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Antioxidants and Diabetic Retinopathy

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Abstract The biochemical perturbations in diabetes mellitus (DM) create the conditions for the production of free radicals, the consequence of which is increased oxidative stress. Evidence has accrued over the past 2 decades that suggests that oxidative stress is an important pathogenetic factor in the development of diabetic retinopathy (DR). Experimental data show that the use of strategies that ameliorate oxidative stress can prevent and retard the development of DR in the animal model. Clinical observations also suggest that reducing oxidative stress may help to reverse pathological manifestations of DR. The present article constitutes an examination of the role of antioxidants in the management of DR and the current state of clinically relevant knowledge.

Keywords Oxidation · Antioxidants · Reactive oxygen species · Retinopathy · Diabetes

Introduction

Research over the past 2 decades has elucidated considerable detail on the mechanistic contribution of oxidative stress in the development of complications in diabetes mellitus (DM) [1, 2••]. Oxidative stress is a state in which the balance between oxidants and antioxidants is shifted in the favor of the former. Oxidation is an important part of normal cellular metabolism and is needed to maintain cell viability and survival. During oxidation, reactive oxygen species (ROS) are produced, which originate either as by-products of normal metabolism or through exposure to exogenous pro-oxidant stimuli such as cigarette smoke, UV, or ionizing radiation [3]. ROS, which include superoxide anion, hydrogen peroxide, and singlet oxygen, as well as reactive nitrogen species (RNS), are necessary for physiological processes such as vascular tone, cell adhesion, and immune function [3]. ROS are normally scavenged by endogenous anti oxidative systems [4]. However, if antioxidant defenses are insufficient, then damage of protein, lipids, and DNA can occur, resulting in altered function and ultimately apoptosis [4].

In DM, free radical load is disproportionately high and markers for oxidative stress are markedly increased [5]. These include lipid peroxidation, increased oxidized to reduced glutathione ratio, and a decreased nitrite/nitrate ratio [5]. The foregoing clearly raises the possibility that amelioration of oxidative stress would have a therapeutic effect in preventing or delaying onset of such complications, and experimental evidence now exists to support this view [6, 7].

Oxidative Status of the Retina in DM

Research has shown that DM results in marked biochemical perturbations and the production of ROS in the retina [8, 9]. In animal models of DM reduced levels of retinal antioxidants have been observed, such as superoxide dismutase, glutathione reductase and glutathione peroxidase as well as nonenzymatic antioxidants such as vitamins C and E, and beta-carotene. Levels of glutathione (GSH), a key scavenger of ROS, are also reduced in the retina of diabetic rats [10]. GSH is regenerated by NADPH. In diabetic animals, supplementation with curcumin improved endogenous antioxidant capacity and prevented formation of diabetes-related structural abnormalities [11]. GSH protects the thiols in structural proteins and enzymes and neutralizes free radicals as well as playing a role in the detoxification of xenobiotics by conjugation. Conversely some studies have demonstrated

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increased superoxide dismutase and anti-oxidant capacity associated with DR [12]. A possible explanation for these disparate findings could be that temporal variation in the retinal redox state exists in DR. As oxidative stress increases, anti-oxidant capacity is upregulated initially, but eventually there is depletion of these protective mechanisms. There may also be regional variation in the retinal redox state, explaining the focal nature of the pathology when it occurs.

The Role of Oxidative Stress in Hyperglycemia-Related Damage

The exact sequence events that follow hyperglycemia, the role of oxidative stress and the pathological steps leading to DR remain incompletely understood [13]. In DM, reactions such as autoxidation of sugars leads to glycoxidation and these products are often found in collagen, though this is thought to be of relevance merely as a biomarker for more extensive hyperglycemia-related oxidative damage [14]. Several pathways have been previously implicated in the pathogenesis of DR as their activity is increased in hyperglycemic conditions. Their relative importance in the development of DR is unclear. Three such pathways are formation of advanced glycation end-products (AGEs) protein kinase C (PKC) activation, and the polyol pathway. In the first, advanced glycation end-products (AGEs) which form through the Maillard reaction, result from non-enzymatic glycosylation of proteins. The glycated proteins may be structural, in the extracellular matrix, may be intracellular or may be plasma proteins. In the second pathway, PKC activation occurs as hyperglycemia is associated with increased de novo synthesis of diacylglycerol, which increases the activity of PKC, which in turn phosphorylates target proteins. The effects of PKC activation on microvasculature include increased permeability, increased flow, apoptosis and proliferation of endothelial cells, all changes seen in DR [10]. Third, the polyol pathway describes, in the context of DM, the reduction of glucose, using NADPH, by aldose reductase to sorbitol, followed by oxidation to fructose. However, in clinical studies, blockage of these pathways individually has not led to clinically applied novel therapies. Thus, a "unified theory" linking these observations has been suggested in that free radical overproduction by mitochondria activates all aforementioned pathways [15]. When glucose is metabolized through the tri-cyclic acid (TCA) cycle, electron donors are generated, the main one being NADH. NADH donates an electron to the mitochondrial electron transport chain, and ultimately ATP is produced. This metabolic process can be fine-tuned according to the need for ATP. In hyperglycemic conditions, more glucose passes through the TCA cycle. Furthermore hyperglycemia results in elevation of the voltage gradient across the mitochondrial membrane, and at a certain threshold gradient electron transfer is blocked [10]. The increased number of accumulating electrons is donated one by one to oxygen, creating superoxide. Superoxide causes damage as it is converted to other more reactive ROS, and by reducing activity of a key glycolytic enzyme, GADPH. It is the inhibition of GADPH through its modification by ROS, which is hypothesised to be the common factor, as reduced GADPH activity can explain increased AGE formation, increased PKC activity and increased polyol pathway activation [15].

The retina is particularly susceptible to oxidative stress given its high concentration of polyunsaturated fatty acids and its high levels of oxygen utilization. Furthermore retinal vascular endothelial cells do not alter the rate of glucose transport across the plasma membrane in conditions of high glucose, the consequence of which is intracellular hyperglycemia [16]. Thus, it is possible that these factors make the retina and its vasculature more susceptible to the effects of oxidative stress. Death of the retinal capillary pericytes and endothelial cells leads to capillary closure and retinal ischemia. These mechanisms are thought to underlie the drive to neovascularization which in turn leads to vitreous hemorrhage, retinal traction and detachment as well as macular ischemia, and ultimately visual loss. Vascular endothelial growth factor (VEGF) is thought to play a central role. VEGF inhibition by drug therapy is known to ameliorate macular edema in DR, and vitreous levels of VEGF are also correlated to the development of proliferative DR [12]. However, although VEGF is a survival factor for retinal vascular endothelial cells, apoptosis of these cells occurs in DR despite increased levels of VEGF. This may be partly because oxidative damage inactivates the VEGF cell-survival pathway [17]. Apoptosis of retinal pericytes, endothelial and neural cells also results from ROS through several other mechanisms. Damage to molecules essential to cell function and signal transduction, and alterations in gene expression may be important, but activation of caspases, a series of proteases, also occurs and this is pro-apoptotic. NF-*k*B, which can also be activated by oxidative stress, is another key mediator of apoptosis in the diabetic retina [10].

In an animal model, diabetic rats had higher vitreous levels of lipid peroxidation products and VEGF levels compared with nondiabetic controls, but the differences were attenuated by antioxidants. The findings illustrated the oxidative damage to lipids observed in DM. Indeed quantitative and qualitative abnormalities of lipid metabolism have also been shown to play a causative role in increasing oxidative stress with consequent damage to the microcirculation [18–20]. The results also suggested a causal relationship between oxidative stress and VEGF expression [21]. In a mouse model of DM, specific inhibition of NADPH oxidase normalized retinal VEGF levels, prevented retinal neovascularisation but also prevented superoxide formation [22]. Thus, NADPH oxidase

may be the bridge between hypoxia and VEGF production, and also contribute itself to an imbalance towards pro-oxidant stimuli.

In some animal models, good glycemic control after a period of hyperglycemia does not reverse pathological effects, an observation illustrating the concept of 'metabolic memory' [23]. Thus, ongoing damage is observed after the initiating hyperglycemic insult has passed, as already damaged molecules are not removed. Ongoing oxidative stress is an important theme in the development of DR, and already-generated ROS may underlie the metabolic memory phenomenon. This persistent pro-oxidant effect may be mediated by several pathways. Superoxide generated within the mitochondria cause breaks in mitochondrial DNA, and eventually electron transfer may become defective in normoglycemic conditions, resulting in promotion of AGE formation, PKC and polyol pathway activity [9]. Also ROS act as second messengers in VEGF induced endothelial migration, a key step in neovascularisation [24]. Oxidative stress also promotes inflammation in the retina, and animal models of DR show that NADPH oxidase activity is associated with inflammatory pathways that promote vascular adhesion and leakiness [25]. Furthermore the hyperglycemia-induced pathways described above all *initially* normalize once euglycemia is achieved, but the legacy of their activation, even if their overactivity has subsided, may be persistent oxidative stress. AGEs are increased in the diabetic retina and are themselves major source of oxidative stress [26]. In an animal model, AGEs resulted in increased VEGF mRNA levels in the retina, an effect ameliorated by antioxidants [27]. Glycated plasma proteins bind to receptors for AGEs on several cells including macrophages, which lead to generation of ROS [28]. Glycated low-density lipoproteins are thought to be more susceptible to oxidation [18]. Increased aldose reductase activity in the polyol pathway has been associated with reduced levels of NADPH, which is required as a cofactor to regenerate GSH [2••]. Persistent damage despite normalization of HbA1c levels may also be due to other factors, such as epigenetic changes affecting gene expression.

Thus, a significant body of evidence implicates oxidative stress both in DM and in the pathogenesis of DR.

Antioxidants as Primary and Secondary Preventative Strategies in DR

Given the evidence from animal models and basic science emphasizing oxidative stress as a significant component in the pathogenesis of diabetic retinopathy, many studies have looked at the role that diet may play in either preventing or slowing progression of the disease in humans. Studies have also evaluated the impact of antioxidant supplementation on diabetes generally and its complications. Extensive reviews have evaluated the evidence to date [29, 30••, 31–33] and a summary of their findings together with those from the most recent studies follows.

Epidemiological Studies

A recent systematic review identified 15 articles on micronutrients and diabetic retinopathy [30••]; the inclusion criteria were observational studies with cross-sectional, case-control or prospective designs on human participants with diabetes published in English. This review focused on diabetic retinopathy, including both proliferative and nonproliferative, as the outcome measure and micronutrient status, measured either as dietary intake or serum levels, as the exposure. The micronutrients evaluated included vitamin C, vitamin E, and magnesium, all of which possess antioxidant activity [34, 35]. In the hospital based cross-sectional studies all of the studies that were evaluated reported that diabetic patients with retinopathy had lower vitamin C levels than those without. However, for vitamin E, 2 studies showed no association and 1 study reported higher vitamin E levels in those with retinopathy than those without. The cross-sectional studies, which enrolled patients from hospital based settings, showed significant associations between diabetic retinopathy and both higher and lower serum magnesium levels. When the population based epidemiological studies were considered, the Atherosclerosis in Communities Study [36] found no association between Vitamin C and E intake. Similarly a Diabetes study carried out in the San Luis Valley [37] found no protective effect of β-carotene or vitamins C or E with diabetic retinopathy while the Third National Health and Nutrition Examination [38] found no association with serum levels of vitamin C or α-tocopherol. The Atherosclerosis in Communities study assessed intake using a food-frequency and supplement questionnaire while the San Luis Valley Diabetes study used a 24-hour dietary recall. Only 1 prospective cohort study evaluated serum magnesium and found a significant association with retinopathy progression [39], however, this effect disappeared after adjustment for appropriate covariates. The systematic review concluded that vitamins C and E or magnesium did not appear to be associated with diabetic retinopathy. The review, however, urged caution regarding the conclusions as there were few published studies and those that were available differed significantly in study design, sample populations, methodologies for exposure, and outcome measurements as well as the degree to which confounding was addressed within the analysis. Although most studies accounted for potential confounding with age and gender within the analysis, other factors such as blood pressure and hyperglycemia were often not considered, leaving the possibility for significant amounts of

residual confounding. In some cases sample size was insufficient to distinguish between an absence of an association from lack of statistical power [36, 38]. Due to the ultimately inconclusive results and the fact that the prevention of sight loss due to retinopathy was so important the authors advocated further high quality prospective studies to properly characterize the associations between micronutrients and diabetic retinopathy.

Supplementation Studies

There are few published studies evaluating the use of antioxidant supplementation specifically for prevention or treatment of diabetic retinopathy, probably reflecting the paucity of evidence for a benefit from an epidemiological perspective. Bartlett and Eperjesi [29] undertook a systematic review investigating nutritional supplementation for type 2 diabetes. Their criteria specified only randomized controlled clinical trials (RCT), and 50 trials were identified that met these criteria. These trials evaluated a variety of nutritional supplements such as alpha-lipoic acid, chromium, folic acid, isoflavones, magnesium, Pycnogenol®, selenium, vitamin C, vitamin E, and zinc. Of those with antioxidant capacity, vitamin E was reported to reduce oxidative stress when taken at levels of 200 mg^{-1} or more [29]. In addition, a Cochrane systematic review looking specifically at supplementation with vitamin C and superoxide dismutase concluded that no RCT had adequately examined their role in the treatment of diabetic retinopathy to be able to establish definitively whether their use would have a significant impact on the clinical course of the disease [33]. However, given the success of the AREDS [40] study in slowing progression of age-related macular degeneration (AMD), researchers are now asking whether resources should be directed towards seriously evaluating the impact of antioxidant supplements on the development of diabetic retinopathy [41].

Dietary and Lifestyle Advice to Modulate Antioxidant Status

Although dietary advice for persons with diabetes is primarily aimed at the maintenance of normal blood sugar, general guidelines are usually provided to encourage a reduction in the intake of carbohydrates, sugar, saturated fats, and salt while encouraging preferential intake of complex carbohydrates and low glycemic index foods and fiber. A recent review of evidence regarding dietary antioxidant intake and glucose metabolism concluded that while the recommendation of antioxidant supplementation is not supported by current evidence, the involvement of ROS in the control of carbohydrate metabolism seems undeniable [42]. Therefore, encouraging an increase in the overall total antioxidant capacity of the diet through eating foods rich in antioxidants may be most prudent for those at risk of diabetic complications.

Evidence is also accumulating regarding the ability of regular physical activity to activate cellular antioxidant systems as well as anti-inflammatory substances [43] and, therefore, should be encouraged, albeit more research is needed to ascertain how exercise intensity and type clinically impacts DR and how these can be optimized for maximal benefit.

Smoking also is known to promote oxidative stress and is also an accepted independent and modifiable risk factor for the development of type 2 diabetes. Its relationship with the development of diabetic nephropathy [44, 45] and macro vascular complications [46] is established, however, the relationship with the development of retinopathy is less clear [47, 48]. However, given the known positive impact of smoking cessation on oxidative status [49, 50] and prevention of other diabetic complications, smoking cessation is generally encouraged in the clinical setting.

Methodological Considerations for Future Studies

Given the lack of evidence from human epidemiological studies or intervention studies, despite the findings of basic science, further high quality studies are required. To ensure this is accomplished the following issues should be considered [30••]:

> *Participant Ascertainment:* for cohort studies, the exposed and nonexposed participants should be drawn from the same target population. For case control studies, the controls should be selected from the community while the cases should be defined by independent validation. The current practice of recruitment of accompanying relatives or other hospital attendees as controls can lead to bias and should be discouraged or avoided.
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> *Data Collection:* procedures for the measurement of micronutrients, whether by dietary intake or serum, should be undertaken using validated or standardized methods. Grading of retinopathy status should be undertaken while masked to exposure status and the use of the International Clinical Diabetic Retinopathy Disease Severity Scale or the ETDRS DR severity scale would make comparisons between studies easier. Vitamin or supplement use should be determined and should be accounted for within the analysis.
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> *Data Analysis:* information on potential confounding factors such as age, gender, blood pressure, history of hypertension, history of cardiovascular disease, and

glycemic control should be collected to enable appropriate adjustment within the analysis.

Supplement Composition: many compounds have been put forward given their putative properties. These include traditional antioxidants such as the AREDS formulation (vitamin A and C, β-carotene and zinc) [51] or other antioxidant mixtures, [13] polyphenols such as curcumin [11], or green tea [52], carotenoids such as lutein and zeaxanthin [53]. Other novel therapeutic approaches such as SOD/catalase mimetics [54, 55] have theoretical advantages over traditional antioxidants in reducing hyperglycemia-induced ROS production [2••]. In a mouse model of DM these latter therapies successfully modulated levels of activity of AGE, PKC, and polyol pathways induced by hyperglycemia. However, acceptance of any of the foregoing will only be possible after large scale clinical trials similar in scale to the AREDS.

Clinical Outcomes: careful consideration will also be required as to the appropriate primary and secondary outcome in clinical trials, and whether it is primary or secondary prevention. The progression of diabetic retinopathy according to a standardized scale is the most obvious but may only be appropriate for very large studies planned over an extended period owing to relatively slow progression rates of DR. In many of the smaller pilot studies carried out to date other surrogate measures of retinopathy progression have been evaluated, such as retinal thickness measured using optical coherence tomography [53], retinal blood flow [56], visual function [53], or antioxidant enzymes [57].

Conclusions

The association between high levels of oxidative stress and DR is not fully understood in terms of the exact pathogenetic mechanisms. Nonetheless, sufficient evidence exists both from experimental work in cells and live animals to support the view that amelioration of oxidative stress, either through the use of exogenous supplements or dietary and lifestyle modification, may be beneficial. However, large well-designed longitudinal cohort studies and clinical trials are needed to confirm their value.

Conflict of Interest Michael Williams declares that he has no conflict of interest.

Ruth Hogg declares that she has no conflict of interest.

Usha Chakravarthy declares that she has no conflict of interest.

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