 25 °C for 40 min. and $O_2^{-\bullet}$ generated from the photochemically reduced riboflavin can reduce NBT to form blue formazan which has an absorbance at 560 nm. This system can be utilized to determine the radical scavenging activity of antioxidants. Antioxidants can be supplemented to the reaction mixture to scavenge $O_2^{-\bullet}$, thereby preventing the NBT reduction. Decreased absorbance of the reaction mixture shows increased $O_2^{-\bullet}$ scavenging activity. The scavenged percentage of O_2^{-}• is calculated by the alterations in the absorption. NBT salt and other tetrazolium salts are chromogenic probes useful for $O_2^{-\bullet}$ determination. These probes are also widely used for detecting redox potential of cells for viability, proliferation and cytotoxicity assays (Lü et al., 2010).

2.1.3. Types of reactive species (ROS/RNS)

Reactive oxygen species can be classified into oxygen-centered radicals and oxygen-centered non radicals. Oxygen-centered radicals are superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), and peroxyl radical ($ROO^{\bullet}$). Other reactive species are nitrogen species such as nitric oxide ($NO^{\bullet}$), nitric dioxide ($NO_2^{\bullet}$), and peroxynitrite ($OONO^{-}$). Oxygencentered non-radicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($1O_2$), Hypochlorous acid and Ozone (Halliwell et al., 1995; Simon et al., 2000).

Superoxide anion ($O_2^{\bullet}$) the most common ROS is generated in mitochondria, in cardiovascular system and other parts of the body (Drew, 2002; Salman et al., 2012). The electron transport chain (ETC) is responsible for most of the superoxide generation through partial reduction of oxygen (Bolisetty et al., 2013). The superoxide anion ($O_2^{\bullet-}$) plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(2.2)}$$ in living systems (Harman, 2001).

The superoxide anion can react with nitric oxide ($NO^{\bullet}$) and form peroxynitrite ($ONOO^-$), which can generate toxic compounds such as hydroxyl radical and nitric dioxide:

$$ONOO^- + H^+ \rightarrow OH^{\bullet} + NO_2^{\bullet} \quad \text{(2.3)}$$ (Stief, 2003).

The hydroxyl radical •OH, is the neutral form of the hydroxide ion. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short in vivo half-life of approximately, 10−9 s (Pastor et al., 2000) . Thus when produced in vivo $OH^{\bullet}$ reacts close to its site of formation. The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation ensures that there is no free intracellular iron; however, in vivo, under stress conditions, an excess of superoxide releases “free iron” from iron-containing molecules. The release of iron by superoxide has been demonstrated for [$4Fe^-{}_4S$] cluster containing enzymes of the dehydratase-lyase family (Liochev et al., 1994). The released Fe2+ can participate in the Fenton reaction, generating highly reactive hydroxyl radical.

$$Fe^{+2} + H_2O_2^{\bullet-} \rightarrow Fe^{+3} + HO^- + HO \quad \text{(2.4)}$$

Nitric oxide ($NO^{\bullet}$) is an abundant reactive radical that acts as an important oxidative biological signalling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation(Beneš et al., 1999). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes. $NO^{\bullet}$ has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu, $NO^{\bullet}$ react with oxygen

and water to form nitrate and nitrite anions. Overproduction of reactive nitrogen species is called nitrosative stress (Klatt et al., 2000).

Peroxyl radicals ($ROO^{\bullet}$) are formed by a direct reaction of oxygen with alkyl radicals ($R^{\bullet}$), for example, the reaction between lipid radicals and oxygen. Decomposition of alkyl peroxides (ROOH) also results in peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals. Irradiation of UV light or the presence of transition metal ions can cause homolysis of peroxides to produce peroxyl and alkoxyl radicals:

$$ROOH \rightarrow ROO^{\bullet+} H^{\bullet}, ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H+ \text{……..(2.5).}$$

Peroxyl and alkoxyl radicals are good oxidizing agents. They can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. Very often the alkyl radical formed from this reaction can react with oxygen to form another peroxyl radical, resulting in chain reaction. Some peroxyl radicals break down to liberate superoxide anion or can react with each other to generate singlet oxygen (Carr et al., 2000).

Singlet oxygen ($1O_2$), it is a non-radical, may be generated with the aid of an input of energy that rearranges the electrons, it is miles alternatively mild and nontoxic for mammalian tissue. It is shaped all through chemical reactions and photosensitization (Salman et al., 2013). Inside the human ($1O_2$) they are a signal and a weapon, with therapeutic potency against numerous pathogens which include viruses, microbes and cancer of the cells. Two one of a kind pathways in biology can produce singlet oxygen. ($1O_2$) can at once oxidize proteins, DNA and lipids also it had been known to be involved in cholesterol oxidation that may be take part in Dielse-Alder reactions. It is able to be generated by chemical processes, consisting of spontaneous decomposition of hydrogen trioxide in water or the reaction of H_2O_2 with hypochlorite.

Hydrogen peroxide (H_2O_2) can be generated through a dismutation reaction from superoxide anion by superoxide dismutase. Enzymes such as amino acid oxidase and xanthine oxidase also produce hydrogen peroxide from superoxide anion. Hydrogen peroxide is highly diffusible and crosses the plasma membrane easily. Hydrogen peroxide is the least reactive molecule among reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions. Hydrogen peroxide is a

weak oxidizing and reducing agent and is thus regarded as being poorly reactive. Hydrogen peroxide can generate the hydroxyl radical in the presence of metal ions and superoxide anion.

$$O2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + O_2 \text{……..(2.6)}$$ (Valko et al., 2007).

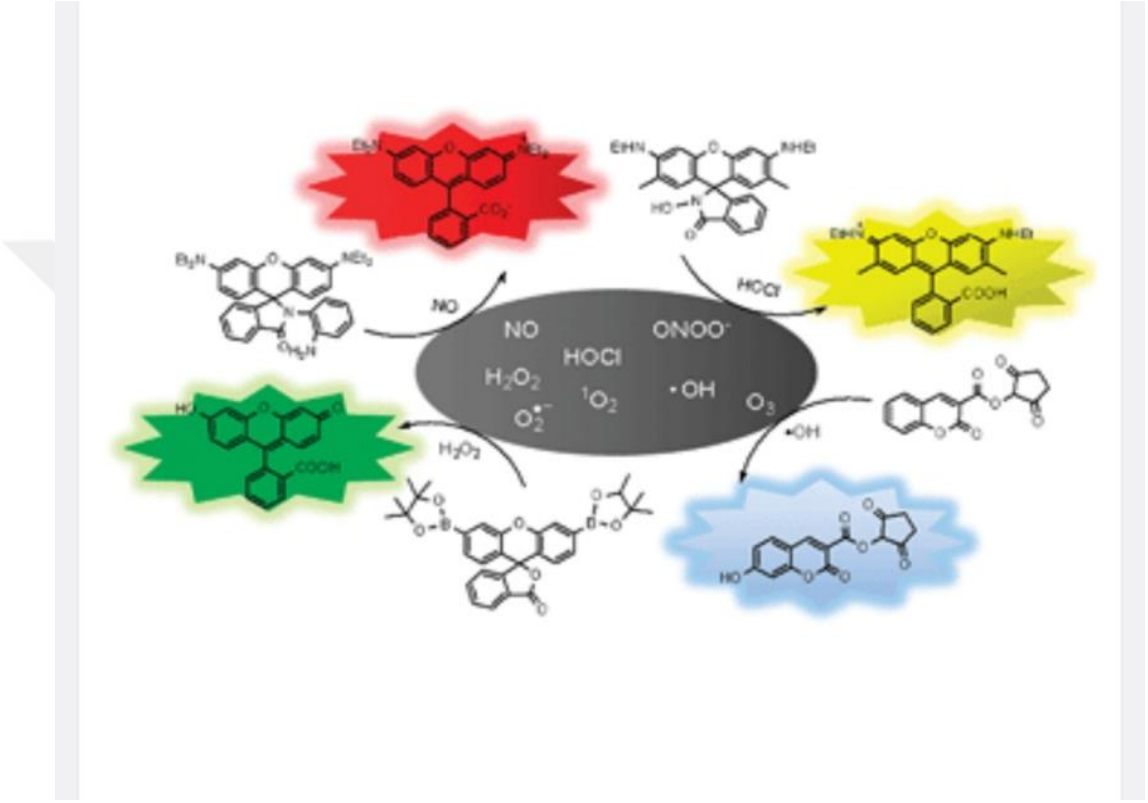

Figure 2.3. Mechanism formating of (ROS) and (RNS) (Chen, X., et al 2011).

2.1.4. Sources of ROS generation

ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Inoue et al., 2003). Mitochondria have long been known to generate significant quantities of hydrogen peroxide. The hydrogen peroxide molecule does not contain an unpaired electron and thus is not a radical species. Under physiological conditions, the production of hydrogen peroxide is estimated to account for about ~2% of the total oxygen uptake by the organism. However, it is difficult to detect the occurrence of the superoxide radical in intact mitochondria, most probably in

consequence of the presence of high SOD activity therein. Generation of the superoxide radical by mitochondria was first reported more than three decades ago (Loschen et al., 1971). Mitochondria generate approximately 2–3 nmol of superoxide/min per mg of protein, the ubiquitous presence of which indicates it to be the most important physiological source of this radical in living organisms (Inoue et al., 2003).

Since mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are present on both sides of their membranes in order to minimise oxidative stress in the organelle (Cadenas et al., 2000). Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the inter membrane space) and Mn-SOD (SOD2, localised in the matrix). Besides mitochondria, there are other cellular sources of superoxide radical, for example xanthine oxidase (XO), a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals (Li, 2002).

Xanthine oxidase is an important source of oxygen-free radicals. It is a member of a group of enzymes known as molybdenum iron–sulphur flavin hydroxylases and catalyzes the hydroxylation of purines. In particular, XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second (Valko et al., 2004).

Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide (Conner et al., 1996). Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle.

In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 concentration produced in vivo at hyperoxia sites (Gupta et al., 1997). Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2 generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H2O2 production as a result of prolonged starvation.

Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. ROS exogenous sources include pollutants in food, environmental pollutants, radiation or they are the by-products of other metabolic processes in the organisms (Poljsak, 2011). The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. For example 2-butoxyethanol is known to produce ROS indirectly, which causes cancer in mice (Klaunig et al., 1997).

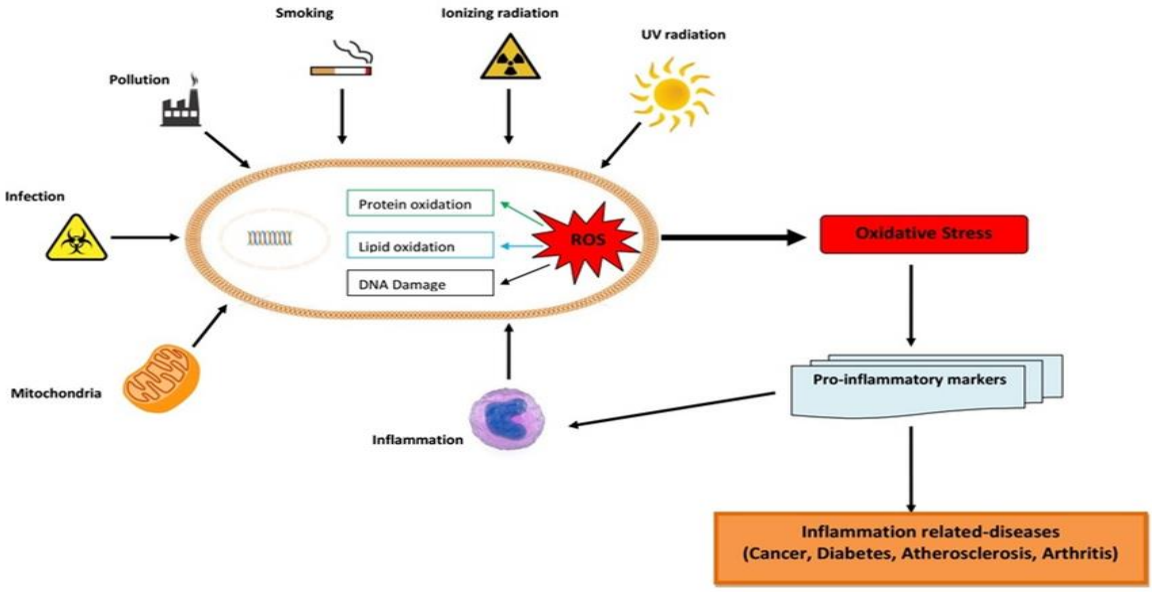

Figure 2.4. ROS/RNS endogenous and exogenous sources.

2.1.5. Physiological functions of reactive oxygen species

Free radicals and other reactive oxygen and nitrogen species play a double role, because they can cause oxidative damage and tissue dysfunction and serve as molecular signals activating stress responses that are beneficial to the organism. Mitochondria have been thought to both play a major role in tissue oxidative damage and dysfunction and provide protection against excessive tissue dysfunction through several mechanisms, including stimulation of opening of permeability transition pores (Di Meo et al ., 2016).

Show interplay between mitochondria and other ROS cellular sources, so that activation of one can lead to activation of other sources. Thus, it is currently accepted that in various conditions all cellular sources of ROS provide significant contribution to processes that oxidatively damage tissues and assure their survival, through mechanisms such as autophagy and apoptosis (Di Meo et al ., 2016).

Free radicals act as an irreplaceable role in phagocytosis as one of the major microbial systems, or in various biochemical reactions such as hydroxylating, carboxylate or peroxidation reactions ,or in the ribonucleotides' reduction (Rahal et al., 2014). ROS also seem to be involved in cell adhesion, a mechanism that plays an important role in embryogenesis, cell growth, differentiation, wound repair, among other processes. The expression of cell adhesion molecules is stimulated by bacteria lipopolysaccharides and by various cytokines such as TNF, interleukin-1a, and interleukin-1b. (Drog, 2002). Also ROS and RNS play an important role as regulatory mediators in signaling processes as Nitric oxide NO is composed from arginine by the action of NO-synthase (NOS) early identified as a signaling molecule in blood vessel modulation NO is formed by the primary NOS during blood vessel softening processes (eNOS) or during transmission of nerve impulses (nNOS) (Ignarro et al., 1987). And now known as a regulator of important physiological processes (Bogdan, 2001), can mediate cellular toxicity damaging metabolic enzymes and generating, by reaction with superoxide, peroxynitrite (Pacher et al., 2007).

2.1.6. Inhibition of free radical generating enzymes

NADPH oxidases are a set of plasma membrane incorporated enzymes that shift one electron from the cytosolic NADPH donor to a molecule of extracellular oxygen, and producing $O_2^{-\bullet}$. XO is an enzyme that is related to the generation of uric acid in the body,

that catalyzes the hypoxanthine and xanthine oxidation to uric acid resulting $O_2^{-\bullet}$ and H_2O_2 and elevates the oxidative level in an organism (Figure 2.5). In phagocytes, $O_2^{-\bullet}$ is produced in huge amounts by NADPH oxidase enzyme for utilization in oxygen-dependent killing mechanisms for attacking pathogens. The controlled formation of reactive oxygen species throughout the respiratory burst is vital for the protection of an organism toward attacking microorganisms without inducing an important damage to tissue functions. Whereas, excessive ROS boost oxidative stress such as low-density.

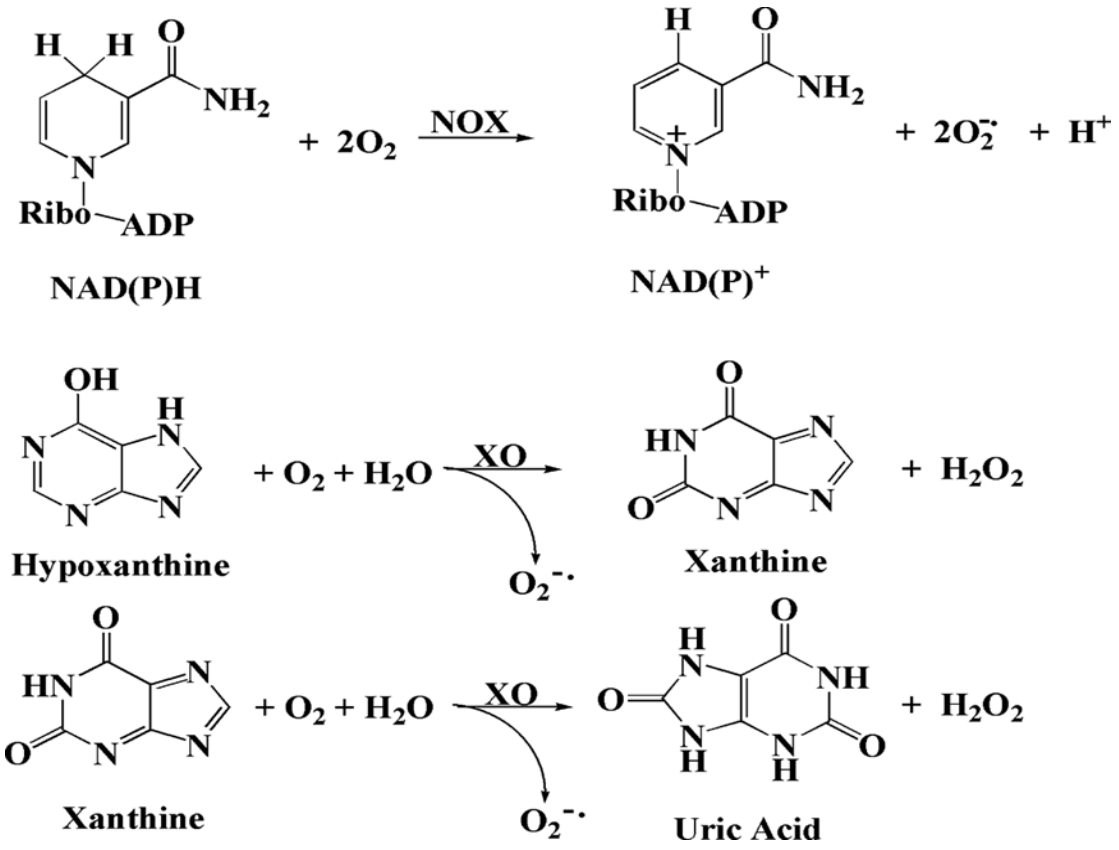

Figure 2.5. Superoxide and hydro peroxide generation (Lü et al., 2010).

2.1.7. Determination of ROS

ROS are highly reactive molecules and are extremely unstable, so detection of ROS relies on measuring the end products that are formed when they react with particular substances. The end products can be measured by changes in their fluorescence, color, or luminescence (Jambunathan, 2010). Identification of free radicals either without delay via

paramagnetic electron resonance like electron spin resonance. And additionally indirectly through identifying some more stable intermediates assessment of the traces of radical assault on biological molecules by means of high overall performance such as (liquid or gas chromatography) and colorimetric investigation .The measurement of antioxidant status may be evaluated via like colorimetric, immune, and enzymatic ways. In vivo, ESR is relatively senseless and needs steady-state concentrations of free radicals in the micromolar range, which restricts its usage in patients for measuring ROS. ESR can be applied only by spin trapping technique. Commonly indirect measures measure the changes in endogenous antioxidant defense systems or measure the ROS-induced damage of cellular components, and additionally we can detection on the oxidative stress that due to high amount concentration of the ROS or that is lowering antioxidant (Poljsak et al., 2013).

2.2. Antioxidant

The term “antioxidant” refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans have evolved highly complex antioxidant systems (enzymic and nonenzymic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. In some other term antioxidant is any substance that when present at low concentrations compared with that of an oxidizable substrate, substantially delays or inhibits oxidation of that substrate (Halliwell et al., 2007).

Antioxidants are categorized into two wide parts: endogenous and exogenous, Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well-being. However, under conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions. The most efficient enzymatic antioxidants involve glutathione peroxidase, catalase and superoxide dismutase (Matés et al., 1999). Nonenzymatic antioxidants include Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds (McCall et al., 1999).

Generally, the enzymic and non-enzymic molecules are distributed in the cytoplasm and different cell organelles. Many basic ubiquitous antioxidant enzymes, such as SOD, catalase, and various peroxidases in eukaryotic organisms catalyze complicated progressive reactions to convert ROS to more stable molecules such as water and O2. In addition to the primary antioxidant enzymes, a large number of secondary enzymes take part in an intimate association with small molecular weight antioxidants to produce redox cycles that maintain essential cofactors for primary antioxidant enzyme functions. Small molecular weight non-enzymatic antioxidants (such as; GSH, NADPH, thioredoxin, vitamins E and C, and trace metals like selenium) also function as direct scavengers of ROS. These enzymatic and non-enzymatic antioxidant systems are required for continuing life by providing a precise intracellular redox balance and diminishing unwanted cellular damage caused by ROS (Duraˇckov´a, 2010).

The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines (McCall et al., 1999). Evidence demonstrate that consumption of fruits and vegetables are better than intake of synthetic antioxidants , people who eat fruits and vegetables have a lower risk of heart disease and some neurological diseases and studies show that some types of vegetables and fruits in general, protect against a number of cancers as epidemiological studies show up, without maintaining the answer whether any specific bioactive molecules within fruit and vegetable have a particular support on lower incidence (Poljsak, 2011).

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German, 1999). Some antioxidants act in a hydrophilic environment, others in a hydrophobic environment, and some act in both environments of the cell. For example, Vitamin C reacts with superoxide in the aqueous phase while Vitamin E does so in the lipophilic phase. In contrast, -lipoic acid is both water and fatsoluble and therefore can operate both in cellular membranes and in cytosol. Certain antioxidants are able to regenerate other antioxidants and thus restore their original function. This process is called an “antioxidant

network"(Sies et al ., 2005). One more vital duty of antioxidants is to regulate ROS-related enzymes and oxidative damage, which can be reduced directly through reacting with free radicals or indirectly by preventing the activity or expression of free radical forming enzymes such as NAD(P) H oxidase or by raising the activity and intracellular antioxidant enzymes' expression like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Lü et al., 2010).

2.2.1. Antioxidant defense system

Reactive oxygen species can cause a lot of damage to cells and tissues, these potentially harmful reactions are countered by a system of enzymatic and non-enzymatic antioxidants that exist in the intracellular and extracellular environment and act as free radical scavengers, hydrogen/electron donors, peroxide decomposers, enzyme inhibitors, and metal-chelating agents (Frei et al., 1988). These antioxidants form the antioxidant defense system (ADS). The enzymatic system such as CAT, SOD, GPx, and GRx helps to defend against ROS "both directly and indirectly". However, it is the non-enzymatic antioxidants that act as scavengers for ROS and RNS, these include glutathione, Vitamin E, and C that prevents oxidation of lipid membranes, uric acid which is the scavenger of proxy-nitrite in the plasma, albumin, and melatonin which reacts with ROS to form disulfides (Buettner, 1993). Besides, the antioxidant systems counteract the impact of harmful oxidants which are the primary cause of cellular damage, and thus, the main mechanism for plenty of diseases including liver diseases, which makes ADS essential for disease prevention.

Table 2.1. Some of enzymatic and non-enzymatic

Enzymatic antioxidants	Non-enzymatic antioxidants
A. Superoxide dismutase (SOD) B. Catalase (CAT) C. Glutathione peroxidase (GP_X) D. Glutathione-S-transferase (GST) E. Glutathione reductase (GSH)	A. vitamins - α- tocopherol - ascorbic acid - carotenoids B. Glutathione C. Trace elements - selenium - copper - manganese, and zinc, D. coenzyme Q, dietary polyphenols E. other, uric acid, flavonoid, melatonin

2.2.2. Endogenous and exogenous enzymes

The body makes endogenous antioxidants, such as superoxide dismutase (SOD), alpha-lipoic acid (ALA), coenzyme Q10 (CoQ10), catalase, and glutathione peroxidase (GPx), and it requires micronutrient cofactors which includes iron, zinc, copper, selenium, and manganese for optimal catalytic activity. An inadequate nutritional intake of these trace minerals can compromise the effectiveness of these antioxidant protection mechanisms (Duthie et al., 1994; Brown et al., 2007). Glutathione a significant water-soluble antioxidant is synthesized from the amino acids glutamate, lysine, and cysteine. Glutathione directly quenches ROS such as lipid peroxides and also performs a major function in xenobiotic metabolism (Jocab, 1995). Lipoic acid also exerts role antioxidant as well as metal chelating capabilities (Kagen, 1992).

Exogenous antioxidants have a powerful effect on the antioxidant response system. They carry out their effects through additional mechanisms of action. By working alongside the endogenous antioxidant response system, exogenous antioxidants provide an improved and more efficient defense against changes that can be deleterious for protein functions. In status as tocopherols and resveratrol can be worked 2 or 3 different jobs together to counter the harmful redox modulation effects (Fraunberger et al., 2016). Melatonin is an endogenous antioxidant that plays a vital role in protecting against oxidative stress. In all organisms, melatonin is generated mainly during night time with

only small amounts synthesized during the day (Fraunberger et al., 2016). Exogenous antioxidants can come from natural sources such as flavonoids, vitamins E and C, and a few mineral compounds. But also can be synthesis compounds, such as butylhydroxyanisole, butylhydroxytoluene with gallates (Litescu et al., 2011).

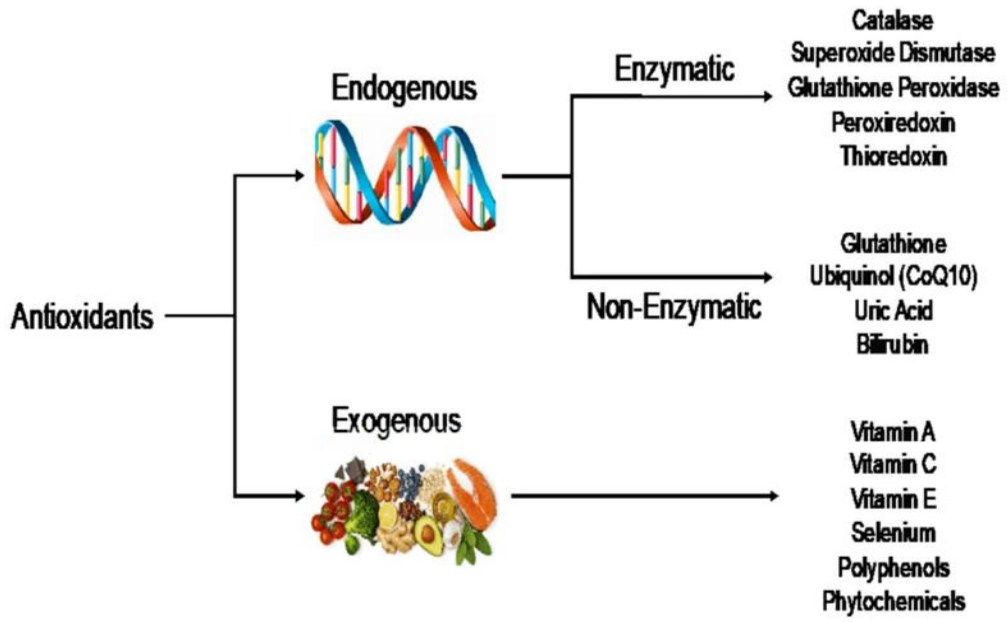

Figure 2.6. Endogenous and exogenous enzymes (Justin Quiles, 2018).

2.2.3. Enzymatic antioxidants

2.2.3.1. Superoxide dismutase (SOD)

Enzyme SOD (superoxide oxidoreductase, EC 1.15.1 .1) is thought to be existing in all oxygen-metabolizing cells, the function is to provide a defense against the potentially damaging reactivities of the superoxide radical (O_2^-)generated by aerobic metabolic reactions (McCord et al., 1971). The superoxide dismutases stimulate the dismutation of superoxide to hydrogen peroxide:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \ldots\ldots\ldots\ldots\ldots (2.7)$$

The hydrogen peroxide should then be eliminated by catalase or glutathione peroxidase, as described above. SOD also seems to be important in the protection of other diseases along with heart diseases, Parkinson's disease, Alzheimer's, and liver diseases.

The response catalyzed by SOD is very fast and in addition to the presence of enough amount of the SOD in cells and tissues, which normally keep the concentration of superoxide anion and it is low (Cases et al., 2017). Superoxide radicals are generated at different sites in the cell, such as the ER, ETC in mitochondria, as well as various enzymatic sources, like NOX and xanthine Oxidase, all of that help to the generation of ROS. Superoxide radicals are detoxified by SODs to form hydrogen peroxide, which is further detoxified by glutathione peroxidase GPx and peroxiredoxin Prx. Glutathione reductase GR is used to regenerate reduced glutathione GSH (Holley et al., 2014).

There are three forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution:

1. Copper zinc superoxide dismutase (CuZn- SOD):

CuZnSOD is containing active copper and zinc atom. Molecular mass is equal to approximately 32 000 kDa. It exists in the cytoplasm and organelles of all mammalian cells (Liou et al., 1993).

2. Manganese superoxide dismutase (Mn-SOD):

MnSOD is containing a lonely manganese atom, and it exists in the mitochondria of almost all cells. And has a molecular mass of 40 000 kDa. It contains four protein subunits into each one there is a manganese atom. Molecular mass is equal to approximately 40 000 kDa. MnSOD activity to have differed from that of CuZnSOD in enzymes mixtures, because of the amino acid sequence of MnSOD is dissimilar from that of CuZnSOD (Weisiger et al., 1973).

3. Extracellular superoxide dismutase (EC-SOD):

EC-SOD was described by Marklund in 1982 and is a secretory copper and zinc containing SOD distinct from the CuZnSOD described above (Marklund, 1982) . EC-SOD is synthesised by only a few cell types, including fibroblasts and endothelial cells, and is expressed on the cell surface where it is bound to heparan sulphates. EC-SOD is the major SOD detectable in extracellular fluids and is released into the circulation from the surface of vascular endothelium following the injection of heparin (Karlsson et al., 1993). EC-SOD might play a role in the regulation of vascular tone, because endothelial derived relaxing factor (nitric oxide or a closely related compound) is neutralised in the plasma by

superoxide (McIntyre et al., 1999). In the (Figure 2.7) shown all form of superoxide dismutase.

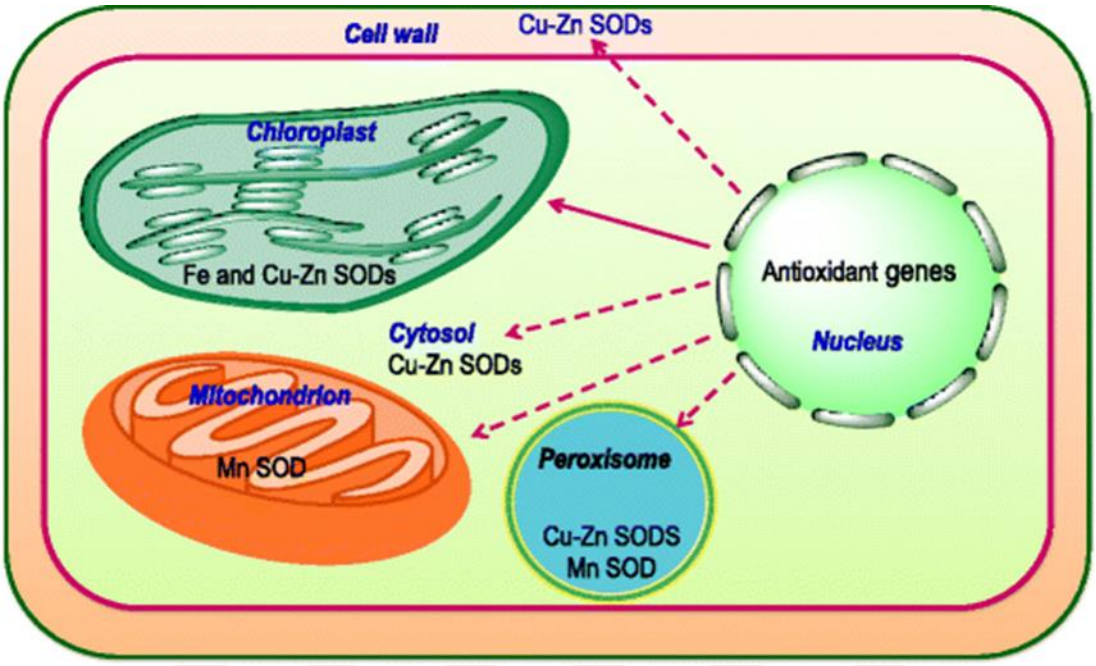

Figure 2.7. Superoxide dismutase three isoenzymes and location (Gill et al., 2015).

2.2.3.2. Catalase

Catalase is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria. Catalase is located in a cell organelle called the peroxisome. The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes one molecule of catalase can convert ~6 million molecules of hydrogen peroxide to water and oxygen each minute:

$$H_2O_2 \text{ cata}\rightarrow\text{lase } 2H_2O + O_2 \quad \text{(2.8)}$$ (Matés et al., 1999).

The enzyme contains 4 ferriprotoporphyrin groups per molecule, which corresponds to a protohaem content of 1.1 % and an iron content of 0.09 %. The physiological function of catalase is still obscure, although various theories have been put forward. Hydrogen peroxide is a harmful by-product of many normal metabolic processes to prevent damage it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Gaetani et al.,

1996). All known animals use catalase in every organ, the catalase activity of tissues varies greatly with particularly high concentrations occurring in the liver (Eisner et al., 1999) .

Catalases are organized into three main groups based on the variety of subunit sizes, different heme prosthetic groups, that is numerous of sequence group. Also diverse group of heme-containing proteins, consist of chloroperoxidase, plant peroxidases, myoglobin and catalatic activity. This may be attributable to the found of heme which alone can exhibit a very low catalase activity (Aebi, 1974; Nicholls et al., 2001).

2.2.3.3. Glutathione peroxidase

Glutathione peroxidase-1 (GPx-1) (glutathione: hydrogen-peroxide oxidoreductase; EC 1.11.1.9) is an intracellular antioxidant enzyme that reduces hydrogen peroxide into water to limit its harmful effects. It was first characterized in 1957 as an erythrocytic enzyme that protects hemoglobin from oxidative damage (Mills, 1957). Glutathione peroxidase-1 (GPx-1) is one antioxidant enzyme in intracellular, which enzymatically decreases hydrogen peroxide to water to reduce its harmful effects. Some reactive oxygen species, like hydrogen peroxide, are also necessary for growth factor-mediated signal transduction, maintenance of normal thiol redox-balance, and mitochondrial function (Lubos, 2011).

Enzyme glutathione peroxidase contains two types of enzymes, one of which is selenium-independent (GST) and the other is selenium-dependent (GPx). These two enzymes also differ in the number of subunits, their catalytic mechanisms, and the bonding nature at the active center with selenium. Glutathione metabolism is one of the important antioxidative defense mechanisms (Matés et al., 1999).

So far, eight different isoforms of glutathione peroxidase (GPx) have been identified in humans which vary in cellular location and substrate specificity:

GPx1; is found in the cytosol of most cells, including red blood cells (RBCs). GPx2; is an intestinal and extracellular enzyme. GPx3; is found in the plasma as a glycoprotein. GPx4; is mitochondrial and interacts with complex lipids like lipoproteins and cholesterol damaged by free radicals. GPx5; is an epididymal androgen-related protein. GPx6; is restricted to embryos and adult olfactory epithelium. GPx7; is one of the

glutathione peroxidase (GPx) family, but without activity. It plays a unique role as a stress sensor or transmitter. GPx8; is a putative GPx. (Espinoza et al., 2008).

All GPx enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH) which have properties that allow them to eliminate peroxides as potential substrates for the Fenton reaction. The substrate for the catalytic reaction of GPx is H_2O_2 or anorganic peroxide ROOH. It acts in conjunction with the tripeptide glutathione (GSH); which presents in high concentration in cells; decomposing peroxides to water (or alcohol) while simultaneously oxidizing GSH:

$$2GSH + H_2O_2 - G \rightarrow Px\ GSSG + 2H_2O \text{……(2.9)}$$

$$GSH + ROOH - G \rightarrow Px\ GSSG + ROH + H_2O \text{……..(2.10)}$$

Significantly, GPx competes with catalase for H2O2 as a substrate and is the major source of protection against low levels of oxidative stress (Matés et al., 1999).

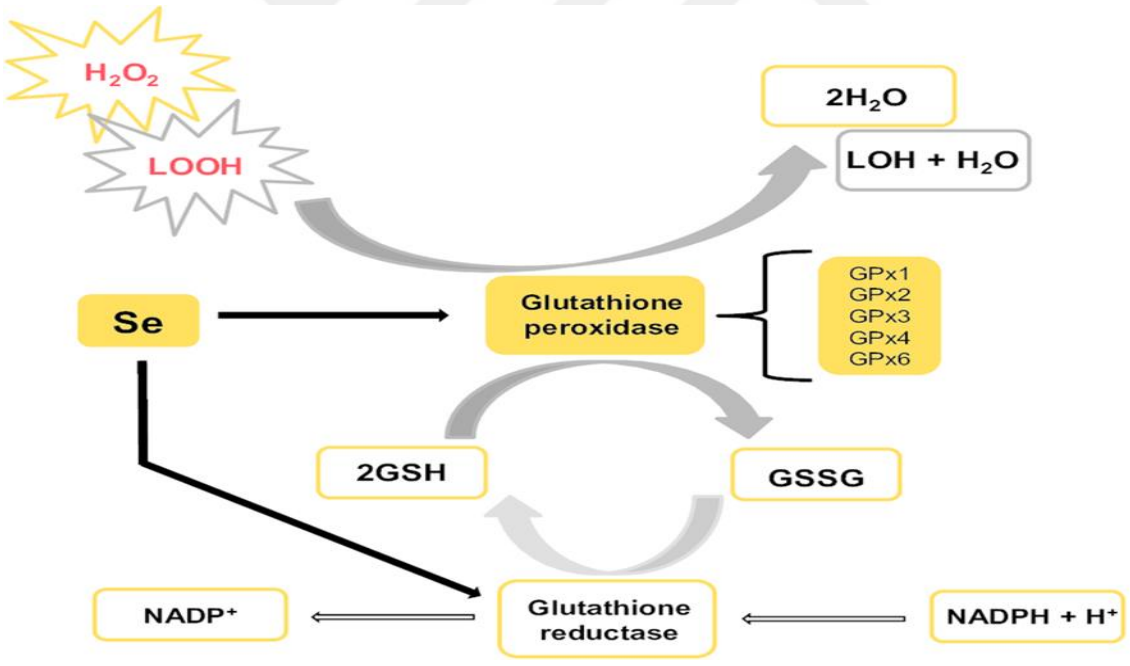

Figure 2.8. Detoxification of hydrogen peroxide (H_2O_2) and lipid peroxides by GPx (Ellwanger et al ., 2016).

2.2.3.4. Glutathione reductase

Reductase glutathione that is a family of the enzymatic antioxidant glutathione reductase (GR, EC 1.6.4.2) is a ubiquitous enzyme, which catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH). Glutathione reductase is essential for

the glutathione redox cycle that maintains adequate levels of reduced cellular GSH. GSH serves as an antioxidant, reacting with free radicals and organic peroxides, in amino acid transport. This enzyme is a family member of flavoprotein disulfide oxidoreductases. GR is that recognize NADPH depended oxidoreductase that alternate GSSG to GSH via using the pentose phosphate reaction pathways.

$$GSSG + NADPH + GR \rightarrow 2GSH + NADP^{+} \text{……..(2.11)}$$

The function of glutathione reductase (GSH) that within the chain of the cellular comprise amino acid its operating the transport. Making the nucleic acid and protein the enzymes activity modulation of carcinogen xenobiotic and reactive oxygen species the biochemical function of the flavoprotein. GSH is an important antioxidant. Hydrogen peroxide generated as a result of aerobic metabolism, may be metabolized by mean of GSH peroxidase inside the cytosol and mitochondria, and via catalase within the peroxisome. GSSG is reduced retrace to GSH with the aid of GSSG reductase at the expense of NADPH, thereby forming a redox cycle. Organic peroxides (ROOH) can be reducing by other GSH peroxidase or GSH S-transferase. To prevent a shift in the redox equilibrium, GSSG can either be actively transported out of the cell or react with a protein sulfhydryl (PSH) to form a mixed disulfide (PSSG) (Lu, 2012).

GR is catalysis of the NADPH-dependent reduction of oxidized glutathione. The glutathione is a tripeptide widely distributed within plant and animal. As a result the enzyme is found in a lot of tissues, allowing the cells to maintain adequate stage of reduced glutathione. Reducing glutathione is a significant cellular antioxidant and likewise a substrate for the glutathione peroxidases, which provide a mechanism for the detoxification of xenobiotic. Oxidative stress has been implicated in ageing and within the pathogenesis of a number of problems. The extent of damage is typically related to an increase or decrease of one or more free radical scavenging enzymes. Excessive range of GR activity had been found in erythrocytes from patients with rheumatoid arthritis. Reductase glutathione content to responsive the environmental factor many distinction condition are known to change intracellular GSH content excessive glucose concentration, heavy metal, exposure to (ROS/RNS) specially hydrogen peroxide and nitric oxide. Mammalian glutathione reductase activity is present in both the cytosol and mitochondria. Due to of the essential role of GSH within removal of deleterious reactive oxygen species

and preservation of the protein thiol redox state, GR is crucial to the cell's antioxidant defense mechanism and maintenance of enzyme activities and protein roles (Lu, 2012).

2.2.4. Non-enzymatic antioxidants

Non-enzymatic antioxidants are small molecules that are soluble in water and lipid, and they also prohibit forming free radicals in the cell partitions of water and lipid. The function of these molecules helps to scavenge and detoxify in the body. The examples of these low molecular weight compounds are β-carotene, uric acid, vitamins C and E and GSH that contain a group of thiol sulfhydryl (Jellinger, 2000).

2.2.4.1. Glutathione

The peptide that contains cysteine and found in most shapes of aerobic life is called Glutathione GSH. The tripeptide glutathione consists of three amino acids (glycine, cysteine and glutamic acid) existent in the tissue of generality mammalian. It's synthesized by amino acids in cells, it's lieu of takes in the diet (Gaballa et al., 2010).
The thiol group in its cysteine moiety behaving as a reducing agent and can be oxidized reversibly and decreased, so it gives an antioxidant properties for glutathione.

Glutathione (Figure 2.9) function is like a free radical scavenger, a detoxifying agent and an antioxidant. The other importance of glutathione is as an agent for the enzyme glutathione peroxidase GPx, in the formation of leukotienes and in the uptake of amino acids. Inside the cells, enzyme glutathione reductase is working at maintained on the glutathione in the reduced form, so it reduces other metabolites and systems of enzym, such as glutathione peroxidases, glutaredoxins, and reaction directly with oxidants (Fairlamb et al., 1992). In the system of glutathione, glucose-6-phosphate dehydrogenase (G-6-PD) and glutathione reductase (GR) are not effecting directly in the action on ROS, but GPx is doing to that function (Weydert et al .,2010). Glutathione is one of the most important cellular antioxidants, according to its high concentration and its basic role in maintaining the cell's redox condition. The substrate for glutathione S-transferase reacts with a group of harmful chemical species, such as epoxides, free radicals, and halides, to form products that are harmless and inactive.

It had been referenced that Glutathione can a helpful ability for cysteine. Glutathione is readily oxidized under the effact of normal oxidative stress. And also, glutathione disulfide (GSSG) is forming from react two molecules of oxidized GSH with each other, forming a disulfide bond. Disulfide bond can be smashen upon reduction. The reduction of GSSG is stimulated by glutathione reductase (GR) using (NADPH) to formtion two molecules of glutathione (GSH) from glutathione disulfide (GSSG) :

GSSG + NADPH →(GR) → 2GSH + NADP……(2.12) (Lu, 1998).

As well high a concentration of Glutathione disulfide (GSSG) may harm numerous enzymes oxidative. GSSG can response with protein sulfhydryl groups to produce protein–glutathione-mixed disulfides. Following reaction:

GSSG + protein-SH ↔ protein-SSG + GSH…….(2.13).

In some organisms glutathione is replaced by other thiols, such as by mycothiol in the actinomycetes, bacillithiol in some Gram-positive bacteria, or by trypanothione in the kinetoplastids (Fairlamb et al., 1992; Meister, 1994).

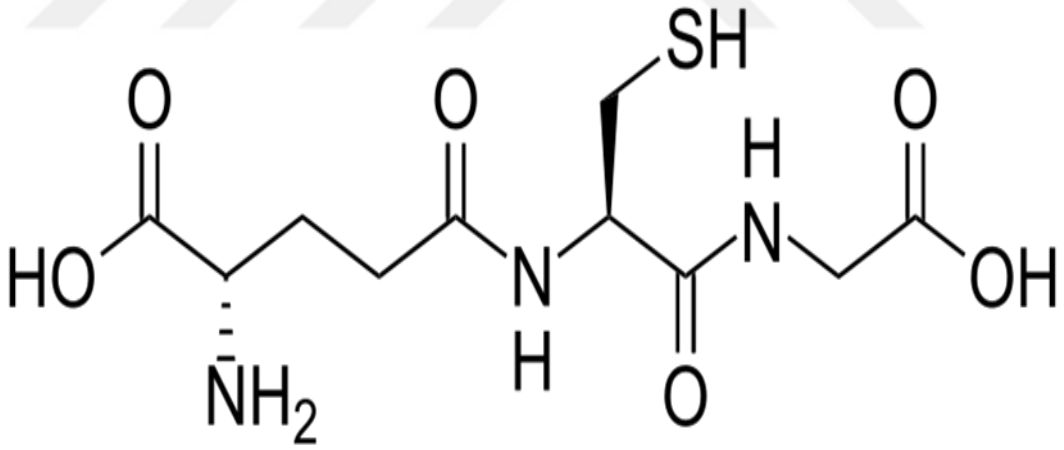

Figure 2.9. Glutathione GSH.

2.2.4.2. Vitamin E

Vitamin E is a fat-soluble vitamin and high efficacy antioxidant. Vitamin E is a chiral composite and it contains eight stereoisomers: α, β, γ, δ tocopherol and α, β, γ, δ tocotrienol. Just α-tocopherol (Figure 2.10) is the most activity from in the human. Studies say that natural dextrorotary d-α-tocopherol is about twice as effective as synthetic racemic dl-α-tocopherol in both animals and humans (Nguyen et al., 2006). On account of

it is fat-soluble, α-tocopherol protects cell membranes from damage by free radicals. Its antioxidant action fundamentally resides in the protection against lipid peroxidation. From mechanisms of α-tocopherol: transfer of a hydrogen atom at 6–hydroxyl group on the chroman ring, and defiance of singlet oxygen and other reactive species (Nguyen et al., 2006).

Tocopherols are renovated in the existence of ascorbic acids. The active chroman ring is carefully put to the surface while the phytyl chain can be suitable in the membrane stratum. This unique structure can tocopherols to work as potent antioxidants and to be renewed through reaction with other antioxidants like ascorbic acid (Papas, 1998).

For the prevention against many diseases (breast cancers, some cardiovascular diseases, cataracts, arthritis and certain neurological disorders, colon, prostate, and ischemia), vitamin E has been recommended. Vitamin E deficiency can occur because of genetic abnormalities in α-tocopherol transfer protein, protein-energy malnutrition, and also fat malabsorption syndromes. Deficiency can result from insufficient dietary intake of the vitamin. Numerous different dietary factors affect the need for vitamin E (IOM, 2000). Vitamin E exists in a lot of like the dietary sources of vegetable oils, wheat germ oil, whole grains, fruits, eggs, nuts, cereals, poultry, meat (Thendral).

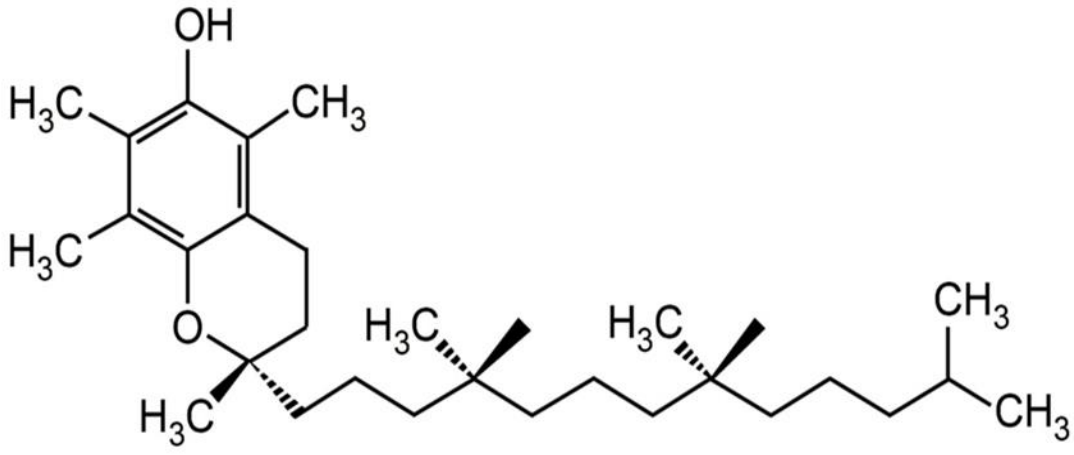

Figure 2.10. α-tocopherol, in humans.

2.2.4.3. Vitamin C

Vitamin C also called ascorbic acid, is one of the vitamins that a water-soluble. It is very important for biosynthesis for each of the collagen, carnitine, and neurotransmitters (Li et al., 2007). Health avails of vitamin C are antioxidants, anti-carcinogenic immunomodulator, anti-atherogenic. Vitamin C is working in decreasing the incidence of stomach cancer and the protection from injury in lung and colorectal cancer. Vitamin C acts synergistically with vitamin E to disable free radicals and also renew the reduced shape of vitamin E. Yet, taking off high potions of vitamin C (2000mg or more daily) can causing disable the action of many drugs necessary for carcinogenesis (Naidu, 2003; Li et al., 2007). Natural sources the most important of vitamin C are green vegetables, acid fruits, tomatoes. Ascorbic acid is an adjustable molecule easily, therefore it can lose from within cooking (Naidu, 2003).

Hydrogen atom awarded to lipid radicals, removal of molecular oxygen, quenching of singlet oxygen are considered as essential antioxidant mechanisms of ascorbic acid. Addition, scavenging aqueous radicals and regeneration of α–tocopherol from the tocopheroxyl are other the antioxidant mechanisms. Ascorbic acid is a superb electron contributor because it enjoys the ability to the low standard 1-electron reduction (282 mV), the generation of relatively steady semi-dehydroascorbic acid, and the easy transformation of dehydroascorbic acid to ascorbic acid (Levine et al., 1999). Vitamin C examine scavenges reactive oxygen and nitrogen species, such as superoxide and hydroperoxyl radicals, singlet oxygen, ozone, aqueous peroxyl radicals, peroxynitrite, nitroxide radicals, nitrogen dioxide, and hypochlorous acid (Halliwell, 1996). Antherefore effectively protection to substrates from oxidative damage.

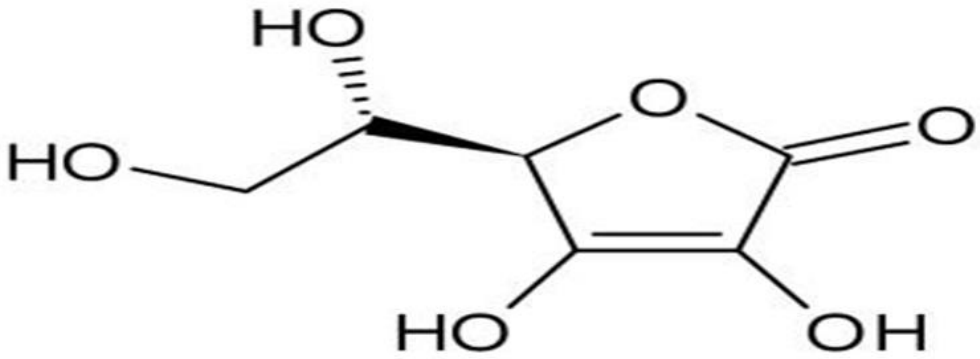

Figure 2.11. Structure vitamin C (Halliwell, 1996).

2.2.4.4. Vitamin A

Vitamin A is an essential nutrient that supports eye, skin, reproductive health, and immune function. There are two types of vitamin A: retinoids and carotenoids. The liver converts both types to retinol. Then, it's either stored or transported by the lymphatic system to cells throughout the body . Skin readily absorbs vitamin A when applied topically, because it's a retinoid-responsive organ (Willcox et al., 2004).

Carotenoids are lipid-soluble antioxidants that play an important role in plant metabolism including oxidative stress tolerance (Maiani et al., 2009). Epidemiological studies have detected that increased consumption of a diet rich in carotenoids is linked with a reduced risk of age-related diseases. Carotenoids' antioxidant activity arises from their conjugated double bonds and their ability to eliminate unpaired electrons (Willcox et al., 2004). Actually, in the last period, several studies have been conducted in rat liver that carotenoids, like β-carotene, lutein, lycopene, and β-cryptoxanthin, have antioxidant effects against lipid peroxidation. And it is responsible for the capacity of carotenoids to suppression singlet oxygen without degradation and react of carotenoids with free radicals.

The efficacy of carotenoids for physical suppressing is connected to the number of conjugated double bonds present in the molecule. Other factors that impact the effectiveness of carotenoids as antioxidants are the concentrations of carotenoids and the

partial pressure of oxygen. β-carotene in particular, exhibit antioxidant properties at low oxygen partial pressure. Whereas at high pressures of oxygen, or high carotenoid concentrations become pro-oxidants, pro-oxidant behavior is displayed (Rice-Evans et al., 1997).

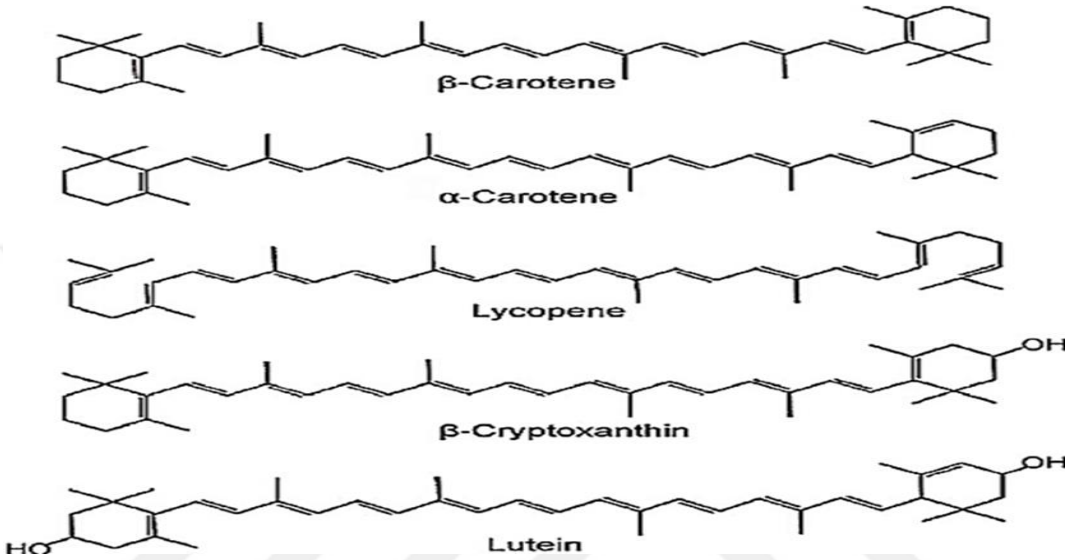

Figure 2.12. Carotenoids structure (Maiani et al., 2009).

2.2.4.5. Trace elements

Zinc, copper, and selenium are trace elements associated with cytoplasmic protection from ROS and RNS. Copper-zinc superoxide dismutase (CuZn-SOD) is an abundant copper- and zinc-containing protein that is present in the cytosol, nucleus, peroxisomes, and mitochondrial intermembrane space of human cells that catalyzes the dismutation of superoxide radicals into hydrogen peroxide which in turn gets reduced (along with other hydroperoxides) by the selenoenzyme glutathione peroxidase (GPx). Cytosolic GPx also acts as a peroxynitrite reductase (Vural et al., 2010).

Furthermore, these trace elements possess antioxidative functions outside the confines of being components within enzymes:

(a) In addition to selenocysteine, selenium also exists in the cell as selenomethionine, which is an analog of methionine, and regularly present in proteins in low amounts. Selenomethionine catalyzes peroxynitrite reduction.

(b) Pharmacological compounds like low-molecular-weight organoselenium catalyze the reduction of hydroperoxides or peroxynitrite with several cellular reducing equivalents.

(c) Copper and zinc ions may stimulate the antiapoptotic phosphoinositide-3-kinase/Akt and other cellular stress-signaling pathways and may stabilize proteins, making them less vulnerable to oxidation. (Klotz, 2003).

2.3. Oxidative stress

Oxidative stress is a dangerous phenomenon resulting from free radicals and oxidants. ROS is working on alteration of the cell membranes and other structures such as deoxyribonucleic acid (DNA), lipids, lipoproteins, proteins (Young et al., 2001; Droge, 2002).

ROS at normal conditions is maintained by different enzyme systems and is a normal fact in the body. Where it is sharing these systems at low levels contribute to the Vivo balance between oxidation and reduction. Therefore, oxidative stress is also known as a disorder between the antioxidants and prooxidants in the body (Rahal et al., 2014). And as oxidative stress occurs because of imbalance between the formation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and the capacity of the vital system to disposal from these reactive mediums. Dangerous substances and compounds arise as a result of Biochemical modifications by ROS and RNS, like lipid peroxidation, which action on oxidize proteins, lipids, and DNA damage (Kukongviriyapan et al., 2016).

Oxidative stress is also an ongoing process in vivo body resulting from normal cellular procedures. As was mentioned before, there is a balance status in biological processes between ROS and antioxidant production (Rahal et al., 2014). This balance can be lost due to some causes but, and Oxidative stress production can surpass normal physiological levels, which in the role can cause cellular damage (Saral et al., 2005).

Oxidative stress can occur on short term result trauma, certain toxins, Infections, overworking, all of that cause to light injuries to different tissues. Tissues forme several enzymes that contribute to radical production like xanthine oxidase, lipogenase, cyclooxygenase, when occurring damage. They work stimulation phagocytes, lead more iron and copper ions to be liberated into circulation, and disable electron transport series.

When these effects continue for a long interval of time, the ROS/antioxidant balance can be lost, that in the role can cause to develop different kinds of cancer, or lead present cancer to prevalence readily. Moreover, some studies were shown a relationship between ROS and diabetes mellitus and various age-associated diseases as such the Parkinson's disease (Rao et al., 2006). ROS also causes chronic fatigue, atherosclerosis, Alzheimer's, malaria disease (Figure 2.13) (Islam, 2015).

The most important endogenous reasons that cause increased ROS production include (elevation in O_2 concentration, inflammation, and increased mitochondrial leakage), and exogenous (active exercise, nutrition, psychological, smoking environmental pollution, chronic inflammation, psychological and emotional stress, and others). Causes of decreased antioxidant defenses include reduced activity of endogenous antioxidative enzymes and reduced eating or intake of antioxidants from food (Poljsak et al., 2013).

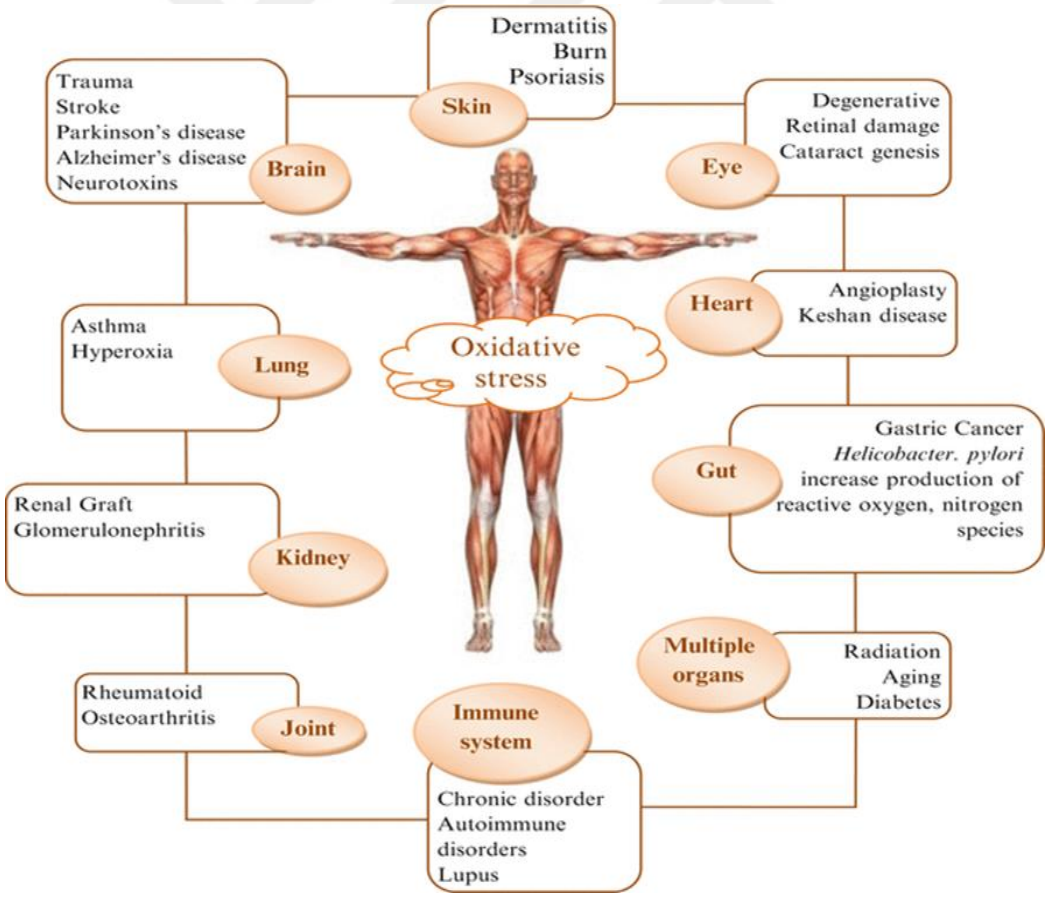

Figure 2.13. Oxidative Stress on Human Health (Islam, 2015).

2.3.1. Nucleic acid (DNA) damage

Oxidative DNA lesions are formed due to constant oxidative damage to DNA, causing major problems and mutations. The body has lots of methods to prevent these damages by using antioxidants or DNA repair enzymes (Willcox ., et al 2004; Halliwell, 2007). If not functioned properly and provided sufficient quantities, oxidative stress can attack DNA and causes some of the chronic and degenerative diseases as well as the aging process and some acute pathologies (trauma, stroke, and Cancer). Reactive oxygen species are formed through a variety of events and pathways. Some reports suggest that every human cell can is exposed to for nearly 1.5×105 oxidative infections daily from hydroxyl radicals and other reactive species (Beckman et al., 1997). Hydroxyl radicals can attack to components DNA and react with it, causing damage both the pyrimidine and purine bases and also the deoxyribose backbone (Dizdaroglu et al., 2002).

ROS-induced DNA damage involves single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA harm can lead either in curb or agitation of transcription, induction of signal transduction course, genomic instability, and replication troubles, all of which are concerned with carcinogenesis (Marnett, 2000; Cooke et al., 2003). The progress of cancer in humans is a complicated procedure including cellular and molecular changes mediated by variety endogenous and exogenous catalysts. It is well discerned that oxidative DNA harm is answerable for cancer development (Valko et al., 2004; Valko et al., 2007). Chromosomal defects and oncogene activation is the beginning of carcinogenesis, and the reason for this is the free radicals. The formation of hydroxylated based is considered from the important cases and common that infection DNA, and be an indication of chemical carcinogenesis (Valko et al., 2004; Halliwell, 2007).

Genetic mutations and altering normal gene transcription be because of adduct formation with normal cell evolution. Base and sugar lesions, strand breaks, DNA-protein cross-links, and base-free sites are the most modifications and changes that occur on the DNA structure. For example, smoking and chronic infection resulting from noninfectious injuries like asbestos are reasons for oxidative DNA damage that can cause to the development of lung cancer and other tumors (Valko et al., 2004; Willcox et al., 2004).

2.3.2. Lipid peroxidation

Lipid peroxidation is considered one of the dangerous conditions that damage cells, cause differences in them and pathological states in adults and babies. It occurs naturally in the body and small amounts because of the influence that it plays reactive oxygen species (hydroxyl radical, hydrogen peroxide, etc). It can be produced via the action of phagocytes (Mylonas et al., 1999). The elimination of the end-compounds of such lipid peroxidation reactions and membrane lipids causes significant damage to viability cells and even tissues.

The great damage that happens to the tissues, results from self-propagating chain-reaction of lipid peroxidation causing initial oxidation of only a few lipid molecules. Despite much research on lipid peroxide it has not yet been arrived at a sufficient result about how much affect lipid peroxide is doing on pathological conditions, but it has been affected in several diseases such as atherosclerosis, IBD, ROP, BPD, Parkinson's disease, kidney damage, asthma, preeclampsia and others (Mylonas et al., 1999).

Peroxyl radicals react with lipids, causing dehydrogenation of fat and production hydroperoxides, lead to an increase propagate the free-radical pathway. Hydroperoxides effect on cells and destroy it. Also, it can interact with transition metals important in the body such as iron or copper to formation aldehydes like malondialdehydes, which will harm cell membranes (Halliwell et al., 1990). Cell membrane phospholipids are more likely to oxide, thus be entered in free radical chain reactions as a result of constant damage that it's exposed by free radicals. Some of the fatty acids are polyunsaturated, possessing a methylene group between two double bonds that gives the fatty acid high sensitive to oxidation. The high concentration of polyunsaturated fatty acids in phospholipids lets them enter in free radical chain reactions (Wang et al., 1996).

2.3.3. Oxidative damage to protein

Proteins are oxidatively exposed in several methods: free radical-mediated peptide cleavage, oxidative amendment of amino acid, and formation of protein cross-linkage through reaction with lipid peroxidation compounds. Proteins that including amino acids such as cysteine, methionine, histidine, and arginine show to be the most affected to

oxidation (Freeman et al., 1982). Oxidative stress is working on oxidizing amino acids that are in proteins, causing the formation of protein-protein cross-linkages, causing a change in the formation of the protein and modify of functioning, absence of enzyme activity, lack of function of receptors and transport proteins (Butterfield et al., 1998).

ROS can ruin proteins and cause of produce carbonyls and alteration on amino acids like the formation of methionine sulfoxide, protein carbonyls, methionine sulfoxide, and protein peroxide. Significant changes occur as a result of oxidations to which proteins are exposed, causing an effect on signal transduction mechanism, heat, enzyme action, and proteolysis susceptibility. Also it's possible to occur increasing in levels of protein carbonyls in many diseases such as, Alzheimer’s disease (Smith et al., 1991), cataractogenesis (Garland et al., 1988), Parkinson's disease (Floor et al., 1998), Rheumatoid Arthritis (Chapman et al., 1989), progeria, atherosclerosis, respiratory destroys syndrome, Werner’s syndrome (Berlett et al., 1997), ageing (Smith et al., 1991), and diabetes (Jones et al., 1993).

2.4. Liver cirrhosis

Cirrhosis appears to a progressive, diffuse, fibrosing, nodular condition that changing the normal form of the liver and disrupts its function. And it is known that the liver acts generally in the maintenance of metabolic homeostasis via processing dietary amino acids, lipids, and vitamins, carbohydrates; producing clotting factors; metabolizing cholesterol and toxins, and storing glycogen (Friedman et al., 2004).

Cirrhosis is indicated to the histological development of regenerative nodules, that is surrounded by fibrous bands as a result of chronic liver injury, causing portal hypertension and end-stage liver disease (Tsochatzis et al., 2014). Cirrhosis affects hundreds of millions of patients worldwide. Liver cirrhosis and digestive diseases the most common cause of death in the US, equivalent 30,000 deaths per year. Also, 10,000 deaths occur due to liver cancer, the majority of which arise in cirrhotic livers, with the mortality rate steadily rising (El-Serag et al., 2000).

There is a variation in clinical manifestations of cirrhosis, from no symptoms to deterioration and liver failure, and are determined by both the nature and severity of liver disease as well as the extent of hepatic fibrosis. About 40% of patients with cirrhosis are

not suffered asymptomatic and probably remain so for many years, but is inevitable progressive deterioration once complications develop including encephalopathy, Ascites, and variceal hemorrhage. In such patients, there is a 50% 5-year mortality, with approximately 70% of these deaths its main reason for liver disease (Fattovich et al., 1997).

Recent advances are helped in access to natural history, know the pathophysiology of cirrhosis and in the treatment of its complications, and therefore in improved management, and life expectancy of cirrhotic patients. At present, liver transplantation is the only treatment currently available for cirrhotic but pharmacological treatments can stop the development of decompensated cirrhosis or even reverse cirrhosis (Tsochatzis, et al 2014).

Figure 2.14. Cirrhosis, scar tissue replaces healthy liver tissue.

2.4.1. Pathophysiology

Cirrhosis occurs from different mechanisms as a result of liver injury that leads to necroinflammation and fibrogenesis. histologically; It is characterized by nodular regeneration surrounded by dense fibrotic septa with subsequent parenchymal extinction and collapse of liver structures, together causing pronounced distortion of hepatic vascular architecture (Figure 2.15). This distortion results in increased resistance to portal blood flow and hence in portal hypertension and hepatic synthetic dysfunction(Schuppan et al., 2008). In the advanced stages of cirrhosis, splanchnic vasodilation becomes more wide and so intense and therefore is helping in determine a hyperdynamic splanchnic and systemic circulation, which has a major role with portal hypertension in the pathogenesis of ascites and hepatorenal syndrome. Also Systemic vasodilation lead to further pulmonary ventilation / perfusion mismatch which causes hepatopulmonary syndrome and arterial hypoxaemia in severe cases (Fernández, 2009).

When endothelial dysfunction occurs in the pulmonary circulation which causes pulmonary vasoconstriction and therefore portopulmonary hypertension. Contributes to variceal bleeding, firstly an increase in size of varices via anatomical factors, angiogenesis dependent on vascular endothelial growth factor, and increased portal pressure, and collateral blood flow. Of causes portal hypertensive and gastropathy, Dilation of gastric mucosal vessels. One of the main determinants of hepatic encephalopathy is shunting of portal blood to the systemic circulation by Portosystemic collateral, decreased reticuloendothelial system function with decreased first-pass effect of orally administered drugs. These changes interfere with effective hepatocyte perfusion so the capillarisation of Sinusoid and Intrahepatic shunts are very important, add to that it is a major determinant of liver failure (García-Pagán, 2012).

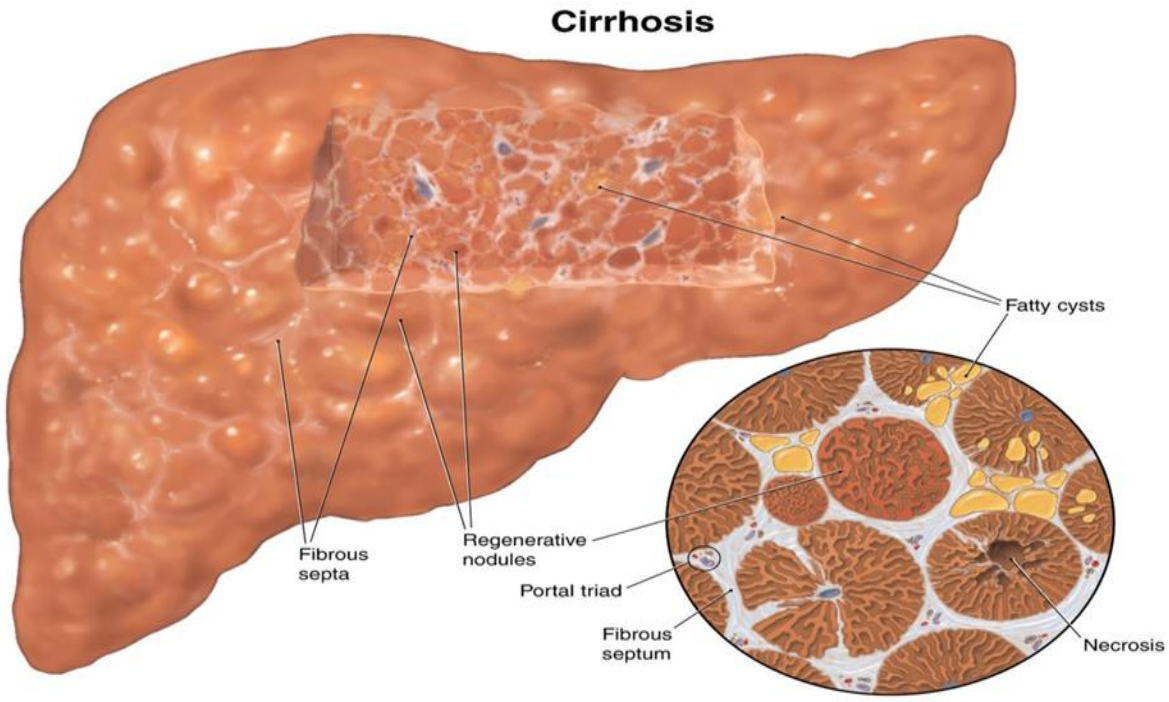

Figure 2.15. Hepatocellular nodules in cirrhosis (Lee et al., 2011).

2.4.2. Diagnosis

In stage Compensated cirrhosis, Diagnosis of asymptomatic cirrhosis is usually made, patients exhibit no symptoms or signs of liver disease, and their test results show that liver synthetic function is intact even when testing such as determination of liver transaminase levels or radiological findings suggest liver disease. but can progress eventually compromising hepatocyte function and hepatic circulation. The first signs of advanced cirrhosis are occurring changes in laboratory test results, which can include thrombocytopenia, prolonged prothrombin time, hyperbilirubinemia, and hypoalbuminemia. All or some clinical manifestations can occur when cirrhosis causes hepatic decompensation (Busuttil et al., 2014).

Although Advances in prevention and treatment of the common complications of cirrhosis such as ascites, variceal bleeding, encephalopathy, and spontaneous bacterial peritonitis. But those complications (e.g., variceal bleeding, spontaneous bacterial peritonitis, hepatorenal syndrome) are dangerous and themselves life-threatening of injured, and in very advanced cases, warrants consideration for liver transplantation, the

prognosis just for any patient with decompensated cirrhosis is not enough (Busuttil et al., 2014). The most common symptoms of cirrhosis:

2.4.2.1. Variceal bleeding and portal hypertension

Variceal hemorrhage is defined Bleeding from a gastric or esophageal varix at the time of endoscopy, and the presence of blood with large esophageal varices in the stomach and no other source of bleeding (Jalan et al., 2000). The rate of variceal bleeding varies from patient to patient but is approximately 10% to 30% per year (Calés et al., 1990). Patients with cirrhosis who have a present upper gastrointestinal bleeding should undergo urgent upper endoscopic evaluation. and that's what it is states the British Society of Gastroenterology guidelines for the management of variceal hemorrhage (Jalan et al., 2000).

The key factor in the pathophysiology of portal hypertension is increased resistance to portal blood flow, and can be determined by the morphological changes occurring in chronic liver diseases. Two factor contributing to aggravate the portal hypertension, the first by a dynamic component, which is decreased synthesis of NO in the intrahepatic circulation (the active — reversible — contraction of different elements of the porto-hepatic bed) , the second factor is significant increase in portal blood flow due arteriolar splanchnic vasodilation and hyperkinetic circulation (Bosch et al., 2000). When occurring endothelial dysfunction in the pulmonary circulation, will pulmonary vasoconstriction, and thus portopulmonary hypertension. And contributes to variceal bleeding several factors including angiogenesis dependent on vascular endothelial growth factor, increase in size of varices is driven by anatomical factors, and increased portal pressure and collateral blood flow (García-Pagán, 2012).

2.4.2.2. Ascites

Splanchnic vasodilatation is the main factor that contributes to ascites (Schrier et al., 1988). cirrhosis leads to an increase in hepatic resistance to portal flow which causes collateral-vein formation with the gradual development of portal hypertension and shunting of blood to the systemic circulation. Increased production of vasodilators like

nitric oxide due develops portal hypertension, leading to splanchnic arterial vasodilatation (Martin et al., 1998).

Splanchnic arterial vasodilatation significantly appears in the advanced stages of cirrhosis causing arterial pressure falls with decrease the effective arterial blood volume. Leads homeostatic activation of vasoconstrictor and antinatriuretic factors to retent sodium and fluid result arterial pressure is maintained. Intestinal capillary pressure and permeability are affected by splanchnic arterial vasodilatation alter and portal hypertension which leads to accumulation of retained fluid within the abdominal cavity. Renal excretion is decreasing significantly and renal vasoconstriction with progression of the disease that lead to the hepatorenal syndrome and dilutional hyponatremia (Schrier et al., 1988; Arroyo et al., 1996).

2.4.2.3. Encephalopathy

One of the most important complications of cirrhosis is Hepatic encephalopathy (HE), which has a affects neuropsychiatric on the patient. Hepatic encephalopathy causes by in depressed level of consciousness, personality changes, and intellectual impairment. Hepatic encephalopathy in cirrhotic patients is characterized by sleep altered and astrocytic changes (rather than neuronal), eventually progressing through asterixis to stupor and coma. These changes are known as Alzheimer type II astrocytosis, which is expressed by astrocytic proteins (Butterworth, 2000). It may result from manganese deposition bilateral signal hyperintensities particularly in globus pallidus on T1-weighted imaging and reveals of them by magnetic resonance imaging (MR). Increases in the glutamine synthesis in the brain results from increased brain ammonia removal, a finding which confirms previous biochemical studies and Proton (1H) magnetic resonance spectroscopy shows its (Butterworth, 2000). Bowel cleansing with lactulose by orally or with enemas helps on toward improving mental status and processing (Angeli P et al., 1999).

2.4.2.4. Jaundice

Bilirubin one of the waste products produced by the body and should get rid of it by the liver. Because it affects the liver and causes jaundice, which refers to discoloration

of the skin and eyes to yellow. And also, urine is dark yellow and that's when the bilirubin levels in the blood increase. Bilirubin generally is transferred from the liver to the intestines, but if there is blockage outside in the bile ducts it causes hepatic cholestasis and if there is a blockage within the liver it is termed intrahepatic cholestasis (Ohkubo, 1994). Long-term abuse of alcohol and hepatitis viruses can cause hepatocellular jaundice besides liver cirrhosis (Ricketts ., 1951). But jaundice is not one of the most important causes of cirrhosis, because more one-thirds of causes are treated during cirrhosis (KIMBALL et al., 1947). Other Symptoms of cirrhosis include:

Spontaneous bacterial peritonitis, Hepatorenal syndrome, Hepatopulmonary syndrome, Hypersplenism, Liver cancer, Fatigue, Weakness, Loss of appetite.

Figure 2.16. Symptoms of liver cirrhosis.

2.4.3. Causes of liver cirrhosis

The main causes of liver Cirrhosis in industrialized countries include chronic HCV , HBV infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH) In addition to the accumulation of some elements such as copper and iron , genetic reasons and reasons unknown.

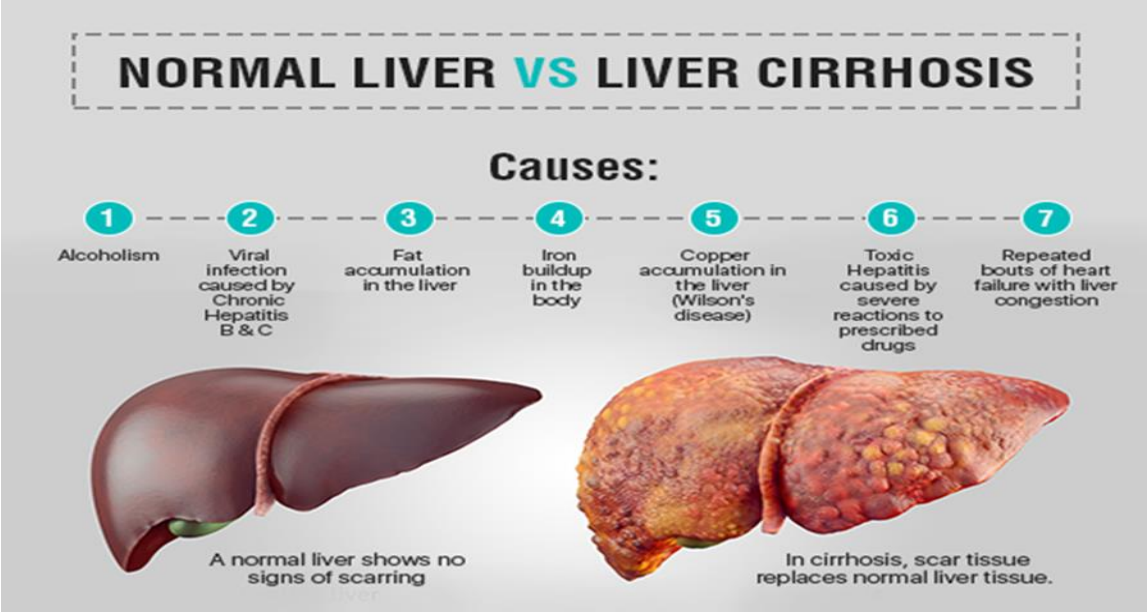

Figure 2.17. The main causes of liver Cirrhosis.

2.4.3.1. Chronic Alcoholism

Alcoholism is considered from factors that negatively affect the liver. Initially, the alcoholic rate will increase in the liver, which causes some problems, besides it's can also worsen other pre-existing liver diseases thus accelerating injury of liver diseases including alcoholic fatty liver, which in turn with the continued drinking of alcohol will develop to alcoholic hepatitis and cirrhosis (Smart et al ., 1992).

May injure in one of those cases without passing or arriving at the other, and continue to along time without developing. However, infected in previous cases not condition, and may injure in one of those cases without passing or arriving to the other, knowing that they are linked together. Studies are showing that it is possible to arrive at cirrhosis without passing through other diseases (Maddrey, 1988).

2.4.3.2. Hepatitis C Infection

HCV forms infections from diseases that injure of liver. Over time it can develop into cirrhosis, where a study showed that each 75 - 80 patients with HCV, between 5 and 20 of them will develop cirrhosis. The hepatitis C virus is considered a serious disease that threatens liver activity and causes cirrhosis (Perz et al., 2006).

The virus is transmitted in several ways contaminated needles, sexual relation, blood, and patient fluids, and from mother-to-child during pregnancy. During HCV infection, collagen dramatically will act to grow thus and it's difficult to dissolve it. It causes it build-up of scar tissue around cells. The blood carries the necessary substances for the liver and difficult arriving it to liver cells (Toshikuni et al., 2014).

2.4.3.3. Hepatitis B Infection

Chronic hepatitis B virus infection is a serious disease injury the liver that causes death nearly a million people worldwide, as a result of the development of the disease to cirrhosis. HBV complications interfere with liver function and cause pathological damage as liver failure and hepatocellular cancer (HCC) (Mast et al., 1999).

Hepatitis B infection is given in several similar ways to the hepatitis C virus. Vaccination can stop HBV infection, that it is one of the chief causes of cirrhotic. Sufferers who are troubled by hepatitis d contamination at the expense of hepatitis b infection are significantly at risk of injuring cirrhosis (Guan et al ., 2011).

2.4.3.4. Non-Alcoholic Steatohepatitis (NASH)

Non-alcoholic fatty liver disease (NAFLD) is the most diffusion of serious liver disease in the world. NAFLD exhibits a histological spectrum, ranging from “bland steatosis” to the more aggressive necro -inflammatory form, in non-alcoholic steatohepatitis, accumulate fibrosis to result in cirrhosis. Data suggests NASH to be the most important histological predictor of liver and non-liver related death. Nevertheless, only a small proportion of individuals develop cirrhosis (Calzadilla Bertot et al ., 2016). NASH occurs following the accumulation of fat in the liver due to causes other than alcoholism. Obesity is a common cause of NASH (Poonawala et al., 2000).

2.4.3.5. Genetic Conditions

The liver is affected by genetic conditions that result in cirrhosis include alpha-1 antitrypsin deficiency, glycogen storage diseases, galactosemia, hemochromatosis, and Wilson disease. The liver cells damaged is causing by abnormal accumulations of certain products of metabolism due to the genetic dysfunction (Scorza, 2014).

3. MATERIALS AND METHODS

3.1. Materials

In our investigation, we took blood from 31 healthy individuals, and 30 patients with Cirrhosis in both male and female children. From each of healthy and patient individuals, we took 4 ml of blood from an antecubital venous vein and added 2 ml to the biochemistry tube and the other 2 ml to the serum tube.

3.1.1. Equipment and materials

Vortex: NMR Core 110

Storage serum tubes

Refrigerated Centrifuge

Test Tubes

Pipette

Spectrophotometer cuvette

Deep Freezing Tubes

Adjustable Automotive Pipettes

Thermostatic Water Bath

Glass pipette

Cooled centrifuge

Deep freeze

Oven

Stopwatch

Sensitive balance

Automated Pipette Tip

PH-meter

Flask

Beaker

Magnet

3.2. Analysis Methods

3.2.1. Sample analysis

The study starts, brachial vein blood samples (4cc) were taken from the cases in the patient group (liver cirrhosis) and the control groups. Blood samples carry up into biochemistry laboratory tubes. The tubes serum were separated from plasma by centrifugation in "Nuve NF 800 centrifuge" at (5000 rpm) for 5 minutes and obtained serums were conserved (at 20-°C) until they are processing. When the adequate numbers of samples were obtained, serum malondialdehyde (MDA) levels and superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) activities were spectrophotometrically measured in the Biochemistry laboratories of the Department of Chemistry, Faculty of Science, and University of Yuzuncu Yil.

3.2.2. Determination of superoxide dismutase (SOD) activity

SOD activity was determined by using the proposed method of (Popov et al., 2004). SOD accelerates the dismutation of hydrogen peroxide and molecular oxygen of superoxide radicals (O2-•) formed during the oxidative energy production. This method is based on the reading of optic density resulted from using of xanthine and xanthine oxidase in which superoxide radicals that generated from the blue colored formazan dye of the nitro blue tetrazolium (N.B.T) in the optical density wavelength of 560 nm. The SOD that exists in the sample serum inhibits the formazan reaction by excluding superoxide radicals from the environment. Under the experimental conditions, 1 unit of SOD is the % 50 inhibition of N.B.T reduction rate.

$$\% \text{ inhibition} = (\text{blank OD} - \text{sample OD}) / \text{blank OD} \times 100$$

3.2.3. Determination of catalase (CAT) activity

One empty tube and one sample tube were achieved for measurement of catalase activity. 2.8 ml of 30 mM hydrogen peroxide (H_2O_2) was placed into the empty tube and 0.2 ml of phosphate buffer was adding. The blend was shaken quickly and spectrophotometrically measured (Ati Unicam UV/VIS-UV2-100, England) two times at 240 nm with thirty-second intervals. Then again, 2.8 ml of 30 mM hydrogen peroxide

(H_2O_2) was placed into the sample tube and 0.2 ml of serum was adding. The combination was shaken quickly and absorbance was read at 240 nm in Hitachi U-2900 (Aebi, 1984).

Activity = (2.3 / Δx) x [(log A1 / log A2)]

Activity; Calculated as in U / L.

Δx = 30 seconds

2.3 = 1 μmol optical density of H_2O_2 in 1 cm light path.

3.2.4. Determination of Glutathione (GSH) activity

All the proteins that don't carry sulfhydryl (SH) group are precipitated with precipitation solution. The glutathione (GSH) level was measured as the final product of the reaction was achieved. That was the formation of the yellow color, of obtained clear liquid of sulfhydryl groups and DTNB (5 ', 5' - (dithiobis 2-nitrobenzoic acid). Measurement of the reduced glutathione level in the EDTA blood was done in 412 nm wavelength in the spectrophotometer within 24 hours (Beutler et al., 1963).

Activity (mg / dl) = [(OD2 – OD1) / 13600 x E1 1.25] x 1000

OD1 = First absorbance before addition of DTNB at 412 nm.

OD2 = Second absorbance after addition of DTNB at 412nm.

E1 = 1 in the calculations.13600 is the molar extinction coefficient of the yellow color that formed during the interaction of GSH and DTNB.

3.2.5. Determination of malondialdehyde (MDA) level

The reaction of fatty acids with free radicals result in malondialdehyde, which is the final product of lipid peroxidation, is measured with thiobarbituric acid that gives a colored form (JM, 1995). 200 μl from the blood is taken and put into 1 tube. 800 μl phosphate buffer, 25 μl BHT solution, and 500 μl of % 30 TCA were added. The tubes were stirred with vortex and kept on ice for 2 hours. Then, it was centrifuged at 2000 rpm for 15 minutes. 1 ml from the supernatant was taken and transferred to other tubes. Then 75 μl of EDTA and 250 μl of TBA were added. Tubes were mixed in the vortex and kept

in a hot water bath for 15 minutes. Then, they were brought to room temperature and their absorbance was read at UV / Vis spectrophotometer at 532 nm.

C = F * 6.41 * A

C: Concentration.

F: Dilution factor.

A: Absorbance.

3.2.6. Statistical data analysis

The defining statistics for the studied parameters were expressed in standard deviation. In paired group comparisons, T-test was utilized where normal deviation was achieved, and Mann-Whitney U statistics was utilized where it wasn't. The significance level was assumed to be 5%, and all calculations were made with SPSS statistics package software.

4. RESULTS

The results obtained in the present study were from a total number of 61 subjects out of which 31 were healthy controls and 30 liver cirrhosis cases. SOD, MDA, GSH, and CAT levels were a statistical significant, and that in the liver cirrhosis patients compared with the healthy controls. These results are an indication of the importance of what has been reached.

Superoxide dismutase (SOD) enzyme level activity was examined, we got values showing the correlation between control and patient group levels (18.213 ± 1.382 U/L; 5.008 ± 1.303 U/L) as in (Table 4.1), where these values were statistically significant ($p<0.05$).

Furthermore, Malondialdehyde (MDA) level activity was examined, we got values giving the correlation between control and patient group levels (0.239 ± 0.045 μmol/L; 0.869 ± 0.256 μmol/L) as in (Table 4.1), was statistically significant ($p<0.05$).

Also, Reduced glutathione (GSH) level activity was examined, we got values showing the correlation between control and patient group levels (0.171 ± 0.017 μmol/L; 0.041 ± 0.022 μmol/L) as in (Table 4.1), where these values were statistically significant ($p<0.05$).

While Catalase (CAT) enzyme level activity was examined, we got values giving the correlation between control and patient group levels (0.254 ± 0.018 U/L; 0.091 ± 0.009 U/L) as in (Table 4.1), where were statistically significant ($p<0.05$).

Table 4.1. Comparison according to the control group and patients with liver cirrhosis

Parameters	Controls (n= 31) **Mean±SD**	Patients (n=30) **Mean±SD**
MDA (μmol/L)	0.239 ± 0.045	0.869 ± 0.256
GSH (μmol/L)	0.171 ± 0.017	0.041 ± 0.022
SOD (U/L)	18.213 ± 1.382	5.008 ± 1.303
CAT (U/L)	0.254 ± 0.018	0.091 ± 0.009

Table 4.1. Contains descriptive statistics and comparative results for SOD, MDA, GSH, and CAT. When samples was examined, the difference between the patient and control group mean SOD, MDA, GSH and CAT was statistically significant (P <0.05).

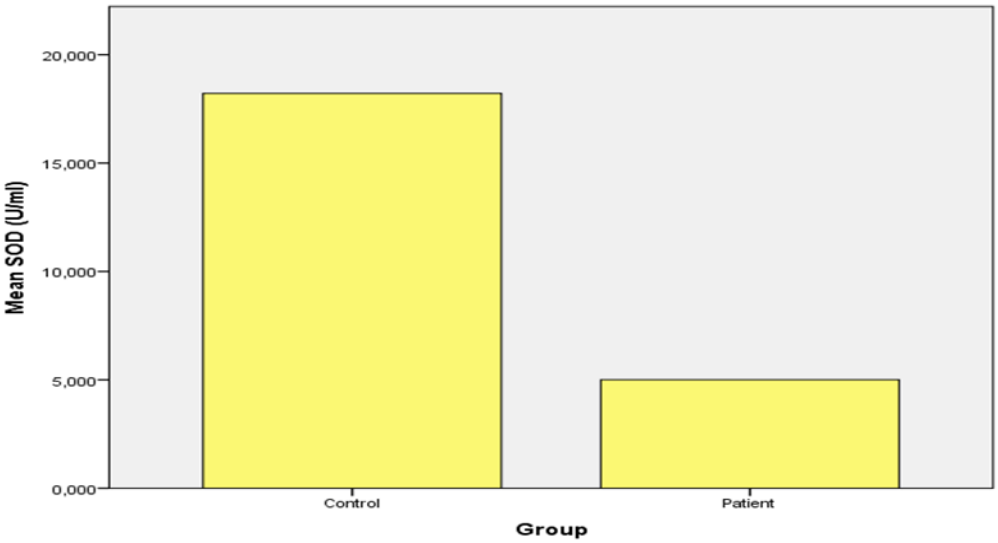

Figure 4.1. The level of SOD enzyme compared between control and cirrhosis patients.

Superoxide dismutase (SOD) enzyme level activity was examined, we got values showing the correlation between control and patient group levels (18.213 ± 1.382 U/L; 5.008 ± 1.303 U/L) as in (Figure 4.1), where these values were statistically significant (p<0.05).

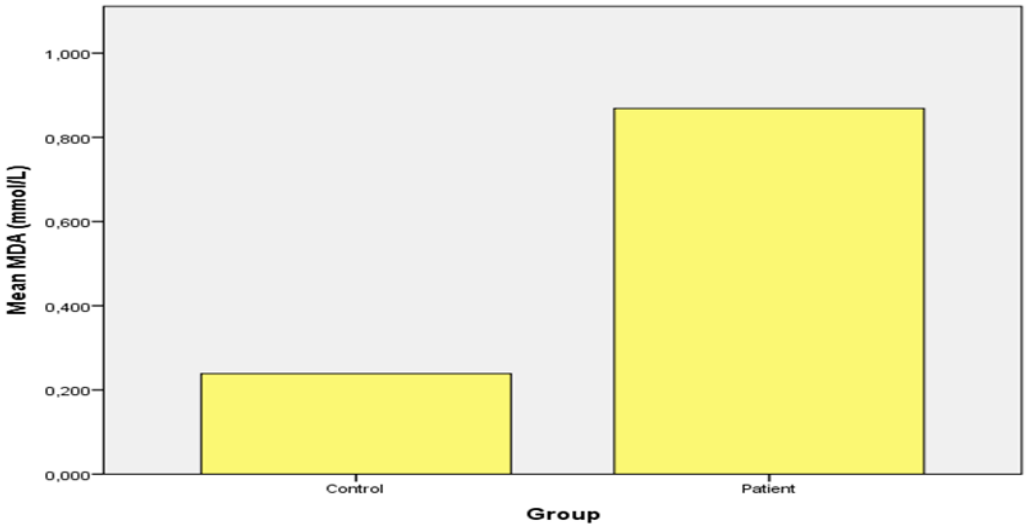

Figure 4.2. The level of MDA compared between control and cirrhosis patients.

Malondialdehyde (MDA) level activity was examined, we got values giving the correlation between control and patient group levels (0.239 ± 0.045 µmol/L; 0.869 ± 0.256 µmol/L) as in (Figure 4.2), where these values were statistically significant ($p<0.05$).

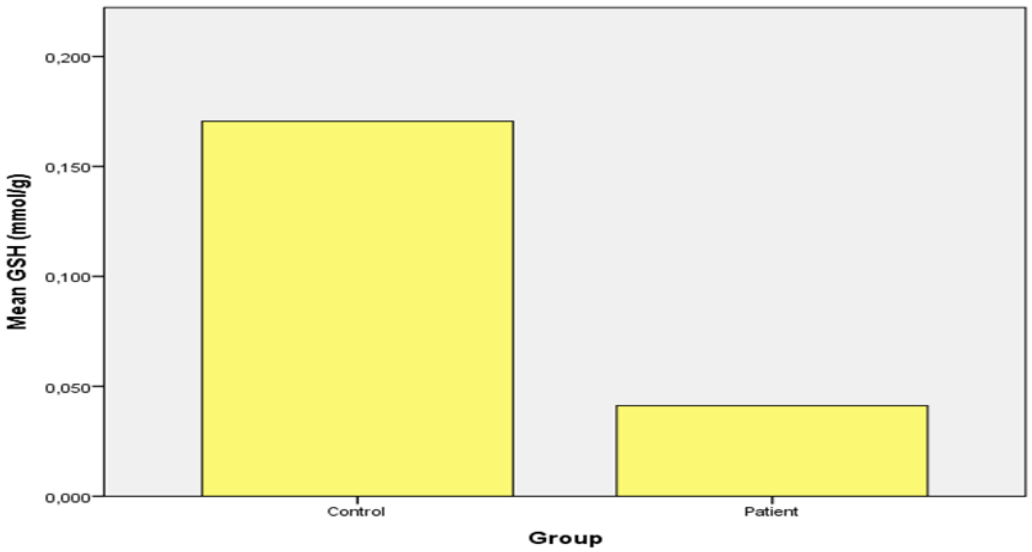

Figure 4.3. The level of GSH enzyme compared between control and cirrhosis patients.

Reduced glutathione (GSH) level activity was examined (Figure 4.3), the relationship between control group levels (0.171 ± 0.017 µmol/L) and patient group levels (0.041 ± 0.022 µmol/L) was found to have a statistically insignificant relationship ($p<0.05$).

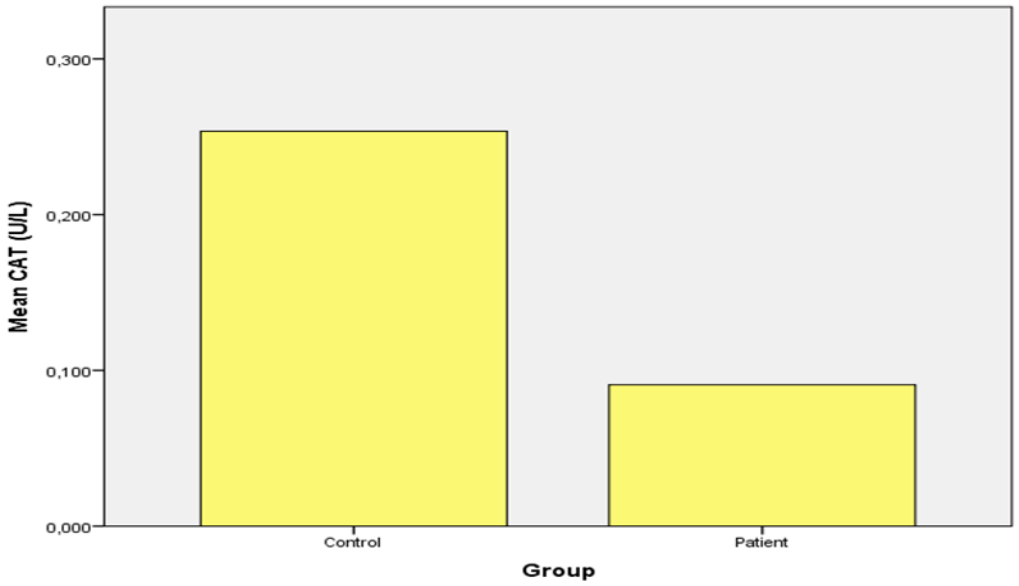

Figure 4.4. The level of CAT enzyme compared between control and cirrhosis patients.

Catalase (CAT) enzyme level activity was examined, we got values giving the correlation between control and patient group levels (0.254 ± 0.018 U/L; 0.091 ± 0.009 U/L) as in (Figure 4.4), where were statistically significant ($p<0.05$).

5. DISCUSSION AND CONCLUSION

Reactive oxygen species overlap with many diseases also potential follow-up diseases, which can cause death. These comprise long inflammation and autoimmune illness like rheumatoid arthritis, diabetes mellitus, and cardiovascular diseases such as atherosclerosis, hypertension, and ischemia. As well oxidative stress is found to have a significant effect over several kinds of cancer, including renal, lung, liver, and breast cancers (Briege et al., 2012; Salman et al., 2012). Oxygen and nitrogen are the basis of reactive species that cause oxidation of cells and tissues. The cytochrome p450 enzymes act on the formation of reactive oxygen species in hepatocytes (mitochondria and endoplasmic reticulum), which attack both proteins and lipids hepatocytic in addition to DNA, due to shortage antioxidants and imbalance with oxidative stress factors (Cichoż-Lach et al., 2014).

Under the right conditions, maintain a balance between oxidant and antioxidant molecules and domination the level of oxidative stress ROS, it requires preparation of the cells with special molecular strategies. ROS is trouble an imbalance between oxidant agents and antioxidants (Cichoż-Lach et al., 2014). Oxygen-free radicals have useful functions in the body like phagocytosis, detoxification reactions, killer of precancerous cells, and apoptosis. Besides, the normal composition of the ROS can control some metabolic cellular functions such as proliferation, migration, immunities, wound curative, and gene expression (Salganik, 2001). Lipids, proteins, carbohydrates and other cell components are exposed to oxidation when increased oxidative stress, which causes significant damage in cell structures. Cumulation of damage is called oxidative stress (Cerutti, 1994). Free radicals work to oxidize unsaturated fatty acids on the membranes catalyzed, this process is called lipid peroxidation. Malondialdehyde (MDA) is a marker for oxidative stress, and one of the end products of lipid peroxidation (Moncada et al., 1991).

Structural abnormalities, functional disorders, and other disorders (such as proliferative, metabolic, and inflammatory) occur in the liver, as a result of the Redox state which is infecting liver cells. And therefore, In light of the diseases that arise as a

result of oxidative stress, should be checked for oxidative stress in liver disease, it may have a major role in fibrosis, as well as can determine the stages of cirrhosis, monitoring cells damage, and follow the actual results of drug treatments (Cichoż-Lach et al., 2014).

Antioxidants help to reach advanced stages of treatment, in addition they have a great ability to protect liver cells from damage caused by free radicals, which causes oxidative stress, that in the case of high levels of ROS leads to damage the cells through necrotic mechanisms and consequently serious liver scarring. When an imbalance between oxidizing agents and antioxidants occurs, oxidative stress will increase, and this has shown a negative role in liver disease and degenerative and chronic disorders (Li et al., 2014). Chronic alcoholism is a major cause of cirrhosis. Where, alcohol will significantly affect enzymatic systems such as catalase and superoxide dismutase non-enzymatic like glutathione, vitamin A, C, and E, that have role in the protection of cells from repeated free radical attacks, as well as its effect on the level of malondialdehyde, according to studies conducted on animals treated with alcohol (Babczyńska et al., 2006; Mallikarjuna et al., 2010; Shanmugam et al., 2011).

We found through the results obtained an increased level of lipid peroxidation (malondialdehyde MDA) in serum due to increased severity of fibrosis and in contrast found a significant reduction in the concentrations of vitamins E and C, which are important indicators of antioxidants. In several studies, compared with healthy controls, were observed a significant decrease in GH levels in patients with alcoholic liver diseases. However, it is possible that the amount and time of alcohol consumption do not interfere in making a significant difference to the activity of SOD and CAT according to some reports that showed increases or the absence of changes or decreases in it, and this has caused controversy in the scientific community (Videla et al., 2004; Das et al., 2008).

Oxidative stress is one of the known pathological mechanisms and as mentioned earlier it has negative roles on the liver and is involved from the beginning of the disease until the development of the disease. Also, other factors affecting the liver such as alcohol, drugs, radiation, and pollutants all increase oxidative stress, which gradually destroys the liver and causes many diseases such as chronic viral hepatitis, alcoholic liver disease, non-alcoholic steatohepatitis, which can develop to cirrhosis (Singal et al., 2011; Li et al., 2015).

As a conclusion of this study, antioxidants level like SOD, CAT and GSH are decreased and oxidative stress (OS) is increased (as evidenced by elevated levels of lipid peroxidation like malonaldehyde (MDA) in patients with liver cirrhosis than healthy controls ($P<0.05$). In liver cirrhosis patients, oxidants in high concentrations, which cause oxidative, are released by stress-activated macrophages and neutrophils. This can lead to damage the DNA, proteins, lipids, and carbohydrates. Lipid peroxidation and MDA react with unsaturated fatty acids (released of cell membranes) that cause damage to cells and tissues (Moncada et al., 1991).

The mean level of SOD activity showed a statistically significant decrease in liver cirrhosis cases when compared to the controls group ($P<0.05$). The decreased SOD activity may be the consequence of SOD degradation as part of the detoxifying process due to the influence of ROS (Jira et al., 2007). Some studies suggested that lowered SOD activity may be caused by inhibitory effects of hydrogen peroxide. This would demonstrate that the increased production of hydrogen peroxide during the dismutation reaction influences the process (Kalpakcioglu et al., 2008).

In this study, the resulting level of CAT activity was statistically and significantly decreased when it is compared to the controls group in liver cirrhosis ($P<0.05$). This decreased catalase activity in the liver cirrhosis group may have occurred due to catalase being inactivated by H_2O_2. Both of these also show reduced catalase activity liver cirrhosis patients serum. The change of H_2O_2 into H_2O and O_2 maybe a cause of reduced catalase. Consequently, it preserves the cells from the harmful effects of accumulated hydrogen peroxide. This result is following other findings (Mohamad et al., 2011).

In this study, our result level of GSH activity was statistically and significantly decreased when it is compared to the controls group in liver cirrhosis ($P<0.05$). Reducing glutathione is a flavoenzyme and it depends on NADPH which takes part in reducing for GSSH into GSH provided by glucose-6-phosphate dehydrogenase. Riboflavin has a great significance for the NADP-NADPH cycle (Feijoo et al., 2010). Glutathione reductase also takes part as a peroxyl scavenging mechanism. GSH is a non-protein sulfhydryl molecule and is considered as a very essential antioxidant defense system for body metabolism. The molecule acts as an intra-cellular reluctant in redox reactions by keeping the cellular element protected against potential damaging ROS.

In conclusion, in this study, antioxidant enzymes such as SOD, CAT and GSH were decreased and increased lipid peroxidation level such as malondialdehyde (MDA) was increased in patients with cirrhosis. And the results show that oxidative stress has related to liver cirrhosis and may increase dangerous of cirrhosis liver. In this study, it shows that oxidative stress affects tissue cellular damage very well in cirrhosis liver patients. While HBV, HCV, and alcohol are the main factors associated with it. ROS are produced in normal metabolism and living cells, also it is considered as signaling molecules that mediate the response. DNA, proteins and lipids are exposed oxidative damage when the ROS level in the cell increases. Other problems include DNA damage, loss of enzyme activity and inhibition of protein synthesis that leads to cell death. ROS is a key product in cells and contributes to the regulation of oxidation and reduction and signal transmission pathways. Further studies are essential to investigate antioxidant enzymes and oxidative stress (OS) status in liver cirrhosis and liver diseases. Liver cirrhosis patients may receive support from antioxidant therapy along with therapeutic drugs and the cessation of drinking alcohol. Combined with catalase and SOD antioxidants helpful effects might be elevated.

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CURRICULUM VITAE

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FEN BİLİMLERİ ENSTİTÜSÜ
LİSANSÜSTÜ TEZ ORİJİNALLİK RAPORU

Tarih: 04./.10./2019

Tez Başlığı / Konusu:
DETERMINATION of SOME ANTIOXIDANT ACTIVITES (SUPEROXIDE DISMUTASE, CATALASE, AND REDUCING GLUTATHIONE) AND OXIDATIE STRESS LEVEL (MALONDIALDEHYDE ACID) IN CIRRHOTIC LIVER PATIENTS

Yukarıda başlığı/konusu belirlenen tez çalışmamın Kapak sayfası, Giriş, Ana bölümler ve Sonuç bölümlerinden oluşan toplam ....69..... sayfalık kısmına ilişkin, ..04.../..10..../..2019.. tarihinde şahsım/tez danışmanım tarafından .....intihal tespit programından aşağıda belirtilen filtreleme uygulanarak alınmış olan orijinallik raporuna göre, tezimin benzerlik oranı % ....8..... (.....) dır.

Uygulanan filtreler aşağıda verilmiştir:
- Kabul ve onay sayfası hariç,
- Teşekkür hariç,
- İçindekiler hariç,
- Simge ve kısaltmalar hariç,
- Gereç ve yöntemler hariç,
- Kaynakça hariç,
- Alıntılar hariç,
- Tezden çıkan yayınlar hariç,
- 7 kelimeden daha az örtüşme içeren metin kısımları hariç (Limit inatch size to 7 words)

Van Yüzüncü Yıl Üniversitesi Lisansüstü Tez Orijinallik Raporu Alınması ve Kullanılmasına İlişkin Yönergeyi inceledim ve bu yönergede belirtilen azami benzerlik oranlarına göre tez çalışmamın herhangi bir intihal içermediğini; aksinin tespit edileceği muhtemel durumda doğabilecek her türlü hukuki sorumluluğu kabul ettiğimi ve yukarıda vermiş olduğum bilgilerin doğru olduğunu beyan ederim.

Hamam Hamam

Gereğini bilgilerinize arz ederim.

Tarih ve İmza

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