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Evaluation of Total Oxidant Status and Total Antioxidant Capacity in the Serum of Type 2 Diabetes Mellitus Male Patients

A Thesis
Submitted to the college of science Baghdad University in partial fulfillment of the requirements for the Degree of Bachelor's in chemistry

By
Abdullah Khamas Abdullah

Supervised by
Assist.lect. Yasser Mohammed Al-Taay

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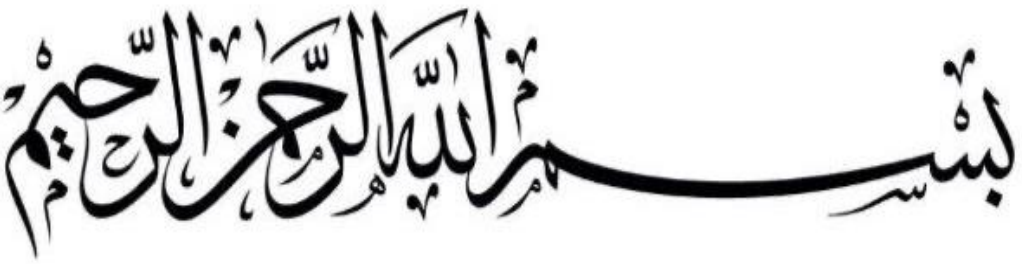

{يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ}

المُجادلة: ١١

قال تَعالى: (وَقُلِ اعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ)
إلهي لا يطيب الليل إلا بشكرك ولا يطيب النهار إلا بطاعتك...
ولا تطيب اللحظات إلا بذكرك...ولا تطيب الآخرة إلا بعفوك...
ولا تطيب الجنة إلا برؤيتك
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الى مــن بلغ الرسالة وأدى الأمانة..ونصح الأمة..الى نبي الرحمة ونور العالمين سيدنا محمد (صل الله عَليه وآلهِ وسَلم).

الى من كلله الله بالهيبة والوقار ... الى من علمني العطاء بدون انتظار ..الى من احمل اسمهُ بكل افتخار ارجو مــن الله أن يحفظك لي ويجعلك سنداً وعوناً لي في حياتي وسأبقى مديناً لكَ طول عُمـري
والدي العَزيز-حَفظك الله

الى ملاكي في الحياة الى معنى الحب الى معنى الحنان والتفاني ...الى بسمة الحياة وسر الوجود
الى مــن كان دعائها سر نجاحي وحنانها بلسم جراحي الى اغلى الحبايب
امي الحبيبة

الى منارة العلم والعلماء الى الصرح الشامخ..جامعة بغداد
الى الذين حملوا اقدس رسالة في الحياة الى الذين مهدوا لنا طريق العلم والمعرفة ..اساتذتنا الافاضل
وبلاخص الأستاذ ياسر محمد الطائي

شكر وتقدير

الحمدلله الذي يسر لنا الدرب
واعاننا على انجاز هذا العمل وانار لنا الطريق في سبيل أدائهُ بكل امانة
أتقدم بخالص شكري وتقديري الى كل مــن ساعدني على اعداد هذا البحث واخص ذلك بالدكتور "ياسر محمد الطائي " الذي كان لي موجهاً ،ناقداً بناءاً فأشكرهُ على الجهد الذي بذله معي ونصائحهُ القيمة التي كانت لي دليلاً خلال عملي .
كما لا انسى اساتذتي الكرام الذين أناروا لي طريقي .

Supervisor Certification

I certify that this research project entitled **"Evaluation of Total Oxidant Status and Total Antioxidant Capacity in the Serum of Type 2 Diabetes Mellitus Male Patients"** introduced by **(Abdullah Khammas Abdullah)** under my supervision, has completed the requirement for Bachelor Degree Science (B.Sc.) in Chemistry**.**

Supervisor Signature:

Name: Yasser Mohammed Khaleel

Title: Assistant lecturer

Date: 2024/5/1

Summary

Diabetes is considered one of the common diseases in this era, especially type 2 (T2DM), as it is considered primarily a hereditary disease, as it is transmitted through generations. This disease affects the body's physiology, causing the pancreas to not secrete insulin sufficiently. In this paper, this study discussed the oxidative stress status in T2DM patients. Total TOS and total antioxidant capacity (TAC) for males, from which 40 samples were collected, 20 for T2DM patients and 20 for healthy subjects (controls), as well as weight, height, and ages for all samples. The average age of the patients was (57.05±11.19) and the average age of the control group was(51.10±16.40)Samples were collected from Imam Ali Hospital, separated, and blood serum was taken, in addition to glucose, cholesterol, and VHDL results. Measurements were conducted at Al-Mustansiriya University, College of Science, Department of Chemistry. After these measurements, it was found that the level of total oxidation state (TOS) in healthy people(14.32±3.86)And patients (17.78 ±5.73)Total antioxidant capacity (TAC) in healthy people(1.26 ±0.11) And patients(1.22 ±0.13)After comparing the values, it was found that the value of TOS and IOS in patients with T2DM was higher than that in healthy people. (Control), and a lower TAC level was observed in T2DM patients compared to healthy subjects (Control).

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List of Abbreviations

DM	diabetes mellitus
WHO	world health organization
ROS	reactive oxygen species
TOS	total oxidant status
TAC	total antioxidant capacity
GDM	gestational diabetes mellitus
IDF	International Diabetes Federation
GLP-I	glucagon-like peptide-1
BMI	body mass index
ADA	American Diabetes Association
PCOS	polycystic ovary syndrome
RNS	reactive nitrogen species
ER	endoplasmic reticulum
MMO	Microsomal monooxygenase
NOX	NADPH oxidase
XOR	Xanthine oxidoreductase
FAD	flavin adenine dinucleotide
NADH	oxidized nicotinamide adenine dinucleotide
LOXs	Lipoxygenases
COX-1	cyclooxygenase-1
TPA	tetradecanoylphorbol-13-acetate
Fe- SOD	iron-superoxide dismutase
Mn-SOD	manganese-superoxide dismutase
Cu/Zn-SOD	copper/zinc- superoxide dismutase
Ni-SOD	last nickel-superoxide dismutase
GPx	Glutathione peroxidases
GSH	Glutathione peroxidases use the reduced glutathione

GSSG	yielding oxidized glutathione
Sec	selenocysteine residue
CAT	Catalases
SPSS	Statistical Package for Social Sciences
SD	standard deviation
AUC	area under the curve
NGT	normal glucose tolerance

Chapter One
Introduction

1. Introduction

1.1. Background

The chronic metabolic disorder, diabetes mellitus (DM), is a fast-growing global problem with huge social, health, and economic consequences. It is estimated that in 2010 there were globally 285 million people (approximately 6.4% of the adult population) suffering from this disease. This number is estimated to increase to 430 million in the absence of better control or cure (Kaul et al., 2013). The world health organization (WHO) estimates that diabetes will be the 7th primary cause of fatality by 2030 (Alam et al., 2021). Multifarious factors including genetic defects, pancreatic obstruction, surgery, organ transplantation contribute to the onset of this type of diabetes (Mathers and Loncar, 2006). DM is classified into several types, where the most common types are type 1 (T1DM) and type 2 (T2DM) (Alam et al., 2021). The latter involved in many health disorders, including hypertension, nephropathies, retinopathies, etc (Zheng et al., 2018).

Oxidative stress is defined as “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” (Sies, 2020). The multitude of oxidant and antioxidant processes are occurring simultaneously in normal and pathophysiological conditions in different cells and organs (Sies and Jones, 2020). Two parts were involved in the definition of oxidative stress, oxidants and antioxidants. The first comprise any material with short half-life, low-stability, high oxidizing ability, and high reactivity. Oxidants are reactive oxygen species (ROS) which can be free radicals such as hydroxyl radical or non-radical species such as hydrogen peroxide (Zorov et al., 2014). One way to determine the overall oxidant effects is the measurement of total oxidant status (TOS) (Erel, 2005). On the other side of the redox balance,

the defense against damaging levels of oxidants consists of several types of antioxidant enzymes in conjunction with their back-up systems, as well as of low-molecular-mass antioxidants, forming an antioxidant network (Sies, 1993). The complement of antioxidant enzymes is subject to regulation by redox master switches as part of the oxidative stress response (Sies et al., 2017). While these two preceding sentences appear to be uncontested, a widespread misconception about “antioxidants” and “antioxidant capacity” deserves a comment (Pompella et al., 2014). In lay language, the term antioxidant seems to be confined solely to exogenous low-molecular-mass compounds, neglecting the much more important contribution of antioxidant enzymes (with their substrates and coenzymes) to cellular defense against oxidants. This misconception also pervades in the scientific community, best exemplified by the term “antioxidant capacity”, or even “total antioxidant capacity (TAC)” (Wayner et al., 1985).

1.2. Aims of Study

The present study was designed to investigate the role of oxidative stress in the pathophysiology of T2DM disease through:

1. Evaluation of TOS and TAC levels in the serum of T2DM patients, and compare the levels to healthy control.
2. Studying the correlation of TOS and TAC with other routine biomarkers that are linked to T2DM.
3. Investigate the prognostic sensitivity of TOS and TAC for T2DM.

Chapter Two
Literature Review

2. Literature Review

2.1. Diabetes Mellitus

The term diabetes describes a group of metabolic disorders characterized and identified by the presence of hyperglycemia in the absence of treatment. The heterogeneous etiopathology includes defects in insulin secretion, insulin action, or both, and disturbances of carbohydrate, fat and protein metabolism. The long-term specific effects of diabetes include retinopathy, nephropathy and neuropathy, among other complications. People with diabetes are also at increased risk of other diseases including heart, peripheral arterial and cerebrovascular disease, obesity, cataracts, erectile dysfunction, and nonalcoholic fatty liver disease. They are also at increased risk of some infectious diseases, such as tuberculosis (Organization, 2019).

Elevated blood glucose is a hallmark of all types of diabetes mellitus, which include T1DM, T2DM, DM characterized by aspects of both type 1 and type 2, gestational diabetes mellitus (GDM), and rare cases of diabetes mellitus caused by, for example, pancreatic trauma. Increased levels of circulating glucose have been proposed to mediate many of the deleterious cellular effects of diabetes mellitus, especially in endothelial cells (Kanter and Bornfeldt, 2016).

2.1.1 Type 2 Diabetes Mellitus

The T2DM accounts for around 90% of all cases of diabetes. In T2DM, the response to insulin is diminished, and this is defined as insulin resistance. During this state, insulin is ineffective and is initially countered by an increase in insulin production to maintain glucose homeostasis, but over time, insulin production decreases, resulting in T2DM. T2DM is most commonly seen in persons older than 45 years. Still, it is increasingly seen

in children, adolescents, and younger adults due to rising levels of obesity, physical inactivity, and energy-dense diets (Goyal et al., 2023).

2.1.2 Epidemiology of T2DM

The International Diabetes Federation (IDF) estimates that worldwide, 415 million people have diabetes, 91% of whom have T2DM (Atlas, 2015). People with diabetes comprise 8.8% of the world's population, and IDF predicts that the number of cases of diabetes will rise to 642 million by 2040 (Einarson et al., 2018). The prevalence of T2DM has been steadily increasing over time. Using data from the Framingham Heart Study, Abraham et al. noted that the overall annualized incidence rates of the disease per 1000 persons increased from 3.0 in the 1970s to 5.5 in the first decade of the 2000s. That change represented an increase in the incidence of T2DM of 83.3% and was higher in males than females by a factor of 1.61 (Abraham et al., 2015).

In 2017, approximately 462 million individuals were affected by T2DM corresponding to 6.28% of the world's population (4.4% of those aged 15–49 years, 15% of those aged 50–69, and 22% of those aged 70+), or a prevalence rate of 6059 cases per 100,000. Over 1 million deaths per year can be attributed to diabetes alone, making it the ninth leading cause of mortality. The burden of diabetes mellitus is rising globally, and at a much faster rate in developed regions, such as Western Europe. The gender distribution is equal, and the incidence peaks at around 55 years of age. Global prevalence of T2DM is projected to increase to 7079 individuals per 100,000 by 2030, reflecting a continued rise across all regions of the world. There are concerning trends of rising prevalence in lower-income countries (Abdul Basith Khan et al., 2020).

Recent study reported that the prevalence of prediabetes among apparently healthy Iraqi individuals was 17%. Prediabetes was more

prevalent in individuals over 40 years of age, especially those who were overweight/obese (Jasim et al., 2022).

2.1.3 Pathophysiology of T2DM

The T2DM is an insulin-resistance condition with associated beta-cell dysfunction. Initially, there is a compensatory increase in insulin secretion, which maintains glucose levels in the normal range (Galicia-Garcia et al., 2020). As the disease progresses, beta cells change, and insulin secretion is unable to maintain glucose homeostasis, producing hyperglycemia. Most of the patients with T2DM are obese or have a higher body fat percentage, distributed predominantly in the abdominal region. This adipose tissue itself promotes insulin resistance through various inflammatory mechanisms, including increased FFA release and adipokine dysregulation (Valaiyapathi et al., 2020). Lack of physical activity, prior GDM in those with hypertension or dyslipidemia also increases the risk of developing T2DM. Evolving data suggest a role for adipokine dysregulation, inflammation, abnormal incretin biology with decreased incretins such as glucagon-like peptide-1 (GLP-I) or incretin resistance, hyperglucagonemia, increased renal glucose reabsorption, and abnormalities in gut microbiota (Westman, 2021).

2.1.4 Signs and Symptoms of T2DM

Patients with diabetes mellitus most commonly present with increased thirst, increased urination, lack of energy and fatigue, bacterial and fungal infections, and delayed wound healing. Some patients can also complain of numbness or tingling in their hands or feet or with blurred vision (Pinchevsky et al., 2020).

These patients can have modest hyperglycemia, which can proceed to severe hyperglycemia or ketoacidosis due to infection or stress.

T1DM patients can often present with ketoacidosis (DKA) coma as the first manifestation in about 30% of patients (Charoenpiriya et al., 2022).

The height, weight, and body mass index (BMI) of patients with diabetes mellitus should be recorded. Retinopathy needs to be excluded in such patients by an ophthalmologist. All pulses should be palpated to examine for peripheral arterial disease. Neuropathy should be ruled out by physical examination and history (Goyal et al., 2023).

2.1.5 Diagnosis of T2DM

Since T2DM represents one of several different diabetes types, diagnosing a child or adolescent with the disease is a two-step process. Firstly, one has to confirm the diagnosis of diabetes and secondly, to establish that it is T2DM (Serbis et al., 2021).

According to the American Diabetes Association (ADA), the criteria used to diagnose diabetes in youth are the same as those used in adult populations (Association, 2018). There are four possible ways to diagnose diabetes and each, in the absence of hyperglycemia symptoms, must be confirmed on a different day by any one of the other three: (1) FPG ≥ 7.0 mmol/L; (2) 2 h post-OGTT plasma glucose ≥ 11.1 mmol/L. It should be noted that OGTT has poor reproducibility in adolescents, with a concordance rate of < 30% between tests performed a few weeks apart (Libman et al., 2008); (3) random plasma glucose ≥ 11.1 mmol/L in the presence of diabetes symptoms. If such symptoms are not present, hyperglycemia diagnosed incidentally or under stress conditions (*e.g.*, acute infection or surgery) may be transitory and should not be regarded as diagnostic of diabetes. In such cases, repeat exam on a subsequent day will help diagnostically; and/or (4) A1C≥ 48 mmol/mol. This criterion remains controversial since, in some but not all, studies it identifies a population that does not overlap entirely with that identified by FPG or OGTT (Chan et al., 2015). In addition, A1C must

be measured by using a laboratory-based National Glycohemoglobin Standardization Program-certified methodology and not a point-of-care device, in order to be reliable.

Once the diagnosis of diabetes is established, the next important step is to differentiate T2DM from T1DM as well as from other more rare diabetes types. This distinction is not merely of academic importance but of clinical importance as well, since different types of diabetes require a different management approach, at least in the long-term (Shah and Nadeau, 2020). Since there is considerable overlap between T2DM and T1DM, a combination of history clues, clinical characteristics and laboratory studies must be used in order to reliably make the distinction, which is not always possible at the beginning. Such clues include: (1) Age. T2DM patients usually present after the onset of puberty, at a mean age of 13.5 years. By contrast, almost one-half of T1DM patients present before 10 years of age (Dabelea et al., 2007); (2) Family history. A reported 75%-90% of patients with T2DM have an affected first- or second-degree relative (Copeland et al., 2011), while the corresponding percentage for patients with T1DM is less than 10%; (3) Ethnic group (Serbis et al., 2021).

Youth belonging to minority groups such as Native American, African American, Hispanic, and Pacific Islander run a much higher risk of developing T2DM compared to Caucasians (Dabelea et al., 2014); (4) Body weight. Adolescents with T2DM are usually obese [body mass index (BMI) $\geq$ 95 percentile for age and sex]. In contrast, children with T1DM are usually of normal weight and may report a recent history of weight loss; although, up to 25% are overweight or obese (Serbis et al., 2021); and (5) Clinical findings. Patients with T2DM usually present with features of insulin resistance and MetS, such as acanthosis nigricans, hypertension, dyslipidemia, and polycystic ovary syndrome (PCOS) (Serbis et al., 2020). Such findings are rarely encountered in youth diagnosed with T1DM. For

instance, a study in the United States showed that up to 90% of youth diagnosed with T2DM had acanthosis nigricans, in contrast to only 12% of those diagnosed with T1DM (Dabelea et al., 2011).

In addition to the above history and clinical clues, laboratory tests that can help include those for: (1) Pancreatic autoantibodies. Since T2DM is not immunologically mediated, the identification of one or more pancreatic (islet) cell antibodies in a diabetic obese adolescent supports the diagnosis of autoimmune diabetes (Klingensmith et al., 2010). Antibodies that are usually measured include islet cell antibodies (against cytoplasmic proteins in the β-cell), anti-glutamic acid decarboxylase, and tyrosine phosphatase insulinoma-associated antigen 2, as well as anti-insulin antibodies, provided that insulin replacement therapy has not been used for more than 2 wk. In addition, a recently described β-cell-specific autoantibody to zinc transporter 8 is commonly detected in children with T1DM and can aid in their differential diagnosis (Rochmah et al., 2020). One should keep in mind, though, that up to one-third of T2DM children can have at least one detectable β-cell autoantibody and, thus, complete absence of diabetes autoimmune markers is not a prerequisite for the diagnosis of T2DM in children and adolescents (Reinehr et al., 2006); (2) Ketoacidosis. Since patients with T1DM are more prone to develop ketoacidosis at the time of diagnosis, measurement of venous pH and urinary ketones could help differentiate between T2DM and T1DM, especially in the presence of typical symptoms (*e.g.*, polydipsia, polyuria, and signs of dehydration). Of course, it should be remembered that up to 10% of adolescents with T2DM can have a similar initial clinical presentation (Rewers et al., 2008); and (3) Insulin and C-peptide levels. A low C-peptide level (< 0.2 nmol/L) detected in newly diagnosed diabetic youth strongly suggests T1DM. Insulin levels can also be used, provided that insulin therapy has not been initiated (Serbis et al., 2021).

2.2. Reactive Oxygen Species

Reactive oxygen species (ROS) is a term used to describe any reactive oxygen containing substances, including free radicals, reactive nitrogen species (RNS) is referred to those with nitrogen, from of which, hydroxyl radical $^{\bullet}OH$, superoxide anion $O_2^{\bullet-}$, hydrogen peroxide H_2O_2, nitric oxide $NO^{\bullet}$, hypochlorous acid HOCl, singlet oxygen $^1O_2$, and peroxynitrite $ONOO^-$ (Table 2-1) (Poprac et al., 2017). ROS, at physiological levels, functionalized as a "redox messengers" in intracellular signaling and regulation but the elevated levels contribute to the oxidative modification of cellular components, inhibit the function of proteins, and leads cell death (Circu and Aw, 2010). The excess amounts of ROS in organisms physiological system is referred to as oxidative stress (Wu and Cederbaum, 2003).

Table 1-4: List of ROS and RNS produced during metabolism (Phaniendra et al., 2015).

ROS	Symbol	RNS	Symbol
Superoxide anion	$O_2^{\bullet-}$	Nitric oxide	$NO^{\bullet}$
Hydroxyl radical	$^{\bullet}OH$	Nitrogen dioxide	$NO_2^{\bullet}$
Alkoxyl radical	$RO^{\bullet}$	Peroxynitrite	$ONOO^-$
Peroxyl radical	$ROO^{\bullet}$	Nitrosyl cation	NO^+
Hydrogen peroxide	H_2O_2	Nitroxyl anion	NO^-
Singlet oxygen	$^1O_2$	Dinitrogen trioxide	N_2O_3
Ozone	O_3	Dinitrogen tetraoxide	N_2O_4
Organic peroxide	ROOH	Nitrous acid	HNO_2
Hypochlorous acid	HOCl	Peroxynitrous acid	ONOOH
Hypobromous acid	HOBr	Nitryl chloride	NO_2Cl

2.2.1 Endogenous sources

Here are an overview of some endogenous sources of ROS:

1- Mitochondria

Mitochondria is a major site for the production of ROS in most cell types. Under normal conditions, up to 4% of oxygen is not fully reduced in the mitochondrial respiratory chain, yielding ROS (de Lucca Camargo and Touyz, 2019). In the inner mitochondrial membrane, a series of protein complexes are responsible of transferring electrons from NADH to oxygen, which create a transmembrane electrochemical gradient by pumping protons across the membrane. The flow of protons back into the matrix via a proton channel in the ATP synthase leads to conformational changes in the nucleotide binding pockets and the formation of ATP. The three proton pumping complexes of the electron transfer chain are NADH-ubiquinone oxidoreductase or complex I, ubiquinone-cytochrome c oxidoreductase or complex III, and cytochrome c oxidase or complex IV. Succinate dehydrogenase or complex II does not pump protons, but contributes to reduced ubiquinone (Sousa et al., 2018). The primary ROS generates within the mitochondria is $O_2^{\bullet-}$ and the major generation is accomplished by complex I and III, in which complex I produces $O_2^{\bullet-}$ only in the mitochondrial matrix, while complex III produces $O_2^{\bullet-}$ to both sides of the mitochondrial inner membrane (Figure 2-1) (Hille, 2005). Several oxidoreductases located in mitochondrial membranes are also shown to generate ROS such as dihydroorotate dehydrogenase, monoamine oxidases, succinate dehydrogenase, α-glycerophosphate dehydrogenase, and α-ketoglutarate dehydrogenase complex (Hauptmann et al., 1996, Andreyev et al., 2005, Quinlan et al., 2013, Sharma et al., 2012).

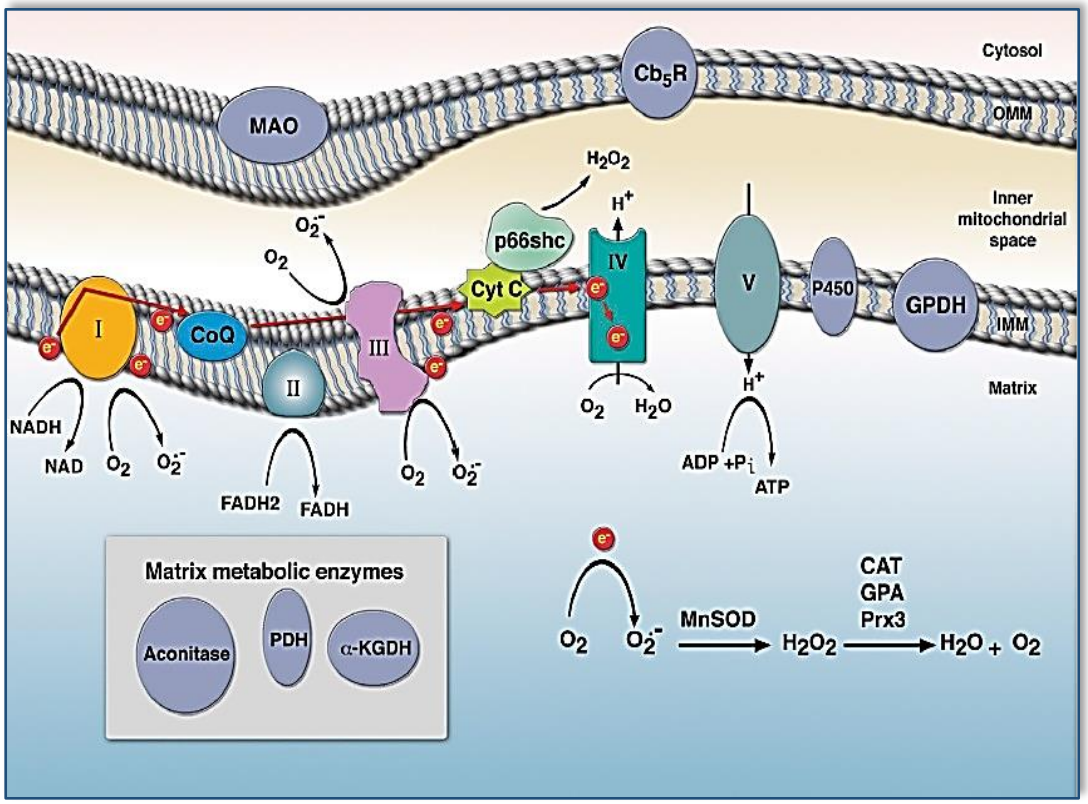

Figure 2-1: ROS generation and disposal in the mitochondria (Finkel, 2011).

2- Peroxisomes

Peroxisomes are considered a major source of intracellular ROS in most eukaryotes (Del Río and López-Huertas, 2016). Within the respiratory pathway of peroxisomes electrons of metabolites are transferred to oxygen leading to the formation of H_2O_2 (De Duve and Baudhuin, 1966). The β-oxidation process is the major H_2O_2 metabolic producing process in peroxisomes. Besides H_2O_2 peroxisomes also generate $O_2^{\bullet-}$ and $NO^{\bullet}$ through their various oxidase enzymes (Phaniendra et al., 2015). Oxidase enzymes found in mammalian peroxisomes and involved in the production of different types of ROS include acyl-CoA oxidases, D-Amino acid oxidase, D-aspartate oxidase, urate oxidase, α-hydroxyacid oxidase, polyamine oxidase, pipecolic acid oxidase, NO synthase, and xanthine oxidase (Schrader and Fahimi, 2004).

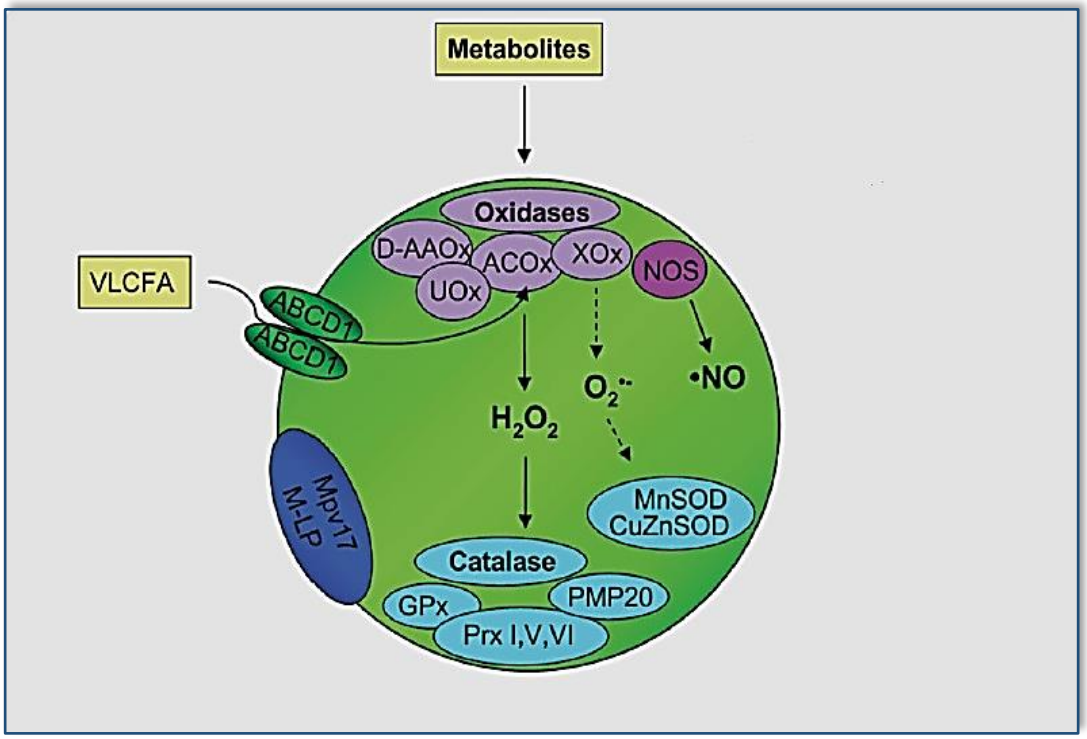

Figure 2-2: An overview of the production and disposing of reactive oxygen species in peroxisomes (Bonekamp et al., 2009).

3- Endoplasmic reticulum

The endoplasmic reticulum (ER) is a delicate network of tubules that carry out multiple functions in the cell. The ER is the primary organelle for secretory pathways in all eukaryotic cells (Schröder and Kaufman, 2005, Chang et al., 2006). ER is also implicated in protein related processes such as biosynthesis, folding, translocation, and post-translational modifications including disulfide bond formation, glycosylation, and chaperone-mediated protein folding processes (Schröder, 2008). Microsomal monooxygenase (MMO) system is considered to be a major source for ROS in ER. The MMO system is a system of multi-enzymes that perform as xenobiotic oxidative metabolism and oxygenate some endogenous substrates, such as 7-dehydrocholesterol reductase, squalene monooxygenase, heme oxygenase, and fatty acid desaturase (Zeeshan et al., 2016). NADPH-cytochrome P450 reductase and cytochrome P450 are located in the core of MMO system and serve as the terminal oxidase (Siegenthaler and Sevier, 2019). The efficiency or degree of coupling of electron transfer from NADPH to p450 is usually

<50%–60% and is often as low as 0.5%–3.0%. This "electron leakage" plays a significant part in ROS generation, while redox cycling occurs between NADPH-cytochrome P450 reductase and eukaryotic p450s (Zeeshan et al., 2016).

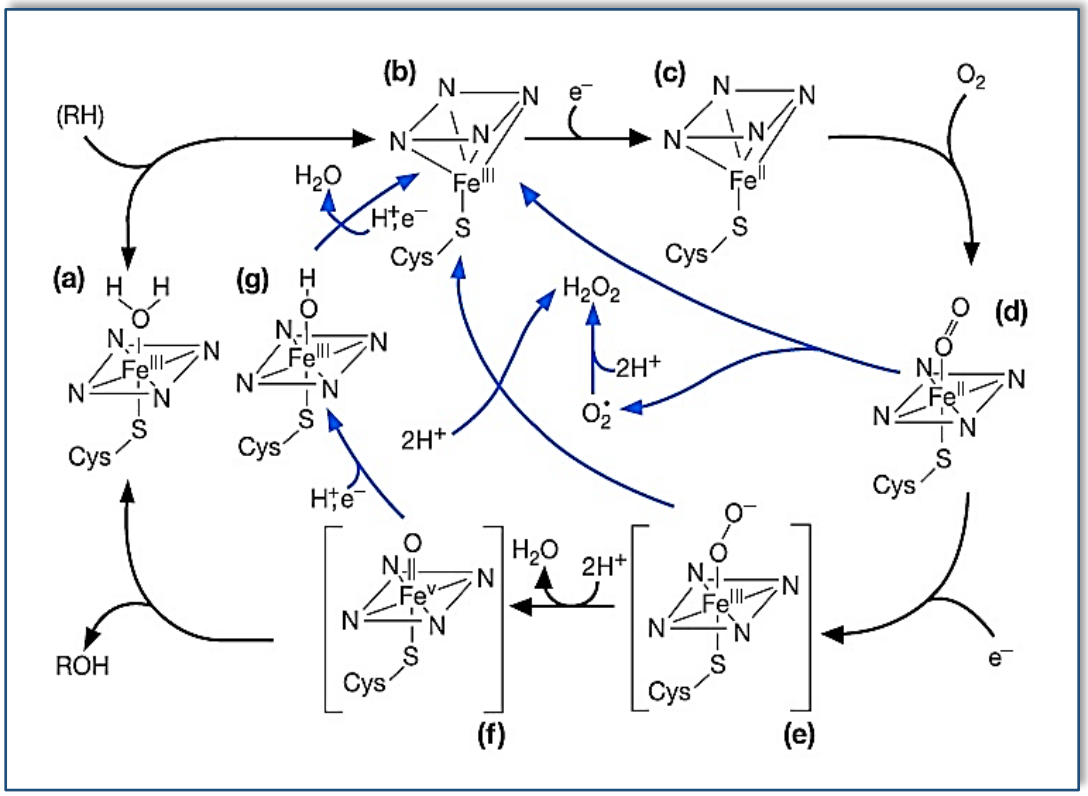

Figure 2-3: Catalytic cycle scheme of cytochrome P450 (Zangar et al., 2004).

4- NADPH oxidase

The NADPH oxidase (NOX) is an enzyme family that contains seven isoform members, NOX1-5, DUOX1, and DUOX2 which named after the catalytic subunit. NADPH oxidase family is the only known enzymes that is exclusively functionalized to produce ROS, in which all isoforms are transmembrane proteins that perform a transferring of electron from NADPH to oxygen, yielding $O_2^{\bullet-}$ which follows a rapid conversion to H_2O_2 (Bedard and Krause, 2007). NOX plays a central role during inflammation as a defender through generation of ROS, during phagocytosis NOX2 produces $O_2^{\bullet-}$ which follows subsequent conversion to H_2O_2 by the action of the enzyme SOD, and used by myeloperoxidase and other peroxidases to

produce strong microbicidal chemicals, such as HOCl (Magnani and Mattevi, 2019).

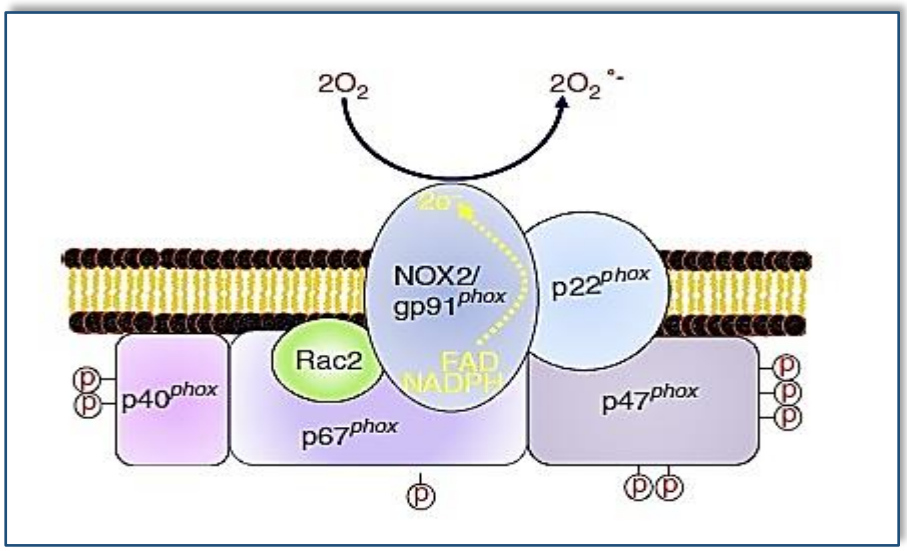

Figure 2-4: The active phagocyte NADPH oxidase complex (El-Benna et al., 2016).

In non-phagocytic process, NOX appears to be activated within discrete subcellular compartments, including lamellipodial focal complexes and focal adhesions, membrane ruffles, caveolae and lipid rafts, endosomes, and the nucleus, thereby facilitating localized reactive oxygen species production (Ushio-Fukai, 2006).

5- Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) is a dimeric metalloflavoprotein comprising two identical subunits of approximately 145 kDa each, including one molybdenum-containing molybdopterin cofactor and one flavin adenine dinucleotide (FAD) coenzyme, as well as two non-identical iron-sulfur redox centers. The purine oxidation occurs at the Mo-co site, while the FAD site is the oxidized nicotinamide adenine dinucleotide (NADH) and O_2 reduction site. The electron flux moves between the molybdopterin cofactor and FAD coenzyme through the two iron-sulfur clusters (Battelli et al., 2016). The enzyme catalyzes the production of uric acid from xanthine/hypoxanthine using O_2 as oxidant, and releases $O_2^{\bullet-}$ and

H_2O_2 as a byproducts (Bergamini et al., 2004). The XOR substrates, xanthine and hypoxanthine, bind to the oxidized enzyme and the molybdenum cofactor is reduced to Mo^{4+} by the action of the two electrons donation of the substrates. H_2O then is used to hydroxylate the substrate at the Mo- site, and the electrons travel via two iron-sulfide residues to FAD. After that, reduced FAD is oxidized by O_2 either univalently in two steps and produce $O_2^{\bullet-}$ or divalently and produce hydrogen peroxide. Under physiological conditions, XOR is account for a minor source of the total ROS production (Vašková et al., 2012).

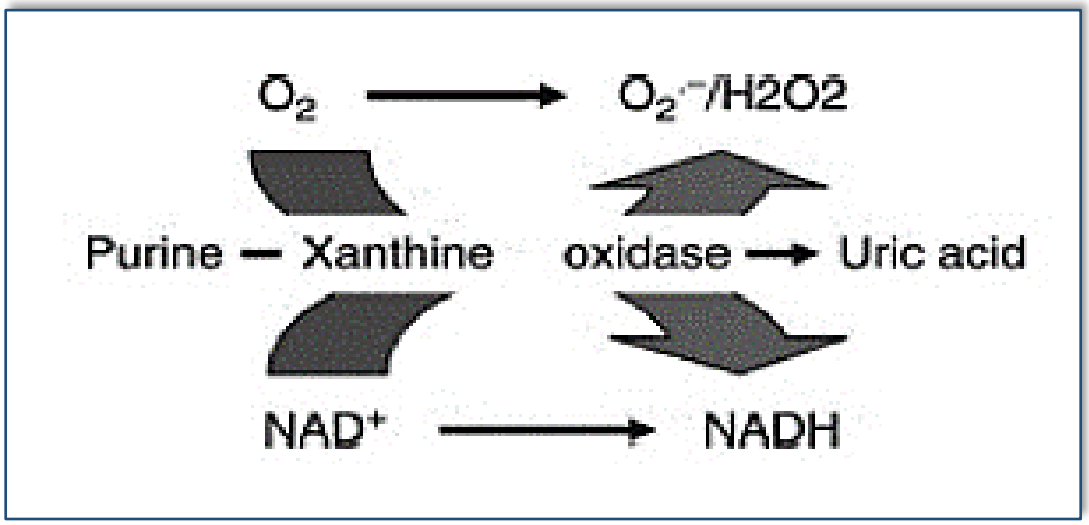

Figure 2-5: The production of ROS by xanthine oxidase (Siraki et al., 2018).

6- Arachidonate enzymes

Lipoxygenases (LOXs) are dioxygenases that catalyze the oxidation of arachidonic acid and other polyunsaturated fatty acids at specific sites to produce hydroperoxide derivatives, according to the site of action on polyunsaturated fatty acids, LOXs are numbered 5-, 8-, 12, and 15-LOX (Kim et al., 2008). 5-LOX has been implicated in the production of ROS in lymphocytes, through the involvement in the production of H_2O_2 by T cells (Vašková et al., 2012). Also, cyclooxygenase-1 (COX-1) has been implicated in the production of ROS in cells stimulated with bacterial

lipopolysaccharides, IL-1, TNF-α , or the tumor promoter 4-O-tetradecanoylphorbol-13-acetate (TPA) (Aggarwal et al., 2017).

2.2.2 Exogenous sources

The generation of ROS from exogenous agents is well established, environmental factors such as pollution,(Lakey et al., 2016) ultra violet (Hanson et al., 2006) and ionizing radiation, (Yamamori et al., 2012) smoking, (Burke and FitzGerald, 2003) and Insecticides (Mangum et al., 2015) are important sources of ROS and free radicals in organisms, which subsequently lead to serious health complications.

2.2.3 Chemistry of ROS and RNS

Aerobic organisms utilize triplet oxygen molecule as an electrons accepter during respiratory chain, yielding H_2O as final product (Eq.1) (Solomon and Stahl, 2018).

$$O_2 + 4e^- \longrightarrow 2H_2O \qquad (1)$$

The univalent reduction of O_2 results in the formation of superoxide anion (Eq.2), (Villamena, 2017) $O_2^{\bullet -}$ has a half-life of about 2-4 µs (Vašková et al., 2012). Superoxide rapidly undergoes one electron reduction in Haber-Weiss reaction and generates more toxic radicals (Eq.3), (Kehrer, 2000) also it can react with $NO^{\bullet}$ and produce $ONOO^-$ (Eq.4), (Huie and Neta, 2002) which can follow subsequent transformation to peroxynitrite acid and then to hydroxyl radical (Eq.5) (Vašková et al., 2012).

$$O_2 + e^- \longrightarrow O_2^{\bullet -} \qquad (2)$$

$$O_2^{\bullet -} + H_2O_2 \longrightarrow HO^{\bullet} + O_2 + HO^- \qquad (3)$$

$$O_2^{\bullet -} + NO^{\bullet} \longrightarrow ONOO^- \qquad (4)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \longrightarrow HO^{\bullet} + NO_2^{\bullet} \qquad (5)$$

Superoxide anion can be protonated and give arise to $HO_2^{\bullet}$, (Hayyan et al., 2016) a membrane-permeable species with higher reactivity than $O_2^{\bullet-}$ (Eq.6) (Edge and Truscott, 2018).

$$O_2^{\bullet-} + H^+ \longrightarrow HO_2^{\bullet} \qquad (6)$$

The divalent reduction of oxygen molecule leads to the formation of hydrogen peroxide (Eq.7), H_2O_2 also produced from one electron reduction of superoxide anion (Eq.8) (Galli et al., 2005). Hydrogen peroxide is a membrane-permeable ROS,(Iwakami et al., 2011) with half-life of about 1 ms (Vašková et al., 2012). In the presence of transition metals, H_2O_2 undergoes Fenton reaction and generates the very reactive hydroxyl radical (Eq.9) (Fischbacher et al., 2017).

$$O_2 + 2e^- \longrightarrow H_2O_2 \qquad (7)$$

$$O_2^{\bullet-} + e^- \longrightarrow H_2O_2 \qquad (8)$$

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+} \qquad (9)$$

Hydroxyl radical also produced by the three-electron reduction of molecular oxygen (Eq.10) (Bergamini et al., 2004).

$$O_2 + 3e^- \longrightarrow {}^{\bullet}OH \qquad (10)$$

2.2.4 Consequences of ROS

The reactive oxygen species perform an essential functions in the living system, one of the well-recognized and beneficial function is cellular signaling (Mittler, 2017). However, ROS are highly reactive substances if they present in levels over allowed physiological limits, their reactions with cellular biomolecules are unavoidable (D'Autréaux and Toledano, 2007).

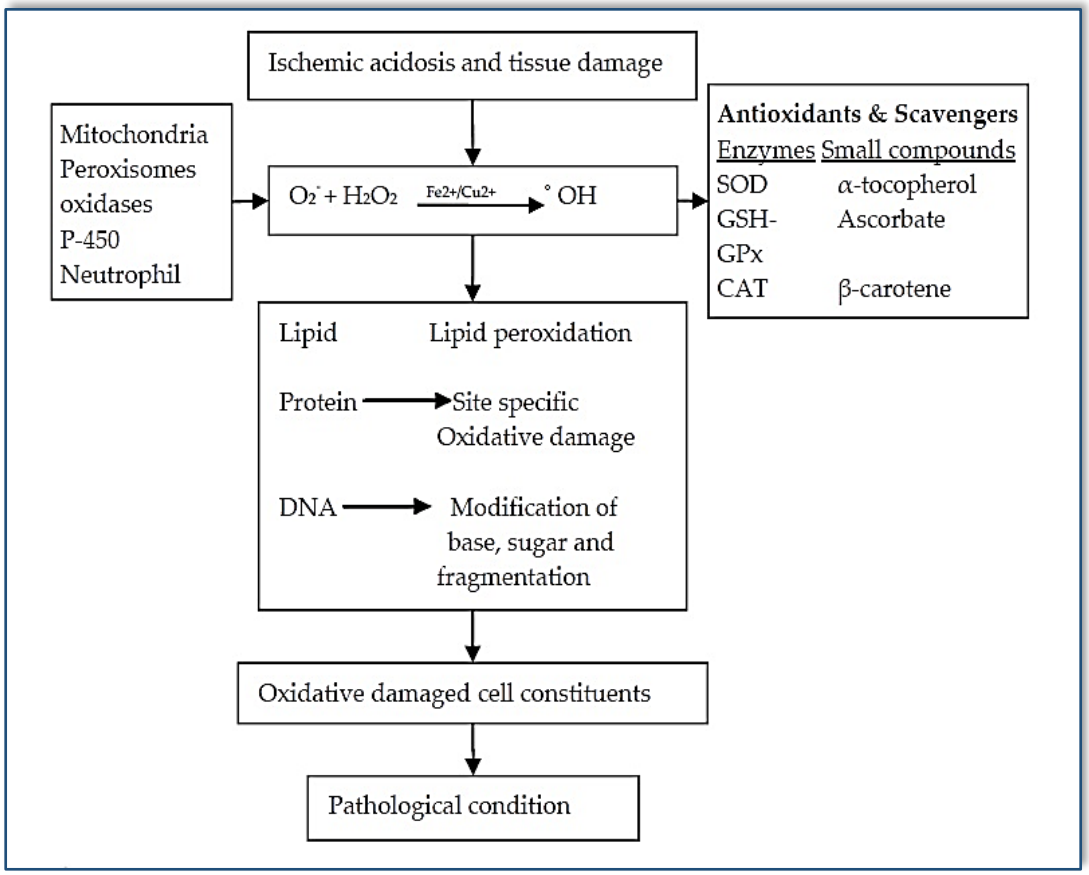

Figure 2-6: An overview of the ROS-induced oxidative damage (Mohammed et al., 2015).

Elevated ROS levels in the living system, either by endogenous production or the exposure to exogenous agents, contributes to the oxidative damage of the system (Di Meo et al., 2016). Reactive oxygen species can attack vital cell components like polyunsaturated fatty acids, proteins, and nucleic acids. To a lesser extent, carbohydrates are also the targets of ROS. These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein synthesis, DNA damage; ultimately resulting in cell death (Krishnamurthy and Wadhwani, 2012).

2.3. Antioxidant Defense System

In order to prevent the oxidative damage of ROS and free radicals, organisms possesses a protecting system made up of antioxidants (Abdollahi et al., 2004). Antioxidant term refers to any substance, when present at low

concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate (Ganaie et al., 2018). Thus it leads to the substantial definition of oxidative stress that is an imbalance between oxidants and antioxidants, where oxidants are level up (Ruiz-Ojeda et al., 2018). Antioxidants function to neutralize free radicals through accepting or donating electron(s) to remove the unpaired status of the radical (Aziz et al., 2019). Antioxidants classified into:

1- Endogenous antioxidants: which follow another sub-classification into: Enzymatic antioxidants: They include the enzymes superoxide dismutase, glutathione peroxidase, and catalase enzyme (Srdić-Rajić and Konić Ristić, 2016).

- Metal-binding proteins antioxidants: These substances represent the main participants of the total capacity of plasma antioxidants. Metal-binding proteins antioxidants include, albumin, metallothioneins, ferritin, myoglobin, transferrin and lactoferrin (Mirończuk-Chodakowska et al., 2018).
- Other endogenous antioxidants: The living system contain substances with antioxidant activity such as uric acid, (Rizzo et al., 2010) reduced glutathione, (Sarangarajan et al., 2017) coenzyme Q, (Hu, 2017) lipoic acid, (Farhat and Lincet, 2020) bilirubin, (Kihara and Higashi, 2019) melatonin, (Reiter et al., 2018) etc.

2- Dietary antioxidants: Many materials have an antioxidant properties found in nature, especially in plants products, (Benzie, 2003) and due to clinical significance of antioxidants, pharmaceutical field have paid a great interest in them. Among them, are vitamins (A, C, and E), (Wu et al., 2017) and polyphenolic compounds (such as proanthocyanidins, and the oligomers of flavan-3-ol) (Huang, 2018).

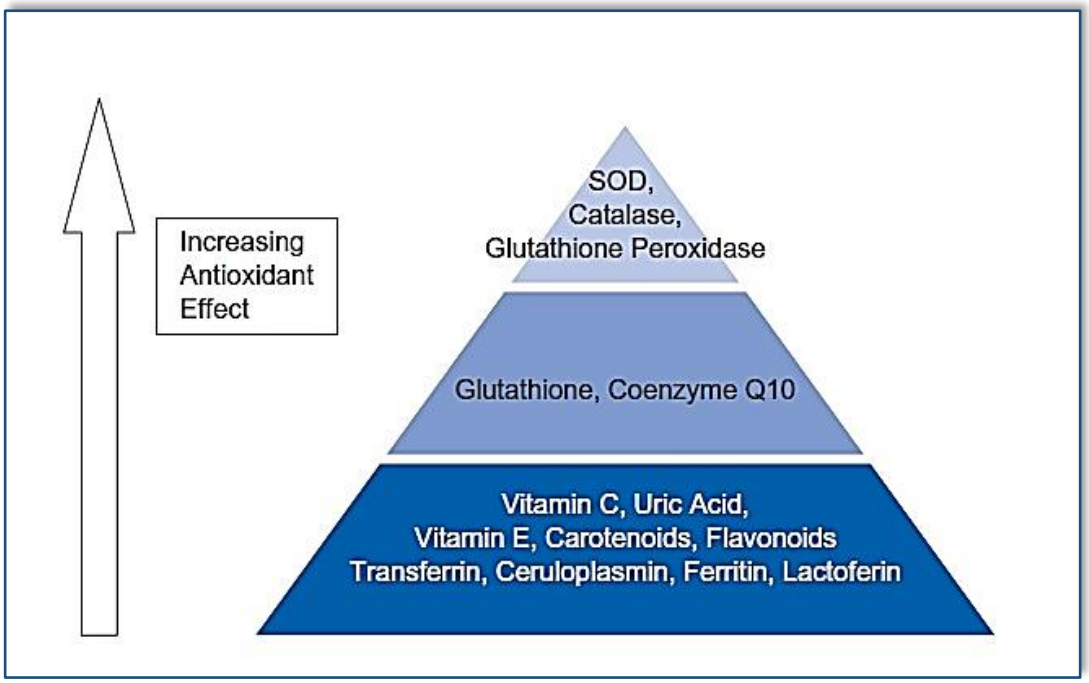

Figure 2-7: Effectiveness of various antioxidants in the human body towered neutralizing free radicals (Dasgupta and Klein, 2014).

1.6. Enzymatic Antioxidants

A. Superoxide dismutases

The superoxide dismutases (SOD, EC 1.15.1.1) considered as the first and most powerful enzyme to detoxify ROS in the cell (Ighodaro and Akinloye, 2018). SOD has been shown to play an important role in protecting cells and tissues against oxidative stress (Rahman and Biswas, 2006). SOD catalysis the conversion of $O_2^{\bullet-}$ to H_2O_2 as the equation, $2O_2^{\bullet-} + SOD + 2H^+ \longrightarrow H_2O_2 + O_2$ (Mruk et al., 2002). The SOD-catalyzed dismutation reaction is extremely efficient, occurring at the almost diffusion-limited rate of $\sim 2 \times 10^9$ $M^{-1}.s^{-1}$, which is $\sim 10^4$ times the rate constant for spontaneous dismutation (Wang et al., 2018). SOD is a family of metalloenzymes with four isoforms, they are iron-superoxide dismutase (Fe-SOD), manganese-superoxide dismutase (Mn-SOD), copper/zinc-superoxide dismutase (Cu/Zn-SOD), and at last nickel-superoxide dismutase (Ni-SOD). All of the four isoforms are found in prokaryotes, (Abreu and Cabelli, 2010) whereas, in eukaryotes there are only three isoforms that have

been found. Cu/Zn-SOD have been found in the cytosols of eukaryotic organisms, the intermembrane space of mitochondria, and chloroplasts. Mn-SOD is located in the mitochondrial matrix, whereas Fe-SOD present in the chloroplasts (Fridovich, 2013).

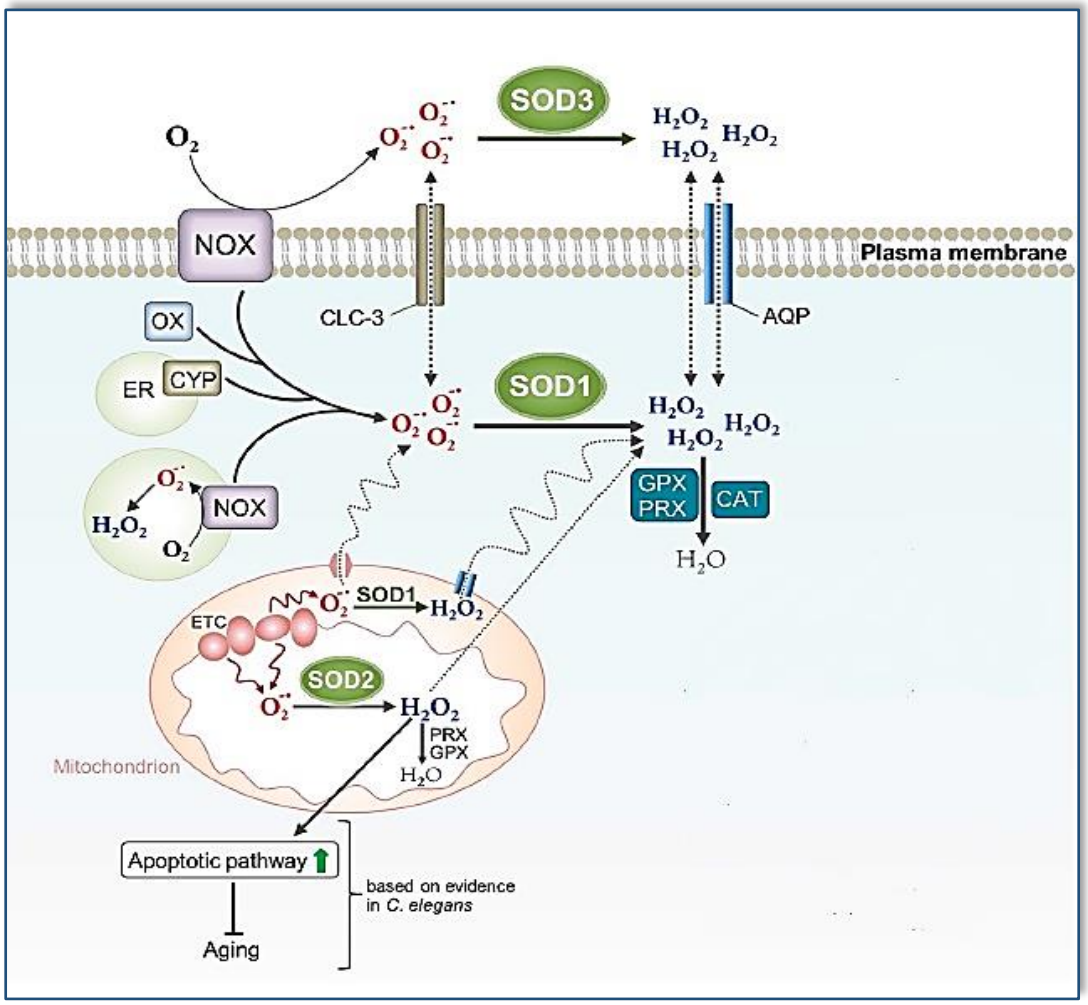

Figure 2-8: The SODs localization and catalytic process in mammalian cells (Wang et al., 2018).

B. Glutathione Peroxidases

Glutathione peroxidases (GPx, EC 1.11.1.9) is an important family of enzymes that catalyze the conversion of hydrogen peroxide to water, also GPx proceed in the conversion of lipid peroxides into corresponding alcohols (Ighodaro and Akinloye, 2018).

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

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

Glutathione peroxidases use the reduced glutathione (GSH) as a substrate (electrons donor) for the reduction of H_2O_2 and ROOH, yielding oxidized glutathione (GSSG) as shown above (Brigelius-Flohé and Flohé, 2019). GPx family is selenium containing enzymes, eight members have been identified in humans (GPx1-GPx8) (Conrad and Friedmann Angeli, 2018). Only five members GPx1-4 and GPx6, being selenoproteins. Their active site contains a selenocysteine residue (Sec), which, together with a Gln, Trp and Asn constitutes a conserved tetrad. In GPx5, GPx7 and GPx8 the active site Sec is replaced by cysteine (Brigelius-Flohé and Flohé, 2019). GPx1 is the first member of the family to be identified, it is a homotetrameric selenoprotein,(Flohé and Brigelius-Flohé, 2016) localized in the cytosol of all cells with a molecular weight of about 22-23 kDa for each subunit of the tetramer,(Arthur, 2001) it reacts with H_2O_2 and soluble low molecular mass hydroperoxides, such as t-butyl hydroperoxide, cumene hydroperoxide, hydroperoxy fatty acids and even hydroperoxy lysophosphatides, but not with hydroperoxides of more complex lipids (Brigelius-Flohé and Maiorino, 2013). GPx2 is another selenoprotein, also known as gastrointestinal glutathione peroxidase,(Kipp et al., 2007) GPx2 is a homotetramer that shares substrate and kinetic characteristics with GPx1 (Burk and Hill, 2010). GPx3 also constitute of four identical subunits that contain selenium of each (also known as plasma GPx), GPx3 is mainly synthesized in kidney, secreted into the plasma, and bound to basement membranes of several tissues (Kipp, 2017). GPx4 is found in most cell types (Lubos et al., 2011). It is a monomer member of the family, which also known as Phospholipid hydroperoxide glutathione peroxidase, GPx4 reacts with phospholid hydroperoxides as well as small soluble hydroperoxides, and is also capable of metabolizing cholesterol and cholesterol ester hydroperoxides in oxidized low density lipoprotein (Brown and Arthur, 2001). Unlike GPx1, GPx4 is capable of

reacting with complex lipid hydroperoxides (Brigelius-Flohé and Maiorino, 2013). GPx5 is a family member that does not contain selenium. It is a homotetramer and is related to GPx3. It is expressed in the epididymis and has been called epididymal Gpx-like protein (Drevet, 2006, Flohe, 2011). Human GPx6 is another homotetrameric selenoprotein member, it appears to be expressed in the olfactory epithelium (Margis et al., 2008). GPx7 is selenium-independent member, with low GPx activity,(Toppo et al., 2008) it is a monomer enzyme that is related to GPx4 and localized in the ER, it also referred to as nonselenocysteine-containing phospholipid hydroperoxide glutathione peroxidase (Conrad and Friedmann Angeli, 2018). The last member of the family in humans is the recently discovered GPx8, (Brigelius-Flohé and Maiorino, 2013) like GPx7, GPx8 also a monomeric free selenium enzyme, (Conrad and Friedmann Angeli, 2018) located in the ER (Fang et al., 2018).

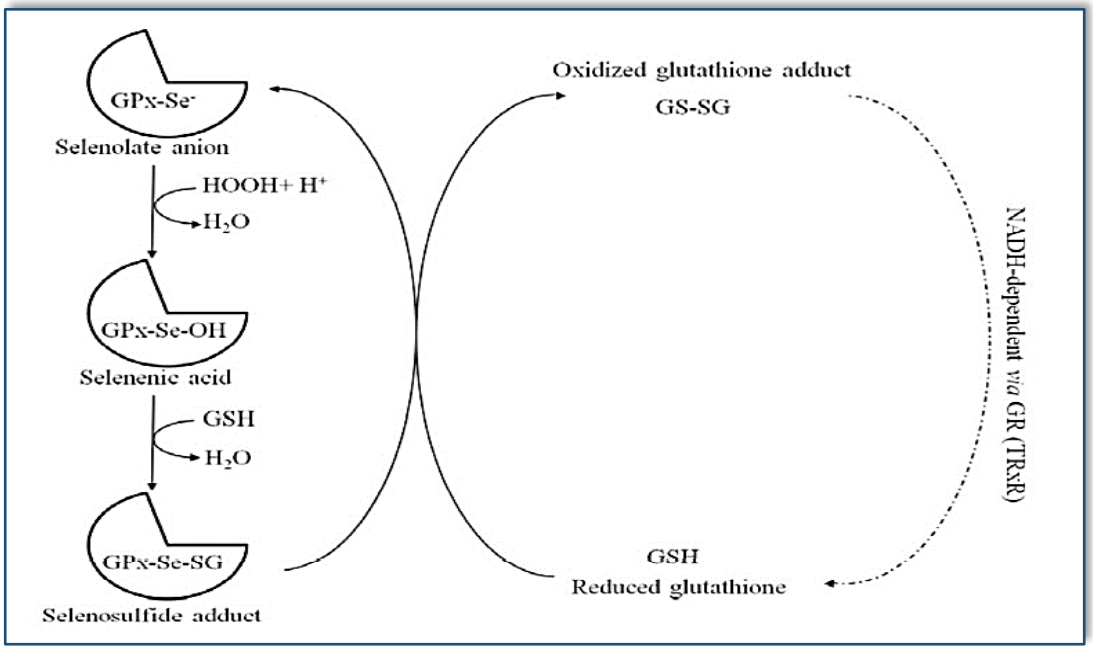

Figure 2-9: Simplified catalytic mechanism of the glutathione peroxidase: in the first step, Sec is oxidixed by hydrogen peroxide to form a selenenic acid, which is then reduced again by glutathione (Mangiapane et al., 2014).

C. Catalases

Catalases (CAT, EC 1.11.1.6) were from the first investigated enzymes in biochemistry, they own the hydrogen peroxide detoxification

activity (Turrens, 2018). CAT role is somewhat auxiliary to that of GPx, they are considered as a second line defense against ROS (Sepasi Tehrani and Moosavi-Movahedi, 2018). Mammalian CAT belongs to a group of mono-functional catalases with small subunit size, (Kirkman and Gaetani, 2007) it consists of four identical subunits, each of which has a molecular weight of about 60 kDa that constitute of 527 amino acid residues, and contains a heme group at the active site (Kodydková et al., 2014).

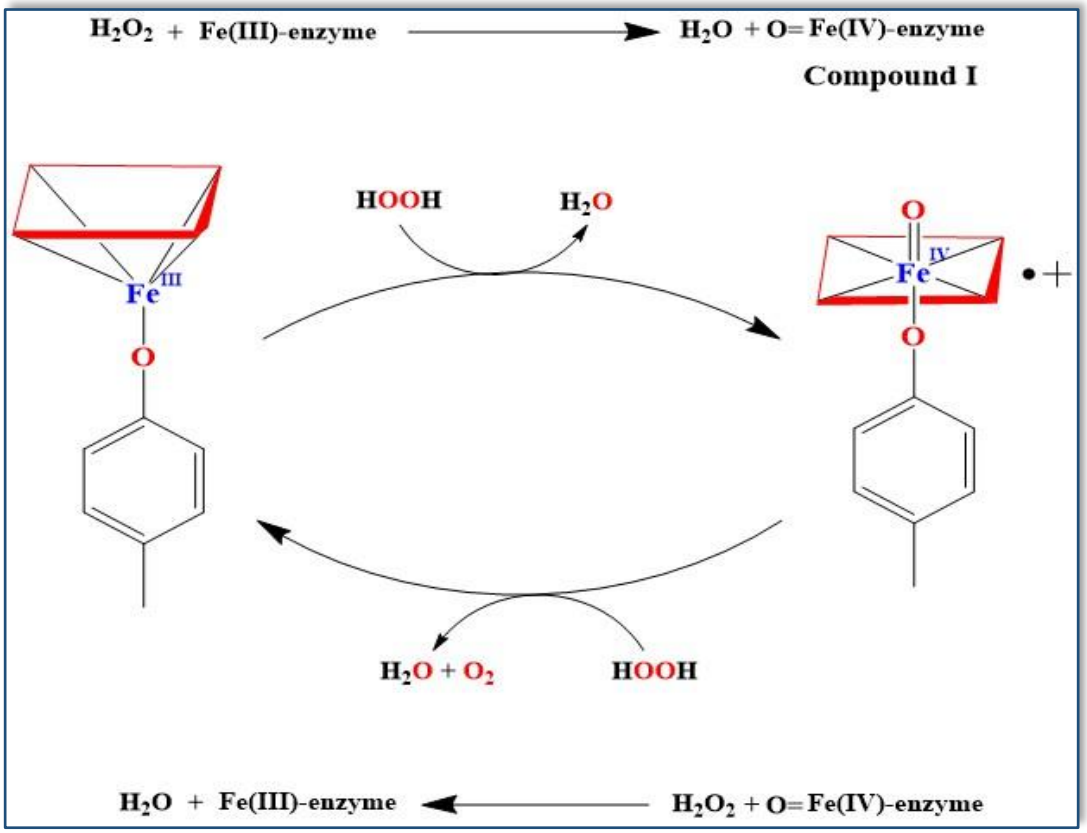

Figure 2-10: The two-stage catalytic reaction of catalase (Goyal and Basak, 2010).

Catalase converts two molecules of H_2O_2 to two molecules of water and one molecular oxygen in two-stage reaction pathway. The first stage involves oxidation of the heme iron by using hydrogen peroxide as substrate to form compound I, the oxygen–oxygen bond in peroxides is cleaved heterolytically, with one oxygen leaving as water and the other remaining at heme iron. In the second stage compound I reduced back to the ferric enzyme by a second molecule of hydrogen peroxide, with the release of molecular oxygen and water (Zamocky et al., 2008).

Chapter Three Materials and Methods

3. Materials and Methods

3.1. Chemicals and Kits

The chemicals that have been used in this study are listed below in Table 3-1.

Table 3-1: The chemicals that have been utilized in the study.

No.	Chemical	Supplier
1	Acetone	BDH Chemicals
2	Ascorbic acid	BDH Chemicals
3	Ferrous ammonium sulfate	BDH Chemicals
4	Glucose kit	MyBioLabo
5	Glycerol	BDH Chemicals
6	Hydrogen peroxide 30%	
7	Hydrochloric acid	BDH Chemicals
8	O-dianisidine	BDH Chemicals
9	Potassium Chloride	BDH Chemicals
10	Sodium chloride	BDH Chemicals
11	Sulfuric acid	BDH Chemicals
12	Xylenol orange	BDH Chemicals

3.2. Instruments

The instruments that have been used in this study are listed below in Table 3-2.

No.	Instrument	Model
1	Centrifuge	Beckman model TJ-6 / Germany
2	Electronic balance	Hawatch Scientific/ China
3	pH meter	Focus/ China
4	Spectrophotometer	Visible PD-303 Apel/ Japan
5	Vortex	VM-10/ China

3.3. General Design

The study was designed to investigate the oxidative stress status in T2DM patients, and the correlation of oxidative stress indicators (TOS, TAC, and OSI) with the blood glucose level of patients.

3.4. Patients and Control

The study included 20 patients who were diagnosed with T2DM from Al-Jawader General Hospital (Baghdad) of age range between 30 and 75 years old. The study controlled with non-diabetic people of matched age range. All of the enrolled subjects were males. The samples were collected from Dec 2023 to Jan 2024.

3.5. Sample Collection

Vein blood was collected from each subject and separated in the centrifuge at 1500 g for 10 to obtain the serum. Then, the serum was stored in two tubes at -20 ºC until the time of analysis. Moreover, height and weight information were collected from each subject.

3.6. Measurement of BMI

The BMI was estimated using mathematical equation that takes both height and weight as parameters:

$$BMI = \frac{Weight\ (kg)}{Height\ (m^2)}$$

3.7. Biochemical Assays

3.7.1 Estimation of Serum Glucose

The level of glucose was measured by spectrophotometric analysis by using commercial kit.

Principle

Trinder method was used for the determination. Glucose is oxidized by glucose oxidase enzyme to gluconic acid and hydrogen peroxide which in conjugation with peroxidase, reacts with chloro-4-phenol and 4-amino-antipyrine to form a red quinoneimine. The absorbance of the colored complex, proportional to the concentration of glucose in the serum and is measured at wave length of 500 nm (Burtis and Ashwood, 1999).

Reagents

Materials supplied in the test kit that was used in the study are listed in Table 3-3:

Table 3-3: Glucose kit components.

Components		Concentrations
Reagent 1	Phosphate Buffer	150 mmol/L
	Glucose Oxidase	≥ 20000 UI/L
	Peroxidase	≥ 1000 UI/L
	4-Amino-antipyrine	0.8 mmol/L
	Chloro-4-phenol	2 mmol/L
Reagent 2	Standard Solution	100 mg/dL

Procedure

1) A volume of 1 mL of reagent 1 was added to sample test tubes and standard test tube.

2) A volume of 10 µL from serums was added to corresponding sample test tubes. And then 10 µL of reagent 2 were added to standard test tube.

3) All tubes were incubated at 37 ºC for 10 min.

4) The absorbance of solutions was measured by spectrophotometer at wave length equal to 500 nm.

Calculations

The concentration of glucose was obtained from the following mathematical equation:

$$\text{Glucose (mg/dL)} = \frac{Abs_T - B}{Abs_S - B} \times S \text{ (mg/dL)}$$

T: Sample concentration.

Abs_T: Sample absorbance.

Abs_S: Standard absorbance.

S: Standard concentration.

B: Blank absorbance.

3.7.2 Estimation of TOS

Principle

The method of Erel was used to estimate the level of TOS. Oxidants present in the sample oxidize the ferrous ion‑o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Equiv./L) (Erel, 2005).

Reagents

Reagent A: Xylenol orange 150 μM; NaCl 140 mM; Glycerol 1.35M, pH 1.75:

The reagent was prepared by mixing 0.025g of xylenol orange + 2.046g of NaCl + 27.71 mL of glycerol and dissolve the mixture in 222.29 mL H_2SO_4 (0.025M).

Reagent B: Ferrous ammonium sulfate 5mM; O-dianisidine 10 mM:

The reagent was prepared by mixing 0.61g of O-diansidine + 0.3575g of ferrous ammonium sulfate in 5 mL acetone, then 245mL of H_2SO_4 (0.025M) were added to the mixture.

H_2SO_4: 18.39M was diluted into 0.025M by mixing 0.68 mL of concentrated H_2SO_4 with 500 mL of deionized water.

Standard solutions:

Series of concentration (250, 125, 62.5, 31.25, and 15.125 µM) of H_2O_2 were prepared from a stock solution of 200 µM H_2O_2.

Procedure

1) In standard tubes, 100 µL of each solution were added to the corresponding tube, followed by the addition of 675 µL of Reagent A.

2) The first absorbance was read at 560 nm.

3) A volume of 33 µL of Reagent B was added to each tube, and the tubes were allowed to stand for 4 minutes.

4) The second absorbance was read at 560 nm. Then ΔA was calculated (A2-A1).

5) Calibration curve was drawn using the collected data from the standard solutions.

6) Serum samples were analyzed using similar instruction from step 1 to step 4.

7) The values of TOS in the serum were estimated using the linear regression equation (y=mx+b), as shown in Figure 3-1.

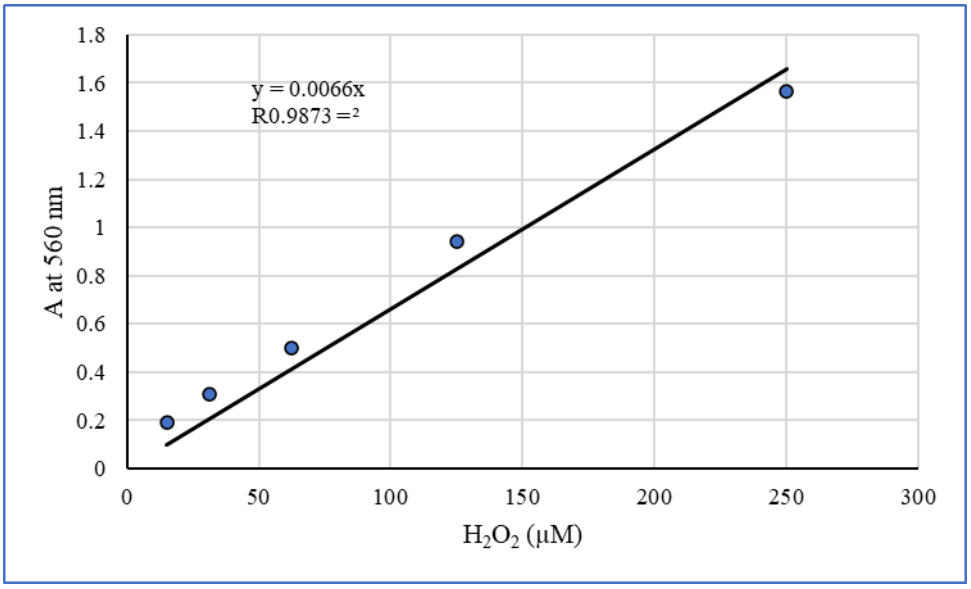

Figure 3-1: The standard curve of H_2O_2 in TOS method.

3.7.3 Estimation of TAC

Principle

Erel method was used to determine the level of TAC in the serum. A standardized solution of Fe^{2+}-O-dianisidine complex reacts with a standardized solution of hydrogen peroxide by a Fenton-type reaction, producing •OH. These potent ROS oxidize the reduced colorless odianisidine molecules to yellow-brown colored dianisidyl radicals at low pH. The oxidation reactions progress among dianisidyl radicals and further oxidation reactions develop. The color formation is increased with further oxidation reactions. Antioxidants in the sample suppress the oxidation reactions and color formation. This reaction can be monitored by spectrophotometry (Erel, 2004).

Reagents

Clark and Lubs: The reagent was prepared by dissolving 5.59g of KCl in 1L deionized water, then pH dropped to 1.8 by using HCl.

Reagent A: The reagent was prepared by mixing 0.61g O-dianisidine + 0.0044g ferrous ammonium sulfate, and dissolve them in 5mL acetone, then 250mL of Clark and Lubs solution.

Reagent B: 65µM H_2O_2 were diluted with in 99.35mL Clark and Lubs solution.

Standard curve: 2, 1, 0.5, 0.25, 0.125, 0 mM of ascorbic acid were prepared from a stock solution of 10 mM ascorbic acid.

Procedure

1) In standard tubes, 25 µL of each solution were added to the corresponding tube, followed by the addition of 1000 µL of Reagent A.

2) The first absorbance was read at 560 nm.

3) A volume of 50 µL of Reagent B was added to each tube, and the tubes were allowed to stand for 4 minutes.

4) The second absorbance was read at 560 nm. Then ΔA was calculated (A2-A1).

5) Calibration curve was drawn using the collected data from the standard solutions.

6) Serum samples were analyzed using similar instruction from step 1 to step 4.

7) The values of TAC in the serum were estimated using the linear regression equation (y=mx+b), as shown in Figure 3-2.

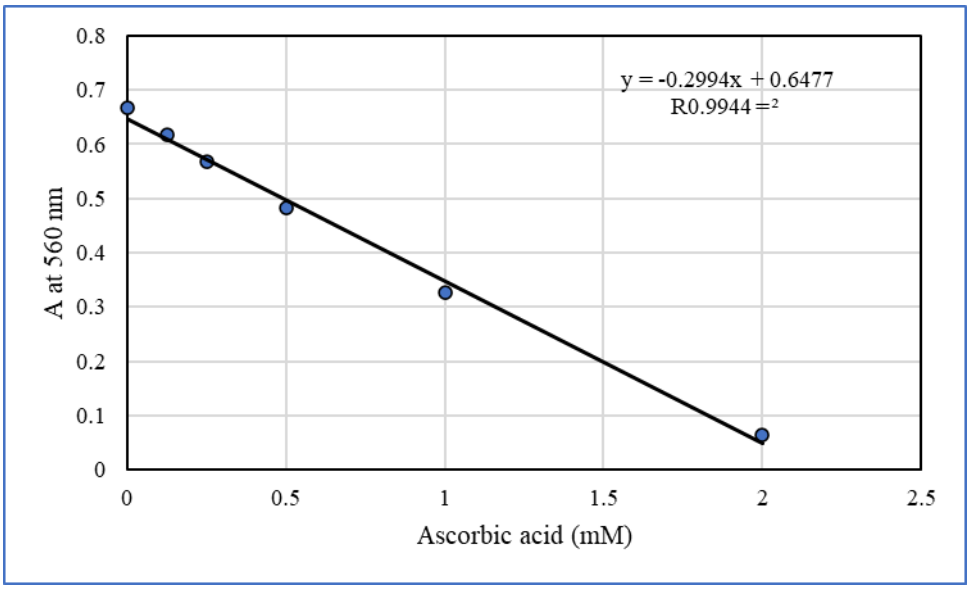

Figure 3-2: Standard curve of ascorbic acid in TAC method.

3.7.4 Calculation of OSI

The level of OSI was calculated using the following mathematical formula (Abod et al., 2021):

$$OSI = \frac{TOS}{TAC}$$

3.8. Statistical Analyses

The collected data were analyzed statistically in Statistical Package for Social Sciences (SPSS) version 26. Means were compared by independent student t-test, and expressed as mean± standard deviation (SD). The relationship between parameters were estimated using Pearson's correlation coefficient (r), and the sensitivity of using parameters as prognostic tools for T2DM was tested using receiver operating characteristic (ROC) analysis, by measuring the area under the curve (AUC) and the cut-off values.

Chapter Four
Results and Discussion

4. Results and Discussion

4.1. Characteristics of Subjects

The information of age and BMI are shown in Table 4-1. The mean age of control was 51.10±16.40 year, which was non-significantly (p>0.05) lower than the mean age of T2DM patients (57.05±11.19 year), as shown in Figure 4-1.

Table 4-1: Characteristics of enrolled subjects.

Variable	Control	T2DM patients	*p*-value
Number	20	20	
Age (year)	51.10±16.40	57.05±11.19	0.152
Height (cm)	176.25±7.50	175.05±6.90	0.601
Weight (kg)	84.52±11.28	88.73±20.26	0.423
BMI (kg.m^{-2})	27.25±3.67	28.95±6.25	0.300

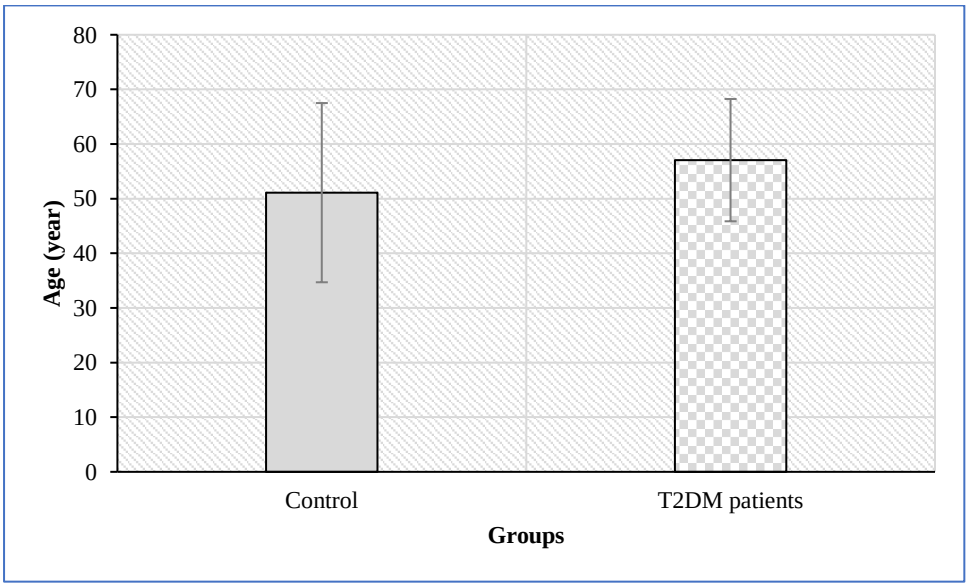

Figure 4-1: The age of control and T2DM patients.

The height of T2DM patients (175.05±6.90 cm) was non-significantly (p>0.05) different compared to the height of control (176.25±7.50 cm). Moreover, the weight of T2DM patients (88.73±20.26

kg) was non-significantly (p>0.05) different compared to the weight of control (84.52±11.28). Therefore, the BMI of T2DM patients (28.95±6.25 $kg.m^{-2}$) was non-significantly (p>0.05) higher than the BMI of control (27.25±3.67 $kg.m^{-2}$), as shown in Figure 4-2.

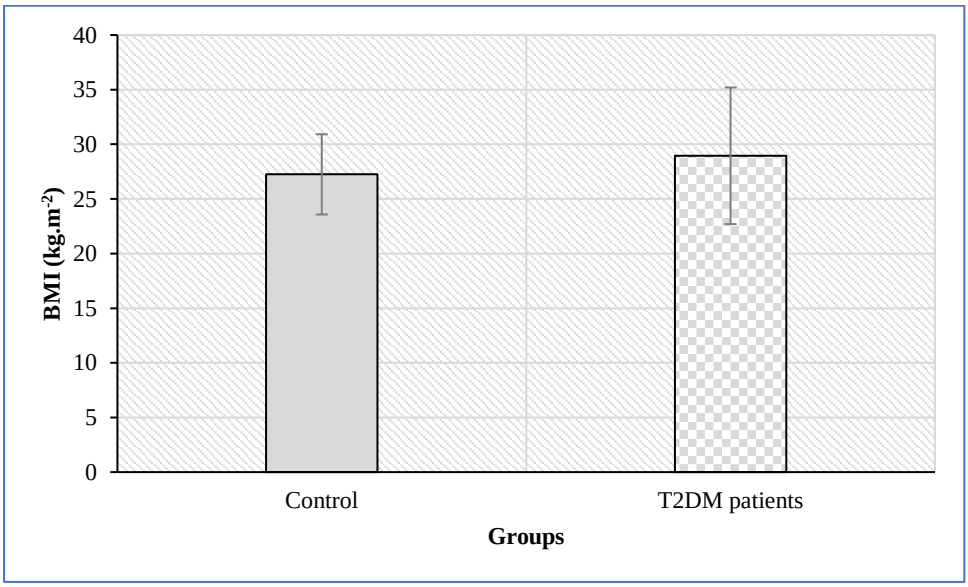

Figure 4-2: The BMI of control and T2DM patients.

Both age and BMI of participants was selected to be non-significant during the collection of samples in this study. This is due to the great influence that can be caused on the levels of TOS, TAC, and OSI, since the age can increase oxidative stress in people (Wu et al., 2021), as well as, obesity (Čolak and Pap, 2021) and overweight (Vincent et al., 2007).

4.2. Glucose

The level of fasting glucose was increased significantly (p<0.05) in T2DM patients (205.60±97.83) compared to control (103.25±12.88), as shown in Table 4-2 and Figure 4-3.

Table 4-2: The level of fasting glucose in control and T2DM patients.

Variable	Control	T2DM patients	*p*-value
Fasting glucose (mg/dL)	103.25±12.88	205.60±97.83	<0.001

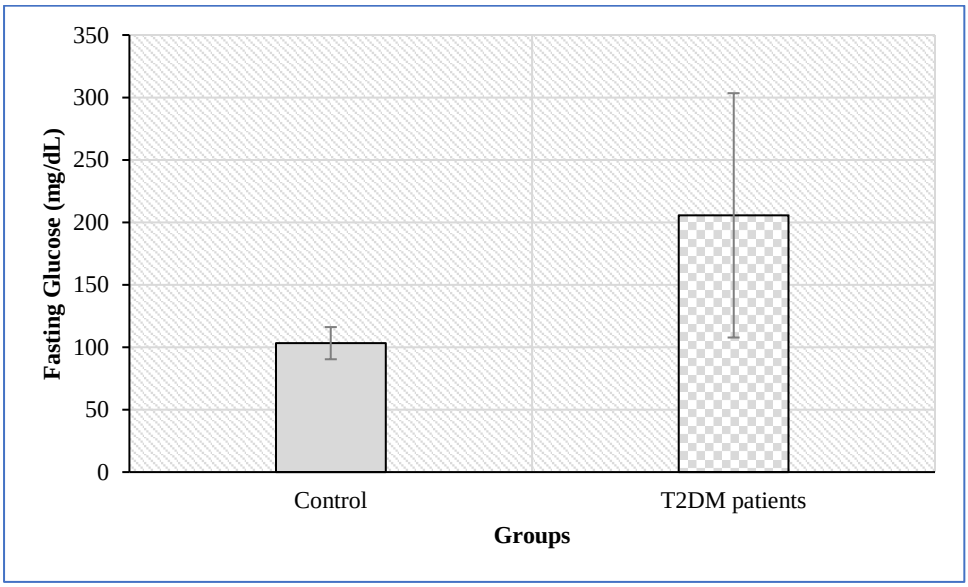

Figure 4-3: The level of glucose of control and T2DM patients.

The level of fasting glucose in T2DM patients indicates poor glycemic control (Khattab et al., 2010). Many factors can influence optimal glycemic control: gender, age, BMI, duration of illness, type of medication, lipid profile and blood pressure (California Healthcare Foundation/American Geriatrics Society Panel in Improving Care for Elders with Diabetes, 2003). Lipid abnormalities are common in patients with diabetes. Studies by Adham et al (Adham et al., 2010) and Benoit et al (Benoit et al., 2005) revealed that factors related to better glycemic control were lower levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides. In patients receiving insulin as mono-therapy were more likely to have poor glycemic control compared to patients who were on oral diabetes medication. This could be due to implementation of an insulin regimen or having an optimal glycemic level that could not be achieved by oral medication alone. The finding is consistent with other reported studies by Khattab et al (Khattab et al., 2010) and Benoit et al (Benoit et al., 2005). As indicated by El-Kebbi et al (El-Kebbi et al., 2001), co-morbidity does not appear to limit achievement of good glycemic control in patients with T2DM (Haghighatpanah et al., 2018).

4.3. Oxidative Stress Biomarkers

The levels of TOS, TAC, and OSI are shown in Table 4-3. TOS level was increased significantly (p<0.051) in T2DM patients (17.78±5.73 µmol H_2O_2 Eq/L) compared to control (14.32±3.86 µmol H_2O_2 Eq/L), as shown in Figure 4-4.

Table 4-3: The levels of oxidative stress biomarkers.

Variable	Control	T2DM patients	*p*-value
TOS (µmol H_2O_2 Eq/L)	14.32±3.86	17.78±5.73	0.031
TAC (µmol vit C Eq/L)	1.26±0.11	1.22±0.13	0.315
OSI	11.43±3.34	14.69±4.79	0.017

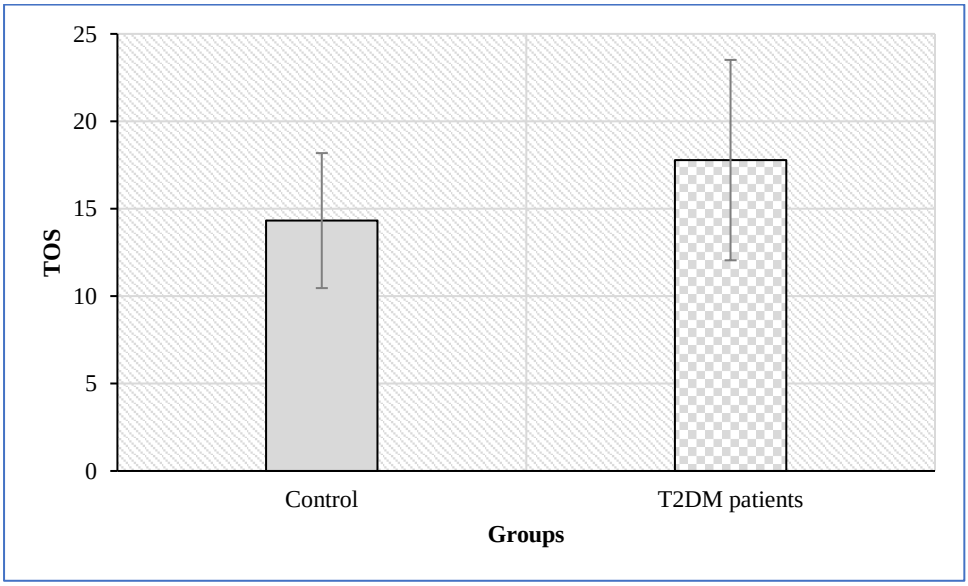

Figure 4-4: The level of TOS of control and T2DM patients.

The level of TAC was non-significantly (p>0.05) decreased in T2DM patients (1.22±0.13 µmol vit C Eq/L) compared to control (1.26±0.11 µmol vit C Eq/L), as shown in Figure 4-5.

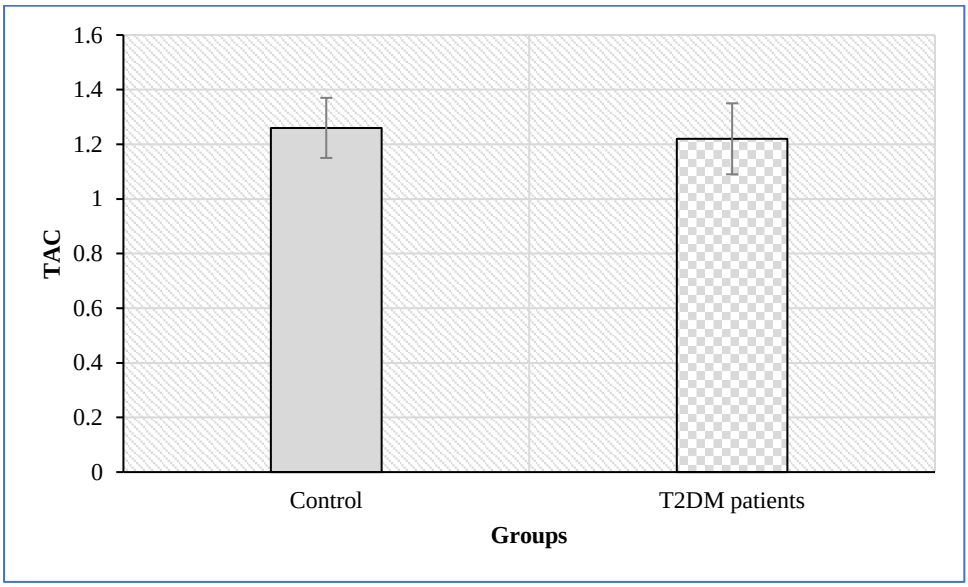

Figure 4-5: The level of TAC of control and T2DM patients.

The OSI level was increased significantly (p<0.05) in T2DM patients (14.69±4.79) compared to the level in control group (11.43±3.34), as shown in Figure 4-6.

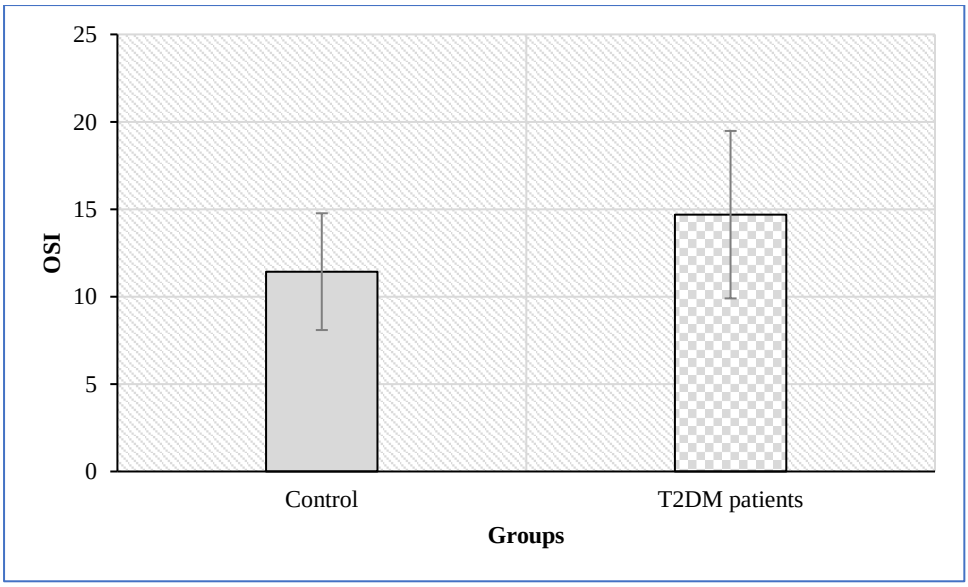

Figure 4-6: The level of OSI of control and T2DM patients.

Aslan et al reported significant increase in the level of TOS in T2DM patients. Also, the authors found a significant reduction in the level of TAC (which disagrees with the current findings) in T2DM patients. They have indicated that the increase in oxidative stress in T2DM patients can lead

to serious health problems including diabetic nephropathy (Aslan et al., 2007).

Sadeghabadi et al reported significant increase in the levels of TOS, OSI, and malondialdehyde (a lipid peroxidation biomarker) in T2DM patients. These elevated levels were corresponded to a reduction in many antioxidant indicators including TAC, SOD, and catalase. They have suggested that increased oxidative stress in T2DM patients increases the inflammation, and therefore, increasing co-morbidities of T2DM (Arab Sadeghabadi et al., 2019).

Picu et al reported significant increase in the level of TOS and OSI in newly diagnosed T2DM patients with a significant reduction in the TAC and glutathione. They have found a negative correlation antioxidant capacity and the respiratory burst in patients. Thus, T2DM patients suffer from a high inflammatory condition due to the increase of oxidative stress (Picu et al., 2017).

Yucel et al reported significant increase of TOS level in T2DM patients, but the reduction of TAC level was non-significant, which agrees with the current findings. They have indicated that elevated ROS in T2DM patients can increase the insulin resistance in T2DM patients (Yücel et al., 2021).

In patients with DM, hyperglycemia can occur via several mechanisms, including hyperglycemia-induced glucose autooxidation, non-enzymatic glycation of proteins and lipids, oxidation of advanced glycation end-products, and increased sorbitol pathway activity, which leads to hyperglycemia-induced tissue damage and oxidative stress. Diabetic patients appear to have significant defects in antioxidant protection when compared to healthy control subjects. This is evident in several studies, which demonstrate a significant reduction in the enzymatic activity of superoxide

dismutase (SOD), catalase, and glutathione peroxidase (GPx), as well as a reduction in some non-enzymatic constituents of the antioxidant defense system, such as vitamin C, vitamin E, glutathione, and protein-bound thiols in type 2 diabetic patients as compared to healthy control subjects (Erdem et al., 2015). This study demonstrated that TOS levels in the diabetic group were significantly higher than those of the control subjects. No differences were found between the TAS levels of the groups. Similarly, Kopprasch et al. found that there were no significant differences in total antioxidant capacity levels among the normal glucose tolerance (NGT), IGT, and diabetic groups (Kopprasch et al., 2002).

4.4. Correlation

The associations between parameters in T2DM patients are shown in Table 4-4, by the means of Pearson's correlation coefficient (r).

Table 4-4: Correlation in T2DM patients.

Parameters	TOS		TAC		OSI	
	r	***P***	**r**	***P***	**r**	***P***
TOS	-	-	0.037	0.876	0.943	<0.001
TAC	0.037	0.876	-	-	-0.286	0.221
OSI	0.943	<0.001	-0.286	0.221	-	-
Glucose	0.133	0.576	0.005	0.984	0.137	0.565
Age	0.190	0.423	0.280	0.231	0.076	0.752
BMI	0.020	0.932	0.146	0.540	-0.039	0.871

According to Pearson's correlation, there was a positive strong correlation observed between TOS and OSI in T2DM patients, as shown in Figure 4-7.

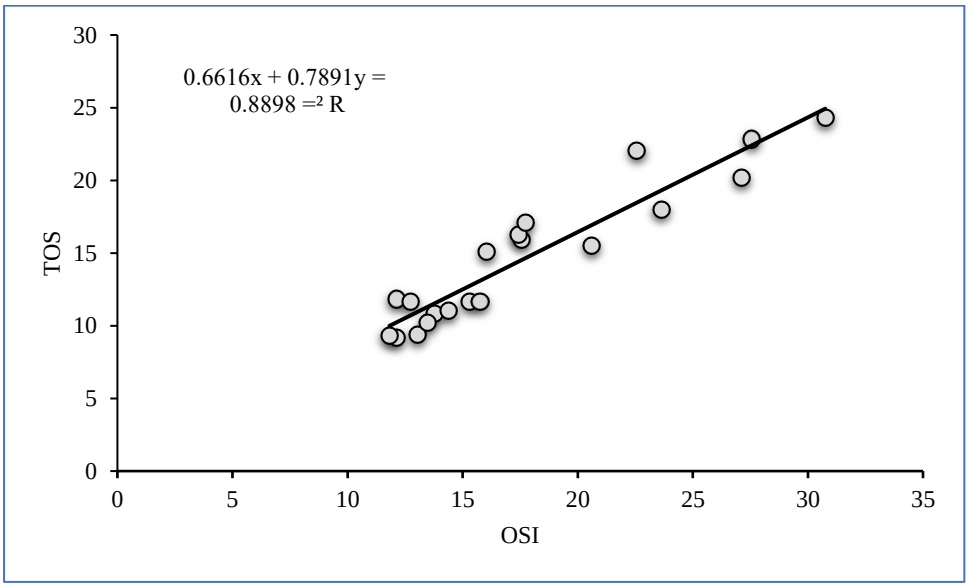

Figure 4-7: The correlation between TOS and OSI in T2DM patients.

4.5. Receiver Operating Characteristics

The possibility of using TOS and OSI as prognostic markers for T2DM patients is shown in Table 4-5 by measuring the area under the curve (AUC) for ROC curve and the sensitivity and specificity for the selected cut-off value.

Table 4-5: ROC curve parameters for TOS and OSI.

Parameter	AUC	SE	*p*-value
TOS	0.623	0.091	0.185
OSI	0.638	0.092	0.137
Parameter	**Cut-off value**	**Sensitivity**	**Specificity**
TOS	15.23	60%	55%
OSI	14.55	50%	90%

According to the ROC analysis, TOS has shown poor sensitivity in the prognosis of T2DM in male patients. the estimated AUC was 0.623 with 0.185 *p*-value. Cut-off value was determined as 15.23 µmol H_2O_2 Eq/L with 60% sensitivity and 55% specificity (Figure 4-8).

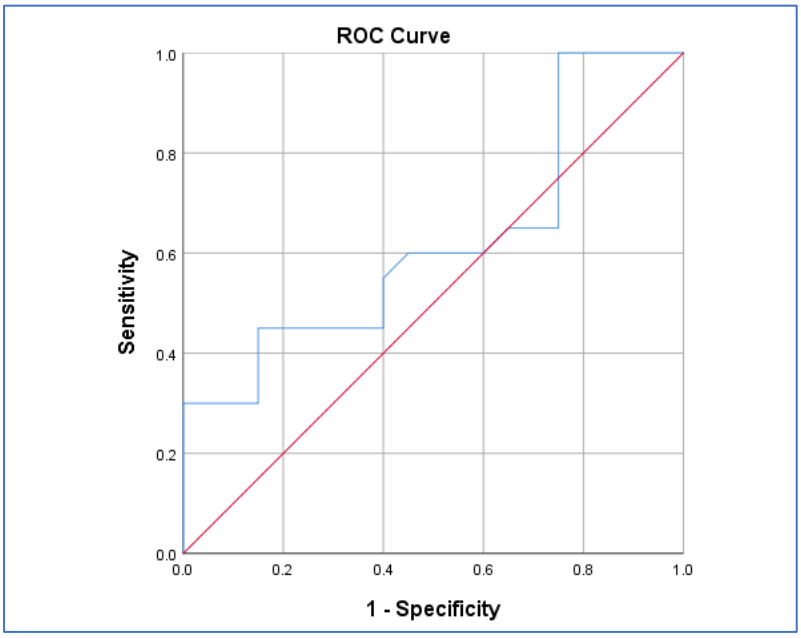

Figure 4-8: ROC curve for TOS in the prognosis of T2DM.

According to the ROC analysis, OSI has shown poor sensitivity in the prognosis of T2DM in male patients. the estimated AUC was 0.638 with 0.137 *p*-value. Cut-off value was determined as 14.55 with 50% sensitivity and 90% specificity (Figure 4-8).

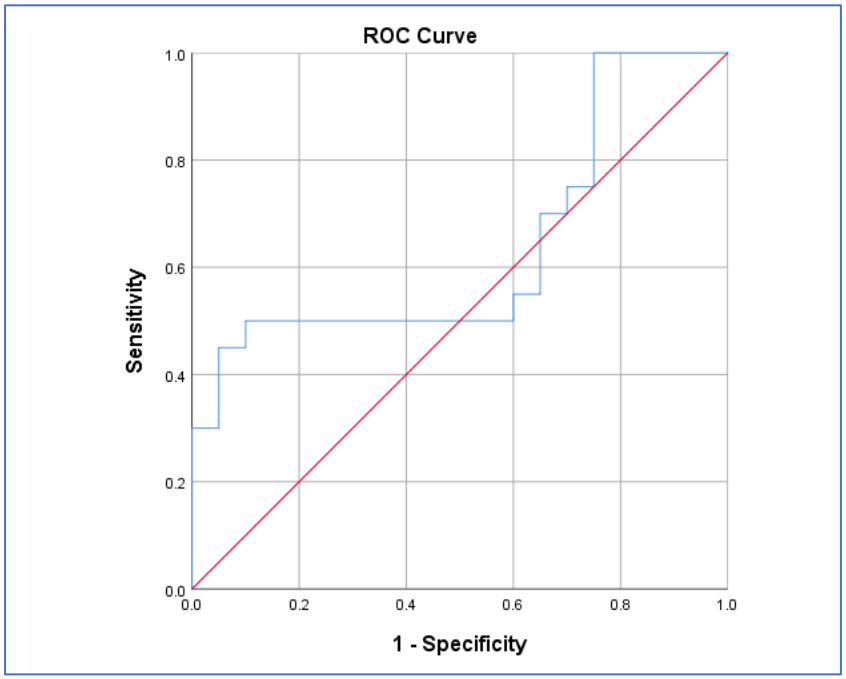

Figure 4-9: ROC curve for OSI in the prognosis of T2DM.

Chapter Five

Conclusions

and

Recommendations

5. Conclusions and Recommendations

5.1. Conclusions

1- The levels of TOS and OSI were elevated significantly in T2DM male patients, indicating an increased oxidative stress caused by increased production of ROS.

2- The non-significant reduction of TAC, indicates either a non-significant role of antioxidants in the elevated oxidative stress in T2DM patients, or T2DM patients have used to get antioxidants from exogenous sources.

3- Oxidative stress biomarkers TOS, TAC, and OSI, are only poor biomarkers for the prognosis of T2DM disease.

5.2. Recommendations

1- According to the results of the study, the use of exogenous antioxidants is important to regulate the redox status in T2DM patients and detoxifying the overproduced ROS.

2- Further investigations are required on other biomarkers of oxidative stress to determine the level of oxidative damage within cells in T2DM patients.

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بغداد
كلية العلوم

تقدير حالة الأكسدة الكلية والسعة الكلية لمضادات الأكسدة في أمصال المرضى الذكور المصابين بداء السكري النوع الثاني

بحث مقدم الى كلية العلوم جامعة بغداد استكمالاً جزئياً لمتطلبات درجة البكلوريوس في الكيمياء

بواسطة:
عبدالله خماس عبدالله

بإشراف :
م.م. ياسر محمد الطائي

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