Chapter 2

Oxidative Stress and Diabetic Complications: The Role of Antioxidant Vitamins and Flavonoids

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1. Introduction

Diabetes mellitus is a group of disorders of multiple aetiologies resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycemia (very high blood glucose levels) with disturbances in carbohydrate, fat and protein metabolism [1]. The two major types of diabetes mellitus (DM) are insulin dependent (IDDM) - type 1 and non -insulin dependent (NIDDM) -type 2. Type 1 DM is characterized by a specific destruction of the pancreatic β cells commonly associated with immune-mediated damage [2]. Individuals with type 2 DM display a gradual change in glucose homeostasis due to insulin resistance and/or decreased insulin secretion [3].

Sustained hyperglycemia leads to the progressive development of long-term microvascular and macrovascular complications which causes morbidity and mortality among those affected [4, 5]. Although glycemic control has long been the mainstay for preventing the progression of diabetic complications, there is far less evidence that these interventions reverse diabetic complications [6]. Also, limitations in intensive glycemic treatment such as difficulty in achieving and/or maintaining tight glycemic control [7], incidence of hypoglycemia and increased mortality [8, 9] suggest an urgent need for alternative and/or complementary therapies to this disorder.

Hyperglycemia-induced oxidative stress is now recognized as the driving force for the development of diabetic complications [10]. Oxidative stress in diabetes results in stimulation of the polyol pathway, formation of advanced glycation end products (AGE), activation of

protein kinase C (PKC) and subsequent formation of reactive oxygen radicals [11, 12]. Hyperglycemia, not only generates more reactive oxygen species (ROS), but also attenuates antioxidative mechanisms by scavenging enzymes and substances [13].

2. The complications of diabetes mellitus (DM)

The injurious effects of hyperglycemia are separated into microvascular (involving small vessels such as capillaries) and macrovascular complications (involving large vessels, such as arteries and veins). Microvascular complications include diabetic nephropathy, neuropathy and retinopathy while macrovascular complications include coronary artery disease, peripheral arterial disease and stroke [5].

Diabetic nephropathy is a major cause of end-stage renal disease worldwide. It is a progressive decline in the glomerular filtration rate, characterized by glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial expansion with the accumulation of extracellular matrix proteins (ECM) [14]. Alteration of the permeability characteristics of the glomerular capillary wall manifests clinically as abnormal albuminuria [15]. Microalbuminuria progresses to end-stage renal disease through a number of stages including normoalbuminuria, microalbuminuria and macroalbuminuria [16].

Diabetic retinopathy results from the damage of the small vasculature of the retina, multi cellular and the light sensitive tissue at the back of the eye. It is a major cause of visual impairment worldwide [17, 18]. The retina capillaries are lined with endothelial cells responsible for maintaining the blood retinal barrier, and are surrounded by smooth muscle cells, pericytes, which provide tone to the vessels [18]. The vascular lesions that are identified at the early stage of diabetic retinopathy include pericytes disappearance from capillaries resulting in pericyte ghosts, obliteration of capillaries and small arterioles, gradual thickening of vascular basement membrane, increased permeability of endothelial cells, and formation of microaneurysms (i.e. weakening of vessel walls that results in the projection of a balloonlike sac), vessel leakage, exudate, and hemorrhage [19, 20].

Neuropathies are characterized by a progressive loss of nerve fiber function. A widely accepted definition of diabetic neuropathy is “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with mellitus after exclusion of other causes” [21]. In the peripheral nervous system, diabetes causes a progressive deterioration of sensory nerves and damage to motor nerves [22]. Diabetic neuropathy is ultimately the leading cause of lower extremity amputation [23]. Peripheral neuropathy is thought to develop because of cellular damage to endothelial cells, affecting nerve blood flow and also damage to the neurons affecting conductivity of impulses [23]. Signs and symptoms of diabetic neuropathy include decrease or no sweating, numbness, or tingling, and some sort of burning sensation, weakness and loss of reflexes [24].

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Both type I and type II diabetes are powerful and independent risk factors for coronary artery disease (CAD), stroke, and peripheral arterial disease [25, 26, 27]. Diabetics have a 2- to 4-fold higher risk for cardiovascular events [28] and nearly 80% of diabetes-associated deaths are caused by cardiovascular disease (CVD) [29]. Atherosclerosis, (excessive accumulation of lipids, cholesterol, inflammatory cells, and connective tissue in the vessel wall) accounts for more than 80% of the CVD-associated death and disability [30, 31]. Formation of atherosclerotic plaques can result in occlusion of vessel lumen and a rapid cessation in blood flow to target tissue [32]. Hyperglycemia, increased free fatty acids, and insulin resistance induce a large number of alterations at the cellular level that contribute to vascular dysfunction and accelerate the atherosclerotic process. These include increased oxidative stress, decreased bioavailability of NO, disturbances of intracellular signal transduction and increased production of several prothrombotic factors [32, 33].

3. Role of oxidative stress in diabetic complications

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants. Biological free radicals are highly unstable molecules which are products of normal cellular metabolism. They have electrons available to react with various organic substrates such as lipids, proteins and deoxyribonucleic acid (DNA). Free radicals are well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems [34]. At low or moderate levels free radicals (ROS and RNS) exerts beneficial effects such as defence against infectious agents, induction of a mitogenic response and the maturation process of cellular structures [35-37]. ROS include superoxide anion ($O_2^{\cdot -}$), hydroxyl ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) while RNS include nitric oxide ($^{\cdot}NO$), nitrogen dioxide ($NO_2^{\cdot -}$) and peroxynitrite ($OONO^-$) [38, 39]. High concentrations of free radicals on the other hand result in deleterious processes that can damage cell structures due to oxidative stress [40, 41].

Free radicals produced under physiological conditions are maintained at steady state levels by endogenous or exogenous antioxidants (externally supplied through foods or supplements) which act as free radical scavengers. However, oxidative stress occurs when the production of free radicals overwhelms the detoxification capacity of cellular antioxidant system causing biological damage [42-44]. The endogenous antioxidants (Table 1) comprise of the enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and non-enzymatic antioxidants including glutathione (GSH), α lipoic acid, vitamins C and E [39, 45, 46]. On the other hand, the exogenous antioxidants include micronutrients and other exogenously administered compounds such as vitamin E, vitamin C, trace metals (selenium, manganese, zinc), carotenoids and flavonoids [39, 44, 47].

Antioxidants	Cellular location	Role	Reference
Enzymatic Antioxidants			
(A) Catalase	Peroxisomes	Decomposition of $H_2O_2$to water and oxygen	[48]
(B) Glutathione peroxidase	Cytoplasm, mitochondria, and nucleus	Detoxifies H_2O_2 and lipid peroxides with simultaneous oxidation of GSH and generation of GSSG	[49]
(C) Glutathione reductase	Cytoplasm, mitochondria, and nucleus	Recycles Glutathione disulfide back to glutathione using the cofactor NADPH	[50]
(D) Superoxide dismutase	Cytoplasm, nucleus lysosomes, mitochondria	Conversion of superoxide radical to H_2O_2	[51]
Non enzymatic antioxidants			
(A) GSH	Cytoplasm, mitochondria and nucleus	Acts as a cofactor for antioxidant enzymes (GPx, GST), regenerates other antioxidants such as Vitamins C and E to their active forms	[52]
(B) Vitamin-E	Membrane	Directly scavenge singlet oxygen, peroxyl and superoxide radicals , protects against peroxidation of membrane lipids	[34]
(C) Vitamin-C	Cytosol	Acts synergistically with vitamin E to terminate radical inducedlipid peroxidation	[34, 53]
(D) α-Lipoic acid	Cell membrane and cytoplasm	Increases glutathione and vitamin C levels	[54]

Table 1. Role of antioxidants in the protection against free radical damage

Numerous experimental evidences have highlighted a direct link between oxidative stress and diabetes through the measurement of oxidative stress biomarkers in both diabetic patient and rodents. As shown in Table 2, a hyperglycemic state can lead to an increase in the levels of oxidative DNA damage markers such as 8-hydroxy-2’-deoxyguanosine (8-OHdG) and 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG); lipid-peroxidation products measured as thiobarbituric acid-reactive substances (TBARS); protein oxidation products such as nitrotyrosine and carbonyl levels and also lower the activity of antioxidant enzymes. Cell culture studies using pancreatic beta cells, aortic smooth muscle cells and endothelial cells have also provided evidence for an increase in ROS production in diabetes [55, 56].

Due to their ability to directly oxidize and damage DNA, proteins, and lipids, free radicals are believed to play a key role in the onset and progression of late-diabetic complications [57]. In the absence of an appropriate condensation by antioxidant defense network, increased oxidative stress leads to activation of stress-sensitive intracellular signaling pathways and the formation of gene products that cause cellular damage and contribute to late diabetic complications [58-61].

	Antioxidants and macromolecules	Evidence of oxidative stress	Target tissue/organ	References
Animals	Enzymatic antioxidants	↓ SOD,CAT,GR, GPX	Liver, Pancreas, Liver, kidney	[62-64]
	Non-enzymatic antioxidant	↓ Vit E and C	Liver, kidney	[64]
		↓ GSH /GSSG,GSH	Kidney, hippocampus. Retina, Heart	[65] [66] [67] [68]
	Lipids	↑ TBARS, lipid peroxides, MDA	Kidney	[69-70]
	DNA	↑ 8-OHdG, 8-OHG	Plasma, Liver, Kidney	[71-72]
	Protein	↑ Nitrotyrosine	kidney Retina	[73] [67]
	Reactive oxygen species	↑ ROS	Hippocampus	[66]
Humans	Enzymatic antioxidants	↑ SOD,CAT, GPX	Erythrocyte	[74]
	Non enzymatic antioxidants	↓ GSH	Erythrocyte	[75]
	Lipid	↑ F2-Isoprostanes ↑ MDA	Urine Erythrocyte	[76] [77]
	DNA	↑ 8-OHdG	Urine	[78-79]
	Protein	↑ Nitrotyrosine Protein carbonyl	Plasma	[80, 81]

Table 2. Experimental evidence supporting the involvement of oxidative stress

4. Pathways of free radical generation in diabetes mellitus and its associated complications

In diabetes, ROS is thought to be generated through increased polyol pathway [82], increased formation of advanced-glycation end products (AGEs) [83] and protein kinase C (PKC) activation [84].

4.1. Aldose reductase pathway and ROS generation

Aldose reductase is the rate limiting enzyme of the polyol pathway. The nicotinamide adenine dinucleotide phosphate (NAD(P)H)-requiring aldose reductase, catalyses the reduction of glucose to sorbitol followed by the oxidation of sorbitol to fructose by NAD+ dependent sorbitol dehydrogenase. At normal blood glucose concentration (5.5 mM), aldose reductase catalyzed reaction represents less than 3% of total glucose utilization [85]. However, hyperglycemia results in saturation of hexokinase and more than 30% of glucose is directed into the polyol pathway [86]. In a diabetic state, polyol pathway increases in tissues that do not require insulin for cellular glucose uptake, such as retina, kidney, peripheral nerves and blood vessels [87].

The overall reaction of the polyol pathway leads to a shortage of intracellular NAD(P)H and a surplus of NADH, i.e, a reductive imbalance. Increased NADH generation during conversion of sorbitol to fructose provides substrate for NADH oxidase to generate ROS [88]. NADH serves as a source of electrons in complex 1 of the electron transport chain resulting in increased mitochondrial generation of superoxide radical. In diabetic cells, oxidative phosphorylation in mitochondria is enhanced due to increase flux of electron donors into the electron transport chain. This drives the inner mitochondrial membrane potential upward causing blockage of electron transfer inside complex III [89]. Electrons back up to coenzyme Q results and electrons are transferred one at a time to molecular oxygen, generating superoxide. DNA damage by superoxide and peroxynitrite results in the activation of poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme. PARP reduces the activity of glyceraldehyde-3- phosphate dehydrogenase (GAPDH) (an enzyme of the glycolytic pathway which catalyses the conversion of glyceraldehydes -3 phosphate to 1, 3 biphosphoglycerate) by ADP- ribosylation [90, 91]. A consequence of GAPDH inhibition by PARP is an increase in triose phosphate pool, upstream of GAPDH and increase flux of intermediates into the damaging pathways of diabetic complications.

The polyol pathway also results in reduction in the bioavailability of NAD(P)H. The reduced bioavailability of NAD(P)H negatively affects the antioxidant defence system by depleting glutathione (GSH) a very important antioxidant. This is because the activity of GSH reductase, an antioxidant enzyme that generates GSH from its oxidized form (GSSH) depends on NAD(P)H. Depletion of NAD(P)H also decreases the synthesis of nitric oxide (NO), a vaculoprotective agent. NAD(P)H serves as a cofactor for nitric oxide synthase (NOS) which synthesizes NO from L-arginine. If endothelial nitric oxide synthase (eNOS) lack its substrate, L-arginine or one of its co-factor, it may produce superoxide radical ($\cdot O_2^-$) instead of NO and this is referred to as ''uncoupled state of nitric oxide''[92]. Nitric oxide performs several physiological roles such as inhibition of platelet activation, vascular relaxation [93] and acts as an anti-inflammatory agent by reducing platelet aggregation and adhesion [94]. These properties inhibit atherogenesis and protect the blood vessel. Reduced bioavailability of NO level will therefore increase inflammation, enhance thrombosis and disrupt the integrity of endothelial cells. Reduction in NO has been documented in diabetes subjects with nephropathy [95]. Superoxide anion directly quenches NO by forming highly reactive peroxynitrite ($ONOO^-$) which initiates lipid peroxidation, oxidizes sulfhydryl group in protein and nitrates amino acids such as tyrosine, thereby affecting many signal transduction pathways. The polyol pathway serves as a main source of ROS generation in the retina [96]. In addition, sorbitol accumulation has been implicated in osmotic swelling of the eye lens and cataractogenesis [97].

4.2. Advanced glycation end product (AGEs) formation and ROS generation in diabetic complications

Glucose can react spontaneously with free amino groups of protein to form Schiff bases. These Schiff bases through complex reactions such as amadori rearrangement, dehydration and condensation forms cross-linked heterogeneous fluorescent derivatives called advanced glycation end products (AGEs). Advanced glycation end products constitute a heterogeneous

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group of molecules formed by non-enzymatic reactions of reducing sugars, ascorbate and other carbohydrates with amino acids, lipids and nucleic acids [98, 99]. Glycation end product's adducts such as pyraline, pentosidine and N- Carboxy- methyl lysine (CML) are found to be elevated in diabetic tissues [100 - 102].

Once formed, AGEs can cause tissue damage by two main pathways which are: (1) formation of cross links that alter protein structure and function and, (2) interaction of AGE with AGE-cell surface receptors on the surfaces of various cells such as endothelial cells, macrophages, neurons, and smooth-muscle cells resulting in activation of cell signaling and gene expression that induces oxidative stress and inflammation [98, 99; 102-105]. Oxidative stress can accelerate AGE formation while AGE formation can also amplify the production of more ROS resulting in a vicious cycle of AGE formation and oxidative stress.

AGE's mediate some of their effect via interaction with some receptors that have been shown to bind to these chemical moieties. Among these receptors, Receptor for Advanced Glycation End products (RAGE) is the most extensively studied [106]. Evidence from numerous studies suggest that AGE's are involved in a vicious cycle of inflammation, generation of ROS and increased production of AGE's. Ligand RAGE interaction results in activation of pathways such as p21ras, erk1/2 (p44/p42), MAP kinases, p38 and SAPK/JNK MAP kinases [107-109]. A consequence of the activation of these pathways is the nuclear translocation of transcription factor, Nuclear Factor Kappa B (NF-KB). Translocation of NF-KB to the nucleus increases the transcription of a number of proteins such as, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, 1L-18 and tumour necrosis factor (TNF)-α which are centrally involved in the endothelial recruitment of neutrophil and subsequent development or progression of atherosclerotic plaque [109-112].

The gene regions of NF-KB are located at the promoter region of RAGE. Moreover, binding of NF-KB to the promoter region of RAGE results in up-regulation of RAGE itsel. Interaction of AGE with RAGE generates more oxidative stress and this further potentiates the formation of AGE's [109, 113]. Generation of ROS by ligand stimulated RAGE activation is mediated at least in part via activation of NADPH oxidase [114]. Other mechanisms by which AGE's may be linked to increased generation of ROS is by reducing the activities of enzymatic antioxidant such as SOD and CAT, lowering of glutathione stores, and activation of PKC [107, 115, 116].

Increased renal AGE in diabetic animals and patients have been linked to structural abnormality observed in diabetic nephropathy such as mesangial expansion, glomerular basement membrane thickening and tubulointerstitial fibrosis [117]. Advanced Glycation End Product's level is increased with decreased renal function in type 1 diabetic patients [118]. Evidence from clinical studies indicates a correlation between progression of diabetic retinopathy and the level of AGE in serum and retinal blood vessels of diabetic patients [100, 119]. In diabetes, increased AGE's are observed within retinal capillary cells and causes pericyte loss in diabetic retinopathy [120]. AGE's induce toxic effects on retinal pericytes by causing oxidative stress and subsequent apoptosis [121].

High levels of serum AGE's have been documented in patients with type 2 diabetes mellitus and coronary heart disease [122]. Glycation increases susceptibility of low density lipoprotein (LDL) to oxidative modification which is considered a critical step in its atherogenicity [123]. Glycation end products can also enhance atherosclerosis by trapping LDL in the subendothelium and decrease the recognition of AGE-modified LDL by LDL receptor [124]. Modification of LDL and its increased localization in vessels increases foam cell production and accelerates atherosclerosis development [125]. Oxidative stress induces AGE's formation on collagen leading to cross-linking which is considered to play a role in diabetic cardiomyopathy [126]. The intermolecular collagen cross-linking caused by AGE increases vascular stiffness and interferes with arterial blood flow [127, 128] and this partly explains the diastolic dysfunction and systolic hypertension seen in diabetic subjects.

4.3. Protein kinase C (PKC) activation and ROS generation in diabetic complications

PKC activation is related to vasoconstriction, proliferation and overgrowth of smooth muscle cells as well as accelerated synthesis of extracellular matrix proteins, and thus plays significant roles in the onset and progression of vascular cell dysfunction in diabetes mellitus [129-131]. Two major pathways have been implicated in the activation of PKC in hyperglycemia. Persistent and excessive activation of several PKC isoforms result primarily from enhanced *de novo* synthesis of diacylglycerol (DAG) from glucose via increase in triose phosphate availability [90, 105, 132, 133]. There is also evidence that the interaction between AGE's and their cell-surface receptors can result in enhanced activity of PKC isoforms [134, 135].

PKC likely regulates diabetic complications on multiple levels such as activation of eNOS, NAD(P)H oxidase, phospholipase A_2 (PLA_2), endothelin-1 (ET-1), Vascular endothelial growth factor (VEGF), Transforming growth factor-β (TGF-β), and by activating NF-KB. Diacylglycerol activated PKC alters the gene expression of key proteins leading to decrease blood flow, capillary occlusion, inflammation, free radicals generation and damage to cellular macromolecule [130-132, 136, 137].

High glucose levels can stimulate ROS production via a PKC-dependent activation of NAD(P)H oxidase in cultured aortic endothelial cells, smooth muscle cells, and renal mesangial cells [84]. Nicotinamide adenine dinucleotide phosphate oxidase, which is primarily found in phagocytic cells, is the main source of ROS in non-phagocytic cells such as mesangial cells, endothelial cells [138], fibroblasts [139], podocytes [140] and smooth muscle cells [141]. The expression of NAD(P)H oxidase components is up-regulated in vascular tissues from animal models of diabetes and in patients with diabetes and coronary artery disease [142-144]. Experimental evidence indicates that NAD(P)H oxidase-dependent production of ROS may cause DNA damage in diabetic renal tissues leading to the development of nephropathy [145]. Increased activity of the NAD(P)H oxidase has also been reported in the retina of diabetic rats suggesting its involvement in the development of diabetic retinopathy [146].

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5. Antioxidant as therapeutic agents in the management of diabetes mellitus

Despite efforts to control blood glucose, tissue and organ damage are cumulative over many years in most diabetic patients. Varying degrees of hyperglycemia are virtually unavoidable in subjects with diabetes mellitus and glycemic memory has been used to describe the development of diabetes-related complications in diabetic patients even after normoglycemia has been restored and initial glycemic environment is remembered in the target organs [105,147]. It is noteworthy that ROS has been implicated as a major cause of the metabolic memory after glucose normalization due to the chains of reactions leading to cell damage and loss of cellular function. Due to the implication of hyperglycemia-induced oxidative stress in diabetes, these patients should in theory benefit from antioxidant supplementation. The beneficial effect of antioxidants has been reported in animal models of diabetes and in diabetic patients [50, 148]

6. Vitamins

Vitamin E is a fat-soluble vitamin. It has been shown that plasma α-tocopherol concentrations are lower in diabetics compared to controls [58] and appear to be even lower in diabetics with complications such as microangiopathy than in diabetics without complications [81]. Administration of Vitamin E has proven to be beneficial in preventing cellular damage by inhibition of lipid peroxidation, protein oxidation, protein glycations and platelet aggregation [149-151] Vitamin E supplementation for two weeks (600 mg/day) lowered urinary F2-isoprostanes (a marker of lipid oxidation) in type 2 diabetics [152]. It was shown in a study that a decrease in plasma F2-isoprostanes was seen in type 2 diabetic patients after six weeks supplementation with Vitamin E [153].

Oxidative stress in the kidney of diabetics is usually associated with tissue damage that interferes with proper organ function, causing an increase in urinary protein excretion and blood urea nitrogen (BUN) [154]. Vitamin E supplementation (1000 IU/kg diet) to diabetic rats for 4 weeks significantly reduced urinary protein excretion and BUN suggesting a beneficial effect on kidney function [154]. Inhibitory effect of Vitamin E on glycation of hemoglobin in type I and type 2 diabetic rats has been documented [151, 155]. The ability of vitamin E to inhibit AGE's might be due to its antioxidant effect on the autoxidative pathways of AGE formation [156]. Vitamin E administration has also reduced oxidation of low density lipoprotein (LDL) and development of atherosclerosis [157].

Numerous studies have shown that vitamin E normalized parameters of oxidative stress and inhibited vascular abnormalities caused by hyperglycemia-induced production of DAG and PKC activation in the retina, glomerulus and macrophages [158-160]. Supplementation with vitamin E reduced basement membrane thickening in diabetic rat retina and reduced vascular endothelial growth factor (VEGF) and aldose reductase activity, the abnormalities associated with diabetic retinopathy [161]. Dietary supplementation of vitamin E (2000 IU/kg) to diabetic

rats for 8 weeks had cardioprotective effects which was simultaneously associated with an ability of vitamin E to blunt diabetes-induced amplification of myocardial 8-*iso* PGF2 and oxidized GSSG formation [162]. Clinical trials with vitamin E provided evidence that vitamin E may improve cardiovascular function [163, 164]. However, most large studies with vitamin E have not yielded positive benefits for decreasing the development or progression of diabetic microvascular and cardiovascular pathologies or mortality [165, 166].

Vitamin C is an antioxidant vitamin which plays an important role in protecting free radical-induced damage and a decrease in basal vitamin C levels has been documented in type 2 DM. Treatment of diabetic rats with vitamin C significantly decreased renal malondialdehyde, albuminuria, proteinuria, glomerular and tubulointerstitial sclerosis, suggesting the role of vitamin C in suppressing the progression of renal injury in diabetic rats [167]. Vitamin C also improved diabetes-induced endothelial dysfunction in a rat model by enhancing NO bioavailability [168].

The beneficial effects of vitamin C supplementation in humans are controversial. A study reported that vitamin C may improve glycemic control, lowering both fasting blood glucose and glycated haemoglobin (HbA1c) [169]. Chronic oral administration of vitamin C to patients with type 2 diabetes causes a decline in plasma free radicals that is associated with improved whole body glucose disposal [170,171] and improved endothelial function [172]. Recently, another study reported a reduction in the malondialdehyde (MDA) level, a major product of oxidative damage in both fasting and postprandial states of type 2 diabetic patients after vitamin C (1000 mg day^{-1}) supplementation for 6 weeks although no effect was observed on lipid profiles [173]. Some studies have indicated that the intra-arterial infusion of vitamin c restores endothelium-dependent vasodilation in patients with type 1 or type 2 diabetes [174, 175] suggesting that hyperglycemia-induced oxidative stress mediates endothelial dysfunction in diabetic patients.

However, in contrast to these promising results, other studies showed no beneficial effect with vitamin C treatment. Chen and colleagues [176] concluded that a high oral dose of vitamin C therapy was ineffective at improving endothelial dysfunction and insulin resistance in type 2 DM. It is important to note that complete replenishment of vitamin C levels was not achieved in the subjects. This is crucial since high concentrations of vitamin C (>80 μM) has been documented as a requirement for the preservation of NO-dependent endothelial function as vitamin C only competes with NO for superoxide anion at these high concentrations [177-178]. Also, in another study, no beneficial effects of oral vitamin C supplementation (1.5 g daily for 3 weeks) was observed on blood pressure, oxidative stress, and endothelial function in type 2 diabetes [179].

7. Flavonoids

Flavonoids (bioflavonoids) are a diverse group of polyphenols (phenyl benzopyrans) which function as phytochemicals [180]. Flavonoids are well-known for their multi-directional biological activities including anti-diabetic efficacy. Experimental evidence has shown that

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flavonoids exhibits anti-inflammatory [181], anticarcinogenic [182], antiviral [183] and antiallergic properties. These effects are generally associated with free radical scavenging activity of flavonoids. The antioxidant effects of flavonoids are enhanced by the number and position of hydroxyl groups in the molecule. The catechol structure, presence of unsaturation and 4-oxo function in the C-ring also contributes to their radical scavenging activity [184, 185]. Flavonoids may be capable of binding the transition metal ions, which play a role in glycoxidation, thus preventing metal-catalysed formation of hydroxyl radicals or related species from H_2O_2 [186].

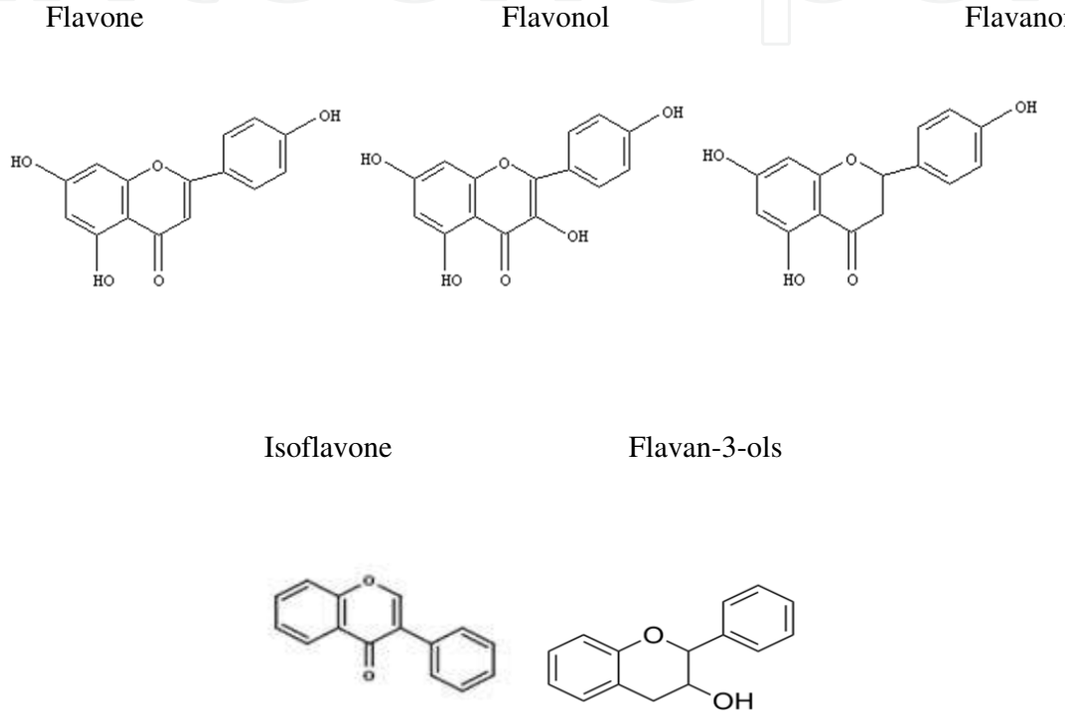

Figure 1. Classes of flavonoids [187]

The potential beneficial effects of flavonoids in the prevention of diabetes mellitus and its associated complications have been investigated both *in vitro* and *in vivo* studies (Table 3). The inhibitory effect of flavonoids on glycation has been demonstrated and it is suggested that this effect is partly due to their antioxidant properties [188]. Epigallocatechin (EGC) has a beneficial effect in a rat model of diabetic nephropathy via suppressing hyperglycemia, proteinuria and lipid peroxidation. EGC also reduced renal accumulation of AGE's and their related oxidative stress [189]. Another study demonstrated the *in vitro* inhibitory effect of different flavonoids on pentosidine formation in collagen in the presence of glucose (250 mmol/L). The decreasing inhibitiory activity was observed from myricetin, quercetin, rutin, catechin and kaempferol in a structure and concentration dependent manner [190]. Kim and colleagues [191] also investigated the effect of quercetin, isoquercitrin, hyperin and cacticin on formation of AGE's *in vitro*. At a concentration of 50 mM, the percentages of inhibition were 1.0, 89.6, 92.0 and 40.5, respectively. The inhibitory effect of hyperin on AGE formation was 6.5 times higher than that

of aminoguanidine, a known AGE inhibitor which showed 14.1% inhibition at a concentration of 50 μM.

Flavonoids, in addition to their antioxidant effect, possess inhibitory activity on aldose reductase pathway and can serve as a potential multifunctional agent in the prevention of diabetic retinopathy. Goodarzi *et al.* [192] showed that oral administration of quercetin and the flavanone, naringin to streptozotocin-induced diabetic rats significantly reduced aldose reductase activity in the lenses compared to control. Oral administration of two isoflavone compounds, tectorigenin and irigenin also inhibited sorbitol accumulation in the lenses of streptozotocin induced diabetic rats [193].

Activation of PKC contributes to the loss of capillary pericytes and thickening of vascular basement membrane (BM) in diabetic retinopathy [194]. Also PKC mediated alterations in vascular permeability, blood flow, formation and response to angiogenic growth factors contribute to retinal leakage, ischemia, and neovascularisation [195]. Therefore, PKC inhibitors can be targeted for the treatment of diabetic retinopathy. Hesperetin (Hsp), a flavanone found in citrus fruits and a potent antioxidant has retina vasculo-protective properties due to its strong anti-angiogenic effect via inhibiting VEGF and PKC-β pathways [196]. Modulation of endogenous biomarkers and inhibition of diabetes induced neuropathic pain was observed in diabetic rats after naringin (4′,5,7-trihydroxy flavonone 7-rhamnoglucoside) administration [197]. In the same study, a dose dependent decrease in the levels of oxidative-nitrosative stress, inflammatory mediators as well as apoptosis was documented in neural cells. The antioxidant properties of naringin may be a factor in the inhibition of neurodegeneration.

The soy isoflavone genistein (3 and 6mg/kg), administered by a subcutaneous injection to diabetic mice relieved peripheral painful neuropathy by reverting the proinflammatory cytokine and ROS overproduction. It also restored the inducible nitric oxide synthase (iNOS) and eNOS content and increased NO production in thoracic aorta although treatment had no effect on hyperglycemia [198]. The flavonoid luteolin (200 mg/kg), when administered to rats orally, protected against the progression of diabetes-induced cardiac dysfunction by attenuation of myocardial oxidative stress probably through its antioxidant properties [199].

In a double blind placebo-controlled study, the effects of daflon 500 (made up of flavonoids diosmin (90%) and hesperidin (10%)) was investigated in a group of 28 type 1 diabetic patients. Treatment with these flavonoids resulted in a decrease in HbA_{1C} which is associated with an increase in the level and activities of thiol-containing antioxidants such as glutathione peroxidase [200]. The *in vitro* protective effect of myricetin on protein oxidation and membrane lipid peroxidation of erythrocytes from diabetic patients was reported in a study by Pandey and co-workers [201].

The treatment of diabetic rats with rutin decreased fasting plasma glucose, glycosylated haemoglobin, thiobarbituric acid reactive substances and lipid hydroperoxides while levels of non-enzymatic antioxidants were increased [202]. In another study, rutin supplementation (500 mg tablets) to diabetic patients for 60 days decreased the levels of fasting blood glucose, blood pressure and improved lipid profiles in the diabetic subjects [203]. Rutin reduced blood glucose, ameliorated oxidative stress and inhibited the accumulation of extracellular matrix

(ECM) component and glomerular basement membrane thickening in the renal cortex of diabetic rats suggesting its renoprotective effect in experimental diabetic nephropathy [204]. The inhibitory effect of rutin on AGE formation in STZ-induced rats has also been shown [205].

Diosmin (DS) (diosmetin 7-*O*-rutinoside) is a natural flavone glycoside which can be obtained by dehydrogenation of the corresponding flavanone glycoside, hesperidin that is abundant in the pericarp of various citrus fruits [206]. Diosmin treatment of streptozotocin-nicotinamide induced diabetic rats, ameliorated oxidative stress in plasma and tissues as evidenced by improved glycemic and antioxidant status along with decreased lipid peroxidation [207]. Experimental evidence showed the potential of rutin, a flavonol to delay glomerulosclerosis of diabetic nephropathy (DN) due to its ability to inhibit cell hypertrophy and the accumulation of ECM mediated by TGF-β1/Smads and ROS signals in mesangial cells cultured by high glucose [208] Quercetin enhances endothelium-derived NO bioavailability, reduced blood glucose levels and oxidative stress in diabetic rats suggesting its beneficial effect in vascular function [209].

Classes of Flavonoid and Food sources	Selected examples	Target organ, tissue or cells	Mechanism of action	Reference
Flavonols (Brussel sprouts, apples, onion, curly kale, leek, beans, cherries, Citrus fruits, Cranberries)	Morin	Liver	Decreased MDA levels , Increased activity of SOD and GSH concentration.	[210]
		Hepatocytes	Decreased ROS production, DNA damage and apoptosis. Modulation of antioxidant enzymes; GSH, CAT, SOD and GPX.	[211]
	Rutin	Kidney, serum, urine	Lowered blood glucose and improved renal function. Increased total antioxidant capability activities of SOD, CAT and GPX. Lowered ECM accumulation and AGE formation. Decreased renal expression of TGF-β.	[204]
	Quercetin	Pancreas, serum, erythrocyte.	Lowered MDA and NO level, increased antioxidant enzyme activity and preservation of islet cells integrity.	[212]
		Kidney , serum, urine	Lowered blood glucose and improved renal function. Reduced renal lipid peroxides and increased activity of anti-oxidative enzymes; SOD and CAT and non-enzymatic antioxidant GSH.	[213]

Classes of Flavonoid and Food sources	Selected examples	Target organ, tissue or cells	Mechanism of action	Reference
Flavanones (Citus peel, Orange juice, grape fruit juice, lemon juice)	Hesperidin & Naringin	Liver, serum	Boost antioxidant system by increasing activities of SOD, GR, GPx, CAT and levels of non-enzymatic antioxidants; GSH, VIT-C and VIT-E. Decreased lipid peroxidation product, MDA and proinflammatory markers, TNF-α, IL-6.	[214]
	Hesperidin	Retina, plasma	Decreased aldose reductase activity and levels of AGE's, VEGF, ICAM-1, TNF-α, IL-1β and MDA while increasing SOD activity.	[215]
	Naringenin	Pancreas, heamoglobin, serum, plasma	Lowered fasting blood glucose, decreased hyperglycemia, glycated haemoglobin, MDA and markers of hepatic damage. Increased levels of insulin and enzymatic and non-enzymatic antioxidants.	[216]
		Kidney, liver serum, urine,	Improved glycemic control and elevated insulin level, reduced plasma levels of kidney dysfunction markers. Lowered renal activity and expression of NF-KB and pro-inflammatory cytokine and chemokine, suppression of PKC activity.	[217]
Flavanolols (Milk thistle, red onion, Siberian larch tree)	Silymarin	Kidney	Increased expression and activity of SOD, GPX, CAT. Decreased high blood glucose	[218]
Flavones (Parsley, pepper celery, broccoli capsicum)	Luteolin	Kidney	Decreased activity of SOD, MDA content and expression of Heme Oxygenase-1 (HO-1) protein.	[219]
		Aortic ring	Aortic Vasorelaxation, decrease in ROS production, increased activity of SOD, NOS and level of NO.	[220]
	Diosmin	Liver and kidney	Decreased TBARS and hydroperoxides. Increased activity of enzymatic antioxidants ;SOD, CAT, GPx, GST and GR and non-enzymatic antioxidants; GSH, Vitamin C and Vitamin E.	[207]

Classes of Flavonoid and Food sources	Selected examples	Target organ, tissue or cells	Mechanism of action	Reference
Flavones	Chrysin and luteolin	Serum and aorta	Aortic relaxation, decreased blood pressure, decreased lipidemia and serum AGE's. Increased NO generation	[221]
	Apigenin	Serum and liver	Increased insulin levels and decreased hyperglycemia. Normalized LPO and endogeneous antioxidants, CAT, SOD, GSH in the liver.	[222]
Flavan-3-ols (Red wine and red grapes, green and black tea)	Catechin	Thoracic aorta	Decreased hyperglycemia NADPH oxidase activity and ROS production. Increased insulin level, lowered blood pressure and improved aortic relaxation.	[223]
	Epicatechin	Pancreatic Islets, plasma, haemoglobin	Increased anti-inflammatory cytokines, IL-10, IL-12. Improved glucose tolerance and insulin levels and lowered HbA1C.	[224]
Isoflavones (Soy foods and legumes)	Genistein	Kidney	Decreased MDA level and expression of PKC and pro-inflammatory proteins such as NF-KB, MCP-1 and Cox-2. Activation of antioxidant enzymes and defense against oxidative damage via increase expression of Nrf2, a transactivator of antioxidant genes.	[225]
	Daidzein	Aorta	Maintenance of endothelium dependent relaxation and attenuation of oxidative stress via decrease MDA levels and increase SOD activity	[226]

Abbreviations: Find all citations in this journal (defECM: Extracellular matrix, MDA: Malondialdehyde, NO: Nitric oxide, NOS: Nitric oxide synthase, NADPH; nicotinamide adenine dinucleotide phosphate, LPO: Lipid peroxidation, Nrf2: NF-E2-related factor-2, Cox-2: Cyclooxygenase-2, TAOC: Total antioxidative capability

Table 3. Beneficial effects of some flavonoids in diabetes mellitus

The inherent antioxidative properties of some common antidiabetic drugs such as aminoguanidine, statins, thiazolidinediones, glibenclamide and repaglinide also provides an additional support to the involvement of oxidative stress in diabetes and therefore suggest that the use of antioxidants as therapeutic agents in diabetes is a promising approach [227-231].

8. Conclusion and future perspective

Increased ROS production has been suggested as a common pathway linking diverse pathogenic mechanisms of diabetic vascular complications. There are numerous evidences from animal studies on the beneficial effect of antioxidant vitamins supplementation in diabetes mellitus, but results from clinical studies are inconclusive. The antioxidant activity of some anti-diabetic drugs has also been shown to contribute significantly to their therapeutic effect. Biflavonoids as antioxidants are promising and attractive natural substances to enrich the current therapy options against diabetes. The overall positive results from animal studies suggest that the role of antioxidants cannot be underestimated in the quest to find effective therapies for diabetic complications. A multi-therapeutic approach to the treatment of diabetic complications might increase the chance of successful therapeutic intervention. In addition to maintaining glycemic control, blockage of pathways involved in the formation of free radicals with antioxidants is a promising approach to the treatment of hyperglycemia-mediated complications in humans. The bioactivity of flavonoids *in vivo* can be greatly influenced by metabolism and bioavailability and this information is limited in many studies. Limited clinical studies have been carried out to support the beneficial effects of flavonoids in the prevention of diabetic complications. In order to achieve the goal of using flavonoids in the management of diabetes mellitus, further investigation on the long and short-term effect of the ingestion of flavonoids in humans is warranted.

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