

# The Role of Oxidative Stress and Antioxidants in Diabetic Complications

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## دور الإجهاد التأكسدي والمواد المضادة للأكسدة في مضاعفات مرض السكري

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الملخص: يُعد مرض السكري أحد الأمراض المزمنة الشائعة في العالم. هناك اهتمام علمي و عام متزايد حول ربط الإجهاد التأكسدي مع مختلف الحالات المرضية بما فيها مرض السكري، وكذلك الأمراض الأخرى التي تصيب الإنسان. ذكرت الدراسات التجريبية والسريرية السابقة بأن الإجهاد التأكسدي يلعب دوراً رئيسياً كمُسبب مرضي وفي تطور مضاعفات كلا النوعين من مرض السكري. من جهة أخرى فإن معرفة الآلية الدقيقة التي يساهم بها الإجهاد التأكسدي في سرعة تطور مضاعفات مرض السكري ناقصة وتبقى بحاجة إلى توضيح أكبر. من جهة أخرى، يُعتبر ارتفاع مستوى السكر في الدم أحد العوامل التي تحتل علي إنتاج الجذور الحرة، ومن ناحية أخرى فإنه يؤدي إلى ضعف النظام الدفاعي لمضادات الأكسدة الداخلي. تشمل آليات دفاع مضادات الأكسدة الداخلية كلا من المسارات الأنزيمية وغير الأنزيمية، ومن أهم وظائفها في خلايا الإنسان هي دورها كمضادات لسُمية المشتقات التفاعلية للأكسجين. تشمل مضادات الأكسدة الداخلية: الفيتامينات أ، ج، إي، جلوتاثيون، والأنزيمات، ومنها أنزيم سوبرأوكسيد ديسميوتيز، كاتاليز، جلوتاثيون بيروكسيديز، وجلوتاثيون ريداكثيز. هذه المراجعة تصف أهمية النظام الدفاعي لمضادات الأكسدة الداخلية وعلاقتها بمختلف العمليات الفسيولوجية المرضية وأثارها العلاجية الممكنة في الجسم الحي.

مفتاح الكلمات: إجهاد تأكسدي، مشتقات الأكسجين التفاعلية، مضادات الأكسدة، مرض السكري، الجذور الحرة، أمراض القلب والأوعية الدموية، مضاعفات مرض السكري، بيروكسيد الدهون.

**ABSTRACT:** Diabetes is considered to be one of the most common chronic diseases worldwide. There is a growing scientific and public interest in connecting oxidative stress with a variety of pathological conditions including diabetes mellitus (DM) as well as other human diseases. Previous experimental and clinical studies report that oxidative stress plays a major role in the pathogenesis and development of complications of both types of DM. However, the exact mechanism by which oxidative stress could contribute to and accelerate the development of complications in diabetic mellitus is only partly known and remains to be clarified. On the one hand, hyperglycemia induces free radicals; on the other hand, it impairs the endogenous antioxidant defense system in patients with diabetes. Endogenous antioxidant defense mechanisms include both enzymatic and non-enzymatic pathways. Their functions in human cells are to counterbalance toxic reactive oxygen species (ROS). Common antioxidants include the vitamins A, C, and E, glutathione (GSH), and the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx). This review describes the importance of endogenous antioxidant defense systems, their relationship to several pathophysiological processes and their possible therapeutic implications *in vivo*.

**Keywords:** Oxidative stress; Reactive oxygen species; Antioxidants; Diabetic mellitus; Free radicals; Cardiovascular diseases; Diabetic complications; Lipid peroxidation.

**D**IABETES IS WIDELY RECOGNISED AS one of the leading causes of death and disability worldwide.<sup>1</sup> The prevalence of diabetes will rise from 6% to over 10% in the next decade.<sup>2</sup> In 2000, the World Health Organization

(WHO) recorded a total of 171 million people for all age groups worldwide (2.8% of the global population) who have diabetes, and the numbers are expected to rise to 366 million (4.4% of the global population) by 2030.<sup>3</sup>

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Diabetes is a group of metabolic diseases characterised by high levels of blood sugar (hyperglycemia). It results from defects in insulin production and/or insulin action, and impaired function in the metabolism of carbohydrates, lipids and proteins which leads to long term health complications.<sup>4,5</sup> In diabetic patients, long-term damage, dysfunction, and failure of different organs, especially the eyes (diabetic retinopathy), kidneys (diabetic nephropathy), nerves (diabetic neuropathy), heart (myocardial infarction), and blood vessels (atherosclerosis) are related to uncontrolled hyperglycemia.<sup>5-7</sup>

However, diabetic patients vary in their predisposition to the development of complications.<sup>8</sup> The genetic hypothesis suggests that complications from diabetes are genetically predetermined as part of the diabetic syndrome, whereas the metabolic hypothesis suggests that complications such as cellular and vascular damage are the effects of long-term hyperglycemia.<sup>9</sup> The Diabetes Control and Complications Trial (DCCT) convincingly showed that complications from diabetes can be delayed and reduced by maintaining tight glycemic control.<sup>9</sup>

## Role of Oxidative Stress in Diabetic Complications

The balance between the rate of free radical generation and elimination is important. Excess cellular radical generation can be harmful;<sup>10</sup> however, if there is a significant increase in radical generation, or a decrease in radical elimination from the cell, oxidative cellular stress ensues.<sup>11</sup> There is convincing experimental and clinical evidence that the generation of reactive oxygen species (ROS) increases in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress.<sup>2,12</sup>

Oxidative stress results from increased ROS and/or reactive nitrogen species (RNS).<sup>13</sup> Examples of ROS include charged species such as superoxide and the hydroxyl radical, and uncharged species such as hydrogen peroxide and singlet oxygen.<sup>2</sup> The possible sources of oxidative stress in diabetes might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defence enzymes such as

superoxide dismutase (SOD) and catalase (CAT).<sup>14</sup> ROS generated by high glucose is causally linked to elevated glucose and other metabolic abnormalities important to the development of diabetic complications. However, the exact mechanism by which oxidative stress may contribute to the development of diabetic complications is undetermined.<sup>15</sup>

In the past few decades, increasing evidence has connected oxidative stress to a variety of pathological conditions, including cancer, cardiovascular diseases (CVDs), chronic inflammatory disease, post-ischaemic organ injury, diabetes mellitus, xenobiotic/drug toxicity, and rheumatoid arthritis.<sup>16,17</sup> Over time, convincing evidence has established the role of free radicals and oxidative stress in the pathogenesis and development of complications from DM,<sup>9,18</sup> including retinopathy, nephropathy, neuropathy, and accelerated coronary artery disease.<sup>19</sup> Several studies have shown that elevated extra- and intra-cellular glucose concentrations result in oxidative stress<sup>20,21</sup> which was reported both in experimental diabetes in animals and in diabetic patients.<sup>21-23</sup> The source of oxidative stress is a cascade of ROS leaking from the mitochondria. This process has been associated with the onset of type 1 diabetes (T1DM) via the apoptosis of pancreatic beta-cells, and the onset of type 2 diabetes (T2DM) via insulin resistance.<sup>21</sup> The underlying mechanisms in the onset of diabetes are complex because hyperglycemia could also be due to the cause-effect relationship of increased oxidative stress.<sup>21</sup> Biomarkers of increased oxidative stress, as measured by indices of lipid peroxidation and protein oxidation, increase in both T1DM, and T2DM.<sup>24</sup>

The aetiology of oxidative stress in diabetes arises from a variety of mechanisms such as excessive oxygen radical production from auto-oxidation of glucose,<sup>25</sup> glycated proteins, and glycation of antioxidative enzymes, which limit their capacity to detoxify oxygen radicals.<sup>20</sup> In addition to these mechanisms, two others have been suggested as being responsible for the generation of oxygen radicals in diabetes. First, Jain demonstrated that high glucose levels could stimulate cytochrome P450-like activity by excessive nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) produced by glucose metabolism.<sup>26</sup> Second, ketosis, a hallmark of T1DM in particular, could increase

oxygen radical production in diabetic patients.<sup>27</sup>

Nowadays, diabetic micro- and macroangiopathy are considered to be poly aetiological multifactorial diseases. A number of studies have evaluated the role of oxidative stress in the aetiology of microvascular and macrovascular complications of diabetes in the fasting state.<sup>28</sup> Furthermore, there is growing evidence suggesting the role of hyperglycemia, hyperinsulinemia and dyslipidaemia in diabetic patients, all of which have been implicated in the development of macroangiopathies, which possibly act upon their ability to induce oxidative stress, leading to endothelial dysfunction and atherosclerosis.<sup>20</sup> Many studies have suggested that oxidative stress is a common pathogenic factor for the dysfunction of beta and endothelial cells.<sup>29,30</sup> Beta cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acid (FFA) levels, or a combination of both.<sup>30</sup> Beta cells are particularly sensitive to ROS because they are low in free-radical quenching (antioxidant) enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD).<sup>31</sup> Therefore, the ability of oxidative stress to damage mitochondria and markedly blunt insulin secretion is not surprising.<sup>32,33</sup> For example, it has been demonstrated that oxidative stress generated by short exposure of beta cell preparations to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) increases the production of protein cyclin-dependent kinase inhibitor 1 (p21) and decreases insulin messenger ribonucleic acid (mRNA), cytosolic adenosine triphosphate (ATP), and calcium flux in cytosol and mitochondria.<sup>34</sup> In other studies, much experimental evidence has been accumulated to show that various types of vascular cells are able to produce ROS under hyperglycemic conditions.<sup>20</sup>

The pathogenesis of diabetic nephropathy remains far from clear. An important role of oxidative stress for the development of nephropathy and neurological complications is suggested by experimental and clinical studies.<sup>35</sup> These studies establish a causal relationship between oxidative stress and diabetic nephropathy by observations that 1) lipid peroxides and 8-hydroxydeoxyguanosine, indices of oxidative tissue injury, increase in the kidneys of diabetic rats with albuminuria; 2) high glucose directly increases oxidative stress in glomerular mesangial cells and target cells of diabetic nephropathy; 3) oxidative stress induces

mRNA expression of transforming growth factor beta 1 (TGF-β1) and fibronectin, which are the genes implicated in diabetic glomerular injury, and 4) inhibition of oxidative stress ameliorates all the manifestations associated with diabetic nephropathy.<sup>36</sup>

Previous studies demonstrated that there is a close relationship between endothelial dysfunction and the development and progression of renal and cardiovascular pathology in patients with T1DM.<sup>37</sup> The combined development of renal and cardiovascular complications is referred to as cardiorenal syndrome. The causes of the development of cardiorenal syndrome in T1DM are poorly understood. Previous studies suggest that endothelial dysfunction and the concomitant atherosclerotic process may lead to simultaneous development and progression of renal and cardiac pathology, since endothelial dysfunction is already present at the early stages of T1DM.<sup>38</sup>

## Reactive Oxygen Species

Although molecular oxygen is required to sustain life, it can be toxic through the formation of ROS.<sup>39</sup> Indeed, the unusual triplet state of the oxygen molecule, due to the presence of two unpaired electrons, confers a remarkable chemical stability, based on the Pauli Exclusion Principle, which forbids reactions between a singlet and a triplet molecule. Due to electron spin constraints, the oxygen molecule cannot readily react with organic substrates.<sup>40</sup> Approximately 1–3% of oxygen consumed by the body is converted into ROS.<sup>41</sup> Activation of oxygen can occur through two different mechanisms. The first mechanism of activation is absorption of sufficient energy to reverse the spin on one of the unpaired electrons, called a monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will become singlet oxygen, in which the two electrons have opposite spins [Figure 1]. This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction). The second mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide

Triplet Oxygen (Ground state)	$\bullet\text{O}-\text{O}\bullet$
Singlet Oxygen	$\text{O}=\text{O}:$
Superoxide	$\bullet\text{O}-\text{O}:$
Perhydroxyl radical	$\bullet\text{O}-\text{O}-\text{H}$
Hydrogen peroxide	$\text{H}:\text{O}-\text{O}:\text{H}$
Hydrogen radical	$\text{H}:\text{O}\bullet$
Hydrogen ion	$\text{H}:\text{O}:$
Water	$\text{H}:\text{O}:\text{H}$

Figure 1: Nomenclature of the various forms of oxygen.<sup>42</sup>

( $\text{O}_2$ ),  $\text{H}_2\text{O}_2$ , hydroxyl radical (OH) and finally water according to the scheme shown in Figure 2. The first step in the reduction of oxygen forming superoxide is endothermic, but subsequent reductions are exothermic.<sup>42</sup>

Humans are exposed to many carcinogens, but the most significant may be the reactive species derived from the metabolism of oxygen and nitrogen known as ROS and RNS. On the one hand, the formation of ROS and RNS in the human body can cause oxidative damage to biological macromolecules, especially the plasma membrane,<sup>43</sup> which may contribute to the development of cancer, CVD, diabetes and other oxidative stress-mediated dysfunctions.<sup>44</sup> On the other hand, ROS are known mediators of intracellular signalling cascades.<sup>45</sup>

Even though ROS are generated under physiological conditions and are involved to some extent as signalling molecules and defence mechanisms as seen in phagocytosis, neutrophil function, macrophages and other cells of immune system,<sup>46</sup> ROS are a heterogeneous group of molecules that are generated by mature myeloid cells during innate immune responses, and are also implicated in normal intracellular signalling. When phagocytes are activated, they produce ROS in amounts high enough to kill intruding bacteria.<sup>47</sup> Also, shear-stress induced vasorelaxation and excess production of ROS may, on the other hand, lead to oxidative stress, loss of cell function, and ultimately to apoptosis or necrosis.<sup>46</sup> ROS are produced by oxidative phosphorylation, NADPH, xanthine oxidase, the uncoupling of lipoxygenases, cytochrome P450 monooxygenases, and glucose autoxidation.<sup>18</sup> Once formed, ROS deplete antioxidant defenses, rendering the affected cells

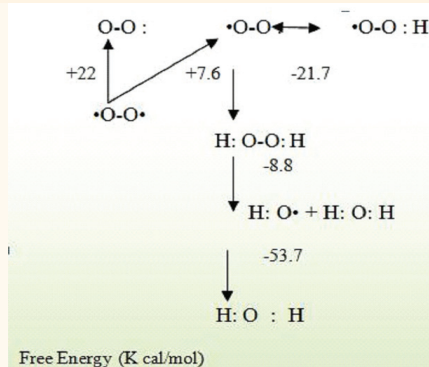


Figure 2: The activation states of oxygen.<sup>42</sup>

and tissues more susceptible to oxidative damage by reacting with lipids in cellular membranes, nucleotides in DNA,<sup>48</sup> sulphhydryl groups in proteins, and cross linking fragmentation of ribonucleoproteins,<sup>49</sup> leading to changes in cellular structure and function.<sup>19</sup>

Levels of ROS are under tight control by the protective actions of antioxidant enzymes and non-enzymatic antioxidants in normal and healthy cells. However, in diabetes, excessive cellular levels of ROS are induced by hyperglycemia causing a major complication of DM.<sup>50</sup> Furthermore, in the case of diabetes or insulin resistance, a higher oxidative glucose metabolism itself increases mitochondrial production of  $\text{O}_2^{\bullet-}$  which will then be converted to  $\text{OH}\bullet$  and  $\text{H}_2\text{O}_2$ .<sup>51</sup> Beyond glucose, ROS formation is also increased by FFAs, through its direct effect on mitochondria.<sup>52</sup> It has been proposed that over-expression and activity of mitochondrial inner membrane uncoupling proteins (UCPs) contribute to an increase in superoxide formation under diabetic conditions.<sup>53</sup>

The ROS<sup>52, 54</sup> listed in Table 1 include free radicals such as superoxide ( $\text{O}_2^{\bullet-}$ ) hydroxyl ( $\text{OH}\bullet$ ), and RNS<sup>52, 54</sup> include free radicals like nitric oxide ( $\text{NO}\bullet$ ). Of these reactive molecules,  $\text{O}_2^{\bullet-}$ ,  $\text{NO}\bullet$  and  $\text{ONOO}^-$  are the most widely studied species and play important roles in diabetic cardiovascular complications.<sup>12</sup>

In diabetes, NADPH oxidase is a major source of the generation of ROS. NADPH oxidase is located in the plasma membrane of various renal cell types, including mesangial and proximal tubular cells, vascular smooth muscle cells, endothelial cells, and fibroblasts. NADPH oxidase-dependent



**Table 1:** Overview of different types of free reactive species<sup>2</sup>

Reactive Oxygen Species	
RADICALS	NON RADICALS
Superoxide, O <sub>2</sub> <sup>•-</sup>	Hydrogen peroxide, H <sub>2</sub> O <sub>2</sub>
Hydroxyl, OH <sup>•</sup>	Hypochlorous acid, HOCl <sup>-</sup>
Peroxy, ROO <sup>•</sup>	Ozone, O <sub>3</sub>
Alkoxy, RO <sup>•</sup>	Singlet oxygen, <sup>1</sup> O <sub>2</sub>
Hydroperoxyl, HO <sub>2</sub>	Peroxynitrite, ONOO <sup>-</sup>
Reactive Nitrogen Species	
Nitric oxide, (nitrogen mono) NO <sup>•</sup>	Peroxynitrite, ONOO <sup>-</sup>
Nitrogen dioxide, NO <sub>2</sub> <sup>•</sup>	Alkyl peroxy nitrates, ROONO
	Dinitrogen trioxide, N <sub>2</sub> O <sub>3</sub>
	Dinitrogen tetroxide, N <sub>2</sub> O <sub>4</sub>
	Nitrous acid, HNO <sub>2</sub>
	Nitronium anion, NO <sup>2+</sup>
	Nitroxyl anion, NO <sup>-</sup>
	Nitrosyl cation, NO <sup>+</sup>
	Nitryl chloride, NO <sub>2</sub> Cl
Reactive Chlorine Species	
Atomic chlorine, Cl <sup>-</sup>	Hypochlorous acid, HOCl
	Chlorine, Cl <sub>2</sub>
	Nitronium (nitryl) Chloride, NO <sub>2</sub> Cl

overproduction of ROS plays a key role in promoting hyperglycemia-induced oxidative stress. The NADPH oxidase increase oxidative stress and finally this increases results in the development of diabetic nephropathy in rats.<sup>47</sup>

## Oxidative Stress-Induced Cellular Damage

The targets of ROS damage include all major groups of biomolecules and can be described as follows:

### A. PROTEINS

Since ROS can target almost all cellular compounds, several studies report that ROS can react with several amino acid residues *in vitro*, generating anything from modified and less active enzymes to denatured, non-functioning proteins.<sup>55</sup> Fragmentation of the peptide chain and aggregation of cross-linked reaction products result in an altered electrical charge and increased susceptibility to proteolysis.<sup>42</sup> The amino acids in a peptide differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. Primary, secondary, and tertiary protein

structures alter the relative susceptibility of certain amino acids.<sup>42</sup> Experimental studies show that the decrease in the total protein concentration in the serum of diabetes-induced rats may be ascribed to: 1) decreased amino acids uptake;<sup>55</sup> 2) a greatly decreased concentration of a variety of essential amino acids;<sup>56</sup> 3) an increased conversion rate of glycogenic amino acids to CO<sub>2</sub> and H<sub>2</sub>O, and 4) a reduction in protein synthesis secondary to a decreased amount and availability of mRNA.<sup>57</sup>

*In vitro* studies show that myeloperoxidase, a heme protein secreted by neutrophils and monocytes, catalyses a very specific reaction, leading to the conversion of L-tyrosine to 3-3-dityrosine.<sup>58</sup> In addition, the oxidative formation of dityrosine in proteins may serve as a crosslink between adjacent polypeptide chains of the same or different proteins. Dityrosine is a very unusual molecule linked not by a peptide, but by a covalent bond resistant to protolytic degradation and acid hydrolysis.<sup>59</sup> This property of dityrosine makes it a convenient marker for protein oxidation. Oxidatively induced cross-linking of proteins with this modified amino acid was suggested to be a signal for rapid and selective *in vivo* degradation by intracellular protease.<sup>60</sup> However, accumulation of oxidatively modified proteins with age was thought to be a result of their increased resistance to degradation.<sup>61</sup>

### B. LIPIDS

In recent years, much attention has been focused on the role of oxidation stress, and it has been reported that the imbalance between oxidative stress measures and antioxidant levels in diabetes is present because of the generation of ROS during glycation, and glucose and lipid oxidation.<sup>62,25</sup> Lipid hydroperoxides (LHP), produced from a variety of long-chain polyunsaturated fatty acid precursors via intermediate radical reactions, involve oxygen and metal cations (iron and copper). The net result of these combined reactions is the generation of highly reactive and cytotoxic lipid radicals, which generate new LHP because of their close proximity in biomembranes to other lipids.<sup>63</sup> In addition, diabetes produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation,<sup>64</sup> which is responsible for an increased incidence of atherosclerosis,<sup>65</sup> a major complication of DM.<sup>66</sup> Lipid peroxidation is a critical biomarker of free radical-mediated oxidative

stress, and it is probably the most explored area of research when it comes to ROS.<sup>67</sup> It is also an important pathological indicator in many diseases.<sup>56</sup> Consequently, mechanisms in the formation of lipid hydroperoxides and biologically active metabolites, together with their effect on cellular structure and function, are of increasing importance to the study of diabetogenesis.<sup>68</sup>

There is much evidence from experimental studies that polyunsaturated fatty acids (PUFA) in the plasma membrane, because of their multiple double bonds, are extremely susceptible to attack by free radicals.<sup>69</sup> Hydroxyl radicals initiate a free radical chain reaction and remove a hydrogen atom from one of the carbon atoms in the PUFA and lipoproteins, causing lipid peroxidation characterised by membrane protein damage through subsequent free radical attacks.<sup>70</sup> One major hypothesis as to why this occurs is that oxidised lipoprotein (oxLDL) contributes to the cardiovascular complications of diabetes. Low-density lipoprotein (LDL) is the major cholesterol carrier in plasma, and elevated levels of circulating LDL are associated with increased risk for atherosclerosis; notably, increased levels of oxLDL have been associated with hypertension in men.<sup>71</sup>

Several studies have demonstrated increased LDL oxidation in diabetic patients when compared to their corresponding controls.<sup>72</sup> Oxidised lipids can affect cell function by accumulating in the cell membrane causing leakage of the plasma lemma and interfering with the function of membrane-bound receptors.<sup>73</sup> In addition, the by-products of lipid peroxidation, such as unsaturated aldehydes and other metabolites, have cytotoxic and mutagenic properties, and oxLDL itself has a specific role in the pathogenesis of atherosclerosis.<sup>74</sup> The effects of oxLDL on the vessel walls themselves include stimulation of cytokine and growth factor production and generation of endothelial dysfunction, including inhibition of endothelial cell vasodilator function, all of which contribute to atherosclerosis.<sup>41</sup> Lipid peroxidation-mediated membrane defects have also been implicated in the decreased reactivity of thiol group membrane proteins.<sup>75</sup> As a consequence, enriching the membranes with PUFA could restore oxidative membrane damage and subsequently normalise decreased membrane fluidity and function. Antioxidants (i.e., dietary supplements) compensating for loss of PUFA may enhance the

activity of transmembrane enzymes, membrane-embedded receptors and membrane transport systems.<sup>52</sup>

## Role of Antioxidant Defense System and Protection Mechanism

In the late 19<sup>th</sup> and early 20<sup>th</sup> centuries, chemists studied antioxidants, a loosely defined group of compounds characterised by their ability to be oxidised in place of other compounds present. At first, their uses ranged from food storage to the vulcanisation of rubber, but it was only later that biologists realised the importance of antioxidants in health with the 1960s publications of vitamins and flavanoids.<sup>39</sup> In the 1970s, Cameron and Pauling, followed by later research, found that ascorbic acid (vitamin C) is a potential human cancer protective agent.<sup>76</sup> With many well-known scientists actively researching antioxidants as protecting agents,<sup>77</sup> explanations for the effects of antioxidants on cancer susceptibility and overall health expanded rapidly in subsequent decades with research into mechanisms, molecular targets, and molecular interactions.<sup>78</sup> In recent years, many conferences and reviews testify to the increasing interest in the roles of the body's antioxidants system working together in human cells against toxic reactive oxygen species, their relationship with several pathophysiological processes and their possible therapeutic implications.<sup>79</sup>

Antioxidant defense mechanisms involve both enzymatic and nonenzymatic strategies. Common non-enzymatic antioxidants include the vitamins A, C, and E, glutathione,  $\alpha$ -lipoic acid, mixed carotenoids, coenzyme Q10 (CoQ10), several bioflavonoids, antioxidant minerals (copper, zinc, manganese and selenium), and cofactors like folic acid, uric acid, albumin, and vitamins B1, B2, B6, and B12. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase.<sup>80</sup> Through normal physiological processes, antioxidants affect signal transduction and regulation of proliferation and the immune response. While ROS have been linked to cancer, diabetic complications and CVD, antioxidants have shown promise as a possible therapy for the prevention and treatment of these diseases, especially given the tantalising links

observed between diets high in fruits and vegetables (and presumably antioxidants) and decreased risks of cancer.<sup>41</sup> Evidence from experimental, epidemiological and clinical studies have proven the utility of antioxidants which might therefore be helpful for treating diabetes and its complication.

## Evidence from Experimental Studies

Worldwide, several animal studies on antioxidants as protective agents have been conducted. The most important studies have been performed utilising antioxidants in experimental diabetic models. The effects of antioxidants on oxidative stress are measured through certain observable biomarkers. These markers include the enzymatic activities of CAT, SOD, GSH-Px, and glutathione reductase (GSH-Rx), as well as thiobarbituric acid reacting substances (TBARS) levels, an indirect measurement of free radical production that has been shown to be consistently elevated in diabetes.<sup>12</sup>

Kunisaki investigated the effects of treatments with classical antioxidants such as vitamin E, vitamin C and lipoic acid. Specifically, vitamin E normalises retinal blood flow and protein kinase C (PKC) activity in the vascular tissue of diabetic rats.<sup>81</sup> One short-term experimental study showed that high doses of vitamin C can improve some aspects of endothelial dysfunction in diabetes.<sup>82</sup> Another experimental study has demonstrated that intraperitoneal administration of  $\alpha$ -lipoic acid to streptozotocin (STZ) diabetic Wistar rats normalises TBARS levels in plasma, and the retina, liver, and pancreas.<sup>83</sup> Furthermore, it has been reported that  $\alpha$ -lipoic acid leads to a decrease in the severity of diabetic neuropathy by maintaining GSH levels and/or by its direct antioxidant properties.<sup>83</sup> However, lipoic acid administration improved endothelial function in subjects with metabolic syndrome.

In another study to determine the effects of vitamin E on oxidative stress and cell membrane fluidity in the brains of diabetes-induced experimental rats, Hong *et al.* reported that vitamin E was found to be effective for strengthening the antioxidant defense system. They noted a reduction of the accumulation of ROS such as superoxide radicals, a decrease in the generation of oxidative damaging substances such as the carbonyl value,

a significantly improved lipid composition, and maintenance of membrane fluidity in the brains of the rats.<sup>84</sup> Coleman suggested that triple antioxidant therapy (vitamin E, lipoic acid [LA] and vitamin C) in diabetic volunteers attenuates the experimental oxidative stress of methemoglobin formation *in vitro* and reduces haemoglobin glycation *in vivo*.<sup>85</sup> Panjwani suggested that vitamin C administered alone or in combination with vitamin E reduced the fall in ulnar nerve conduction velocity.<sup>86</sup>

Sivan and Reece investigated whether dietary supplementation with vitamin E would reduce the incidence of diabetic embryopathy in an *in vivo* rat model.<sup>87</sup> Fifty pregnant rats were designated as the control group and received a normal diet while the diabetic group received vitamin E supplements (400 mg/day). This experimental study found that supplementation with vitamin E reduces the incidence of neural tube defects by more than 75%.<sup>87</sup> These findings suggest that vitamin E reduces this oxidative load, confers a protective effect against diabetic embryopathy, and thus may potentially serve as a dietary prophylaxis in the future.

These results are in line with the reports from Chang and others which showed that diabetic embryopathy of rat or mouse embryos is prevented by vitamin C, vitamin E, SOD, N-acetyl-cysteine, or glutathione ethyl ester.<sup>88</sup> Another study by Otero *et al.* showed the effects of supplementation with vitamin E to diabetic mice. The beneficial effect of vitamin E observed in this model was shown to retard coronary atherosclerosis accelerated by DM, and was demonstrated to be due to a reduction in oxidative stress, and not secondary to a decrease in plasma glucose or cholesterol since their respective plasma concentrations remained unchanged in the diabetic mice supplemented with vitamin E.<sup>89</sup>

Furthermore, it has been recently reported that macrophages from diabetic mice are under excess oxidative stress, and that the antioxidant vitamin E can attenuate macrophage oxidative stress which exists in DM and leads to accelerated atherosclerosis development.<sup>90</sup> In addition, there have been other experimental studies assessing the prophylactic effects of vitamin E against heart failure in type 1 diabetic cardiomyopathy. These studies have found that supplementation of streptozotocin-induced (STZ)-diabetic rats with 2000 IU of vitamin E/kg of feed beginning immediately after induction of DM and continuing for 8 weeks provided a

significant protection against cardiac dysfunction induced by T1DM. This cardioprotective effect was simultaneously associated with the ability of vitamin E to blunt diabetes-induced amplification of myocardial infarction.<sup>91</sup> These findings suggest the usefulness of vitamin E supplementation during the early phases of T1DM for the prophylaxis of cardiomyopathy and subsequent heart failure.

Ozkan *et al.* examined the protective effects of a triple antioxidant combination (vitamins E and C plus  $\alpha$ -lipoic acid) on the total lipid and cholesterol levels and the fatty acid composition of brain tissues in experimental diabetic and non-diabetic rats. Vitamin E and  $\alpha$ -lipoic acid were injected intraperitoneally (50 mg/kg) four times per week and vitamin C was provided as a supplement dissolved in the rats' drinking water (50 mg/kg). The result of this study confirms that treatment with a triple combination of antioxidants protects the arachidonic acid level in the brains of diabetic and non-diabetic rats. In addition, both antioxidants and insulin treatment have beneficial effects on the D-6 desaturase system and unsaturated fatty acid levels. Antioxidants may also have protective effects on unsaturated fatty acids, and reduce oxidative stress, thus impairing the progression to LDL oxidation, cell membrane lipid peroxidation and decreased endoneurial blood flow, thereby reducing peripheral nerve and vascular dysfunction.<sup>92</sup>

## Evidence from Clinical Studies

Early interest in the use of vitamin E supplementation in patients with CHD came from the enthusiastic reports of a Canadian physician, Evan Shute, who recommended vitamin E for all his patients with coronary heart disease (CHD) and presented a case series showing its significant benefit in reducing symptoms of angina pectoris.<sup>93</sup> In a clinical trial study conducted by Bursell, *et al.*, they evaluated 36 patients with T1DM. In their crossover trial, individuals were divided into two groups; one group received a placebo while the other group received high dose vitamin E supplementation (1,800 IU/day). The major aim of this study was to determine the effectiveness of vitamin E in normalising retinal blood flow and renal function in patients with T1DM. They found that oral vitamin E treatment appears to be effective in normalising retinal

haemodynamic abnormalities and improving renal function in patients with T1DM, especially those with short disease duration, without inducing a significant change in glycemic control. This suggests that vitamin E supplementation may provide an additional benefit in reducing the risks for developing diabetic retinopathy or nephropathy.<sup>94</sup>

In terms of vitamin E supplementation in diabetes, Ross and others have demonstrated beneficial effects on cataract genesis in diabetic rats.<sup>95</sup> Furthermore, in a study done over a four month period, Paolisso, *et al.* evaluated the efficacy of oral supplementation of vitamin E (900 mg/day) in T2DM patients. They found that supplementation of vitamin E decreased lowered insulin resistance and improved glucose uptake as shown by euglycemic glucose clamp assays performed both before and after the supplementation period.<sup>96</sup> In addition, they also concluded that vitamin E may be a useful adjunct in reducing oxidative stress in T2DM. These findings confirmed an earlier study by Ceriello *et al.* in which glycosylated haemoglobin and other proteins were significantly decreased after two months' treatment with either 600 or 1200 mg/day of vitamin E.<sup>97</sup> The potential for vitamin E in pharmacological doses to be used as a means to delay or prevent secondary complications of diabetes seems well established.

Vitamin C supplementation is effective in reducing sorbitol accumulation in the red blood cells of diabetics. These findings have been confirmed by a clinical study carried out by Cunningham, *et al.*, who investigated the effect of two different doses of vitamin C supplements (100 and 600 mg) during a 58 day trial on young adults with T1DM. The results of this study showed that within 30 days, vitamin C supplementation at either dose normalised sorbitol levels in those with diabetes. Furthermore, vitamin C also helps to reduce capillary fragility which also contributes to complications from DM. Moreover, several studies suggest that chronic vitamin C administration has beneficial effects on glucose and lipid metabolism in T2DM patients.<sup>98</sup> In addition, vitamin C supplementation improved glycemic control, fasting blood glucose levels, cholesterol and triglycerides.<sup>99</sup>

In a placebo-controlled, randomised trial of diabetic patients, Reaven investigated the effect of 1600 IU of RRR- $\alpha$ -tocopherol supplementation daily for 10 weeks on hyperglycemia-induced LDL



**Table 2:** Selected controlled clinical trials of antioxidant supplements' effect on preventing diabetic complications

Study name with type of supplementation	Number	Sex	Age	Dose	Duration in years (Y) or months (M)	Study Outcome
Dietary supplementation (GISSI). 1999 <sup>106</sup> Vitamin E	11,324	M, F	No age limits	300 mg	3.5 Y	No effect on MI + CVD death + stroke
Yusuf, <i>et al.</i> 2000 <sup>107</sup> Vitamin E	9541	M, F	≥55	400 IU	4.5 Y	No effect on MI, CVD death, or stroke
Pruthi, <i>et al.</i> 2001 <sup>108</sup> Vitamin E	39,910	M	No age limits	100 IU/day	2 Y	37% lower risk of CHD
Kritharides, <i>et al.</i> 2002 <sup>109</sup> Vitamins A and C, folic acid, niacin,β-carotene, selenium, zinc	87,000	M, F	No age limits	Not available	10 Y	Significantly lower risk of CVD, mortality
Deepak, <i>et al.</i> 2003 <sup>105</sup> Vitamins E and C, and β-carotene	20,500	M, F	40–80	VE 600 mg, VC 250 mg, β-carotene 20 mg	5 Y	No significant difference in cardiovascular mortality and incidence of vascular events between vitamin and placebo groups
Lee, <i>et al.</i> 2005 <sup>110</sup> Vitamin E	40000	F	≥45	600 IU	10 Y	No effect on cardiovascular events
Costacou, <i>et al.</i> 2006 <sup>111</sup> Vitamin supplementation with multivitamin pills		M, F	<17	Multivitamin pills	10 Y	Significant decreased risk of CAD incidence in T1DM
Milman, <i>et al.</i> 2008 <sup>112</sup> Vitamin E	1434	M, F	≥55	400 U/day	18 M	Reduced cardiovascular events in individuals with DM
Hodis, <i>et al.</i> 2009 <sup>113</sup> Folic acid, Vitamin B12, and B6	506	M, F	40–89	Folic acid 5 mg,VB12 0.4 mg, VB6 50 mg	3.1Y	Significantly reduced progression of early-stage subclinical atherosclerosis and low risk for CVD
Shargorodsky, <i>et al.</i> 2010 <sup>114</sup> Combined antioxidant (Vitamins E and C, coenzyme Q10)	70	M, F	No age limits	VE (400 IU/day),VC (1000 mg/d), Q10 (120mg\ day)	6 M	Positive effects on cardiovascular risk factors

modifications. The result of the study pointed to a reduction of approximately 60% of plasma LDL oxidation in diabetic patients, which was statistically significant when compared to healthy controls.<sup>100</sup> These results are supported and confirmed by other similar clinical data. Salonen, *et al.* found that doses equal to or higher than 450 IU are sufficient to significantly ameliorate the susceptibility of LDL to oxidation, indicating that relatively high doses of RRR-α-tocopherol for supplementation are needed.<sup>101</sup> Furthermore, Bellomo and others have shown that the effects of RRR-α-tocopherol supplementation on LDL oxidation are accompanied by a concomitant reduction in autoantibody levels against hyperglycemia-induced LDL modifications. Moreover, clinical studies have shown an inhibitory

effect of RRR-α-tocopherol supplementation on the hyperglycemia-induced LDL modifications in T1DM and T2DM diabetic patients.<sup>100</sup>

Based on nutrition recommendations and interventions for diabetes, there is no clear evidence of benefits that can be derived from vitamin or mineral supplementation in people with diabetes. Routine supplementation with antioxidants, such as vitamins E and C and carotene, is not advised because of lack of evidence of efficacy, and concern related to long term safety, and therefore cannot be recommended.<sup>102</sup> The majority of studies included in this review support a possible role of antioxidant supplementation in reducing the risk of CVD and diabetic complications. However, the results of the majority of the prospective randomised controlled

antioxidant clinical trials have failed to demonstrate a significant benefit in antioxidant supplementation in the prevention of atherosclerotic events, as shown in Table 2. As also supported by more recent meta-analysis studies of antioxidant-intervention trials in humans, doses of vitamin E supplements (400 or 800 IU) given in many clinical trials are associated with adverse effects,<sup>103</sup> or are associated with an increased risk of death.<sup>104</sup> Furthermore, the results of the majority of the prospective randomised controlled antioxidant clinical trials have failed to demonstrate a significant benefit of antioxidant supplementation (vitamins C, E and  $\beta$ -carotene) in primary patients (i.e. those without clinical evidence of CVD) or secondary patients (i.e. those with clinical evidence of CVD) in the prevention of cardiovascular events.<sup>105</sup> Moreover, in clinical trials the supplementation of vitamins C and E, and/or  $\beta$ -carotene was associated with an increased risk for all-cause mortality, a finding also supported by a recent meta-analysis.<sup>104</sup>

## Conclusion

In conclusion, there is considerable evidence that induction of oxidative stress is a key process in the onset of diabetic complications. The precise mechanisms by which oxidative stress may accelerate the development of complications in diabetes are only partly known. Evidence for the protective effect of antioxidants has been presented in experimental, clinical, and epidemiological studies, which have demonstrated that antioxidants might be helpful in treating diabetes and its complications. In contrast, the results of the majority of the prospective randomised controlled antioxidant clinical trials have failed to demonstrate a significant benefit, in the prevention of cardiovascular events, of antioxidant supplementation (vitamins C, E and  $\beta$ -carotene) in primary patients without clinical evidence of CVD, or in secondary patients with clinical evidence of CVD.

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