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Gluco-Oxidation of Proteins in Etiology of Diabetic Retinopathy

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1. Introduction

Diabetes mellitus a chronic slow progressing catastrophe and is a major medical problem throughout the world. Diabetes causes an array of long-term systemic complications that have considerable impact on patient as well as society, as the disease typically affects individuals in their most productive years (Federman et al., 1994; Bhavsar et al., 2010). In addition, this increase appears to be greater among certain ethnic groups and in developing countries. Diabetic retinopathy is one of the main causes of diabetic complications. It causes visual impairment and finally blindness, a result of long-term accumulated damage to the small blood vessels in the retina. The proportion of blindness due to diabetic retinopathy ranges from close to 0% in most of Africa, to 3–7% in much of South-East Asia and the Western Pacific, to 15–17% in the wealthier regions of the Americas, Europe and the Western Pacific (Resnikoff et al. 2004; Zhang et al., 2010). According to the WHO fact sheet Aug. 2011, 346 million people worldwide have diabetes (World Health Organization [WHO], 2011). About 50% of persons with diabetes are unaware that they have the condition, although about 2 million deaths every year are attributable to complications of diabetes. After 15 years, about 2% of persons with diabetes become blind, and about 10% develop severe visual loss (WHO 2011). After 20 years, more than 75% of patients will have some form of diabetic retinopathy (Barcelo et al., 2003).

Post onset diabetes with increasing age, there is a higher risk of developing diabetic retinopathy and its complications, including diabetic macular oedema or proliferative diabetic retinopathy increases. The exact mechanism by which diabetes causes retinopathy remains unclear, but several theories have been postulated to explain the typical course and

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history of the disease (Crawford et al. 2009). Chronic hyperglycemia exerts protein gluco-oxidation, a process involving the non-enzymatic modification of tissue proteins by physiologic sugars in vivo, appears to play a central role in the pathogenesis of diabetic complications. One mechanism linking uncontrolled hyperglycaemia with tissue damage such as that in diabetic retinopathy is the formation and accumulation of advanced glycation end-products (AGE) (Hammes et al, 1996). *Ex vivo* and in vivo studies have indicated that AGE induce irreversible cross-links in long-living extracellular matrix (Brownlee et al., 1988; Fu et al., 1992; Sell et al., 1992; Sell et al., 1993) and, upon binding to specific cellular proteins, change the local concentrations of cytokines, growth factors and other bioactive molecules (Schleicher & Nerlich 1996; Vlassara et al, 1985). Accumulation of AGEs depends on both sugar concentration and the rate of protein turnover. Thus, some proteins that reach critical levels of AGE modification in sites where diabetic complications occur may turnover too quickly for normal levels of blood glucose to cause functional alterations, while proteins with a longer half-life would continue to be modified over a longer period of time (Brownlee 1995).

The relation between diabetes mellitus and oxidative stress is well known. With the onset of diabetes, persistent and chronic hyperglycemias causes increased production of free radicals through auto-oxidation of glucose, via nonenzymatic protein glycation and enhanced flux of glucose through the polyol pathway (Giugliano et al., 1996). The generation of reactive oxygen species and protein glycation are strictly interconnected (Palm et al, 2003). Levels of serum AGE are increased in diabetes mellitus before they have developed microvascular complications (Berg et al., 1997). These increased serum levels of AGE can predict changes in microvascular morphology in patients with diabetic retinopathy. Proteins containing AGE are highly immunogenic (Reddy et al., 1995) and anti-AGE antibodies were found in the sera of patients with diabetes (Shibayama et al, 1999; Baydanoff et al., 1996). Our research team has hypothesized that increase in the titre of anti-AGE antibodies has a direct role in the pathogenesis of diabetes microvascular complications especially diabetic retinopathy. Detection and characterization of antibodies against gluco-oxidative modified proteins could help in understanding the exact aetiology of gluco-oxidation of protein and diabetic retinopathy. Anti-gluco-oxidative modified proteins antibodies may potentially help in the prediction and /or prognosis of diabetes retinopathy. However the exact pathophysiology is yet to be ascertained.

2. Glycation

Reducing sugars such as glucose (or other reducing sugars as fructose, pentoses, galactose, mannose, ascorbate, xylulose) reacts nonenzymatically with free ε-amino groups in protein, lipids and nucleic acids through a series of reactions forming Schiff's bases and Amadori products to produce AGEs; and this process, also known as the Milliard reactions. In theory, every protein can be modified by glycation. Indeed, many protein-AGE adducts have been identified, *e.g.* glycated fibrinogen, collagen, albumin, herpes simplex glycoprotein B, hemoglobin, β2-microglobulin, and low density lipoprotein (Raj et al., 2000; Cribbs et al., 2000). Albumin is the most abundant protein in human serum, about 35-50 g/liter, and it is prone to glycation (Carter & Ho 1994). Non-enzymatic glycation of albumin occurs at multiple sites; glucose can attach to Lys199, Lys281, Lys439, and Lys525 as well as some other lysine and arginine residues and also at the N-terminal residues of polypeptides (Iberg & Fluckiger 1986). In fact, only a small number of factors are known to result in the variation

of serum albumin. The alteration in the structure of albumin due to uncontrolled hyperglycemia causes vascular complications (Bourdon et al., 1999).

Glycation is a classical covalent reaction in which, by means of N-glycoside bonding, the sugar-protein complex is formed through a series of chemical reactions described for the first time by a chemist Louis Camille Maillard in 1912 (Sing et al., 2001). Maillard reactions are complex, multilayered, and can be analyzed in three steps. (i) The sugar-protein complex is formed first (Amadori rearrangement), an early product of non-enzymatic glycation leading to intermediary products which are precursors of all later compounds. (ii) Formation of numerous intermediary products, some of which are very reactive and continue the glycation reaction. (iii) Final phase consists of polymerization reaction of the complex products formed in the second step, whereby heterogeneous structures named advanced glycation end products (AGE) are formed (Fig. 1).

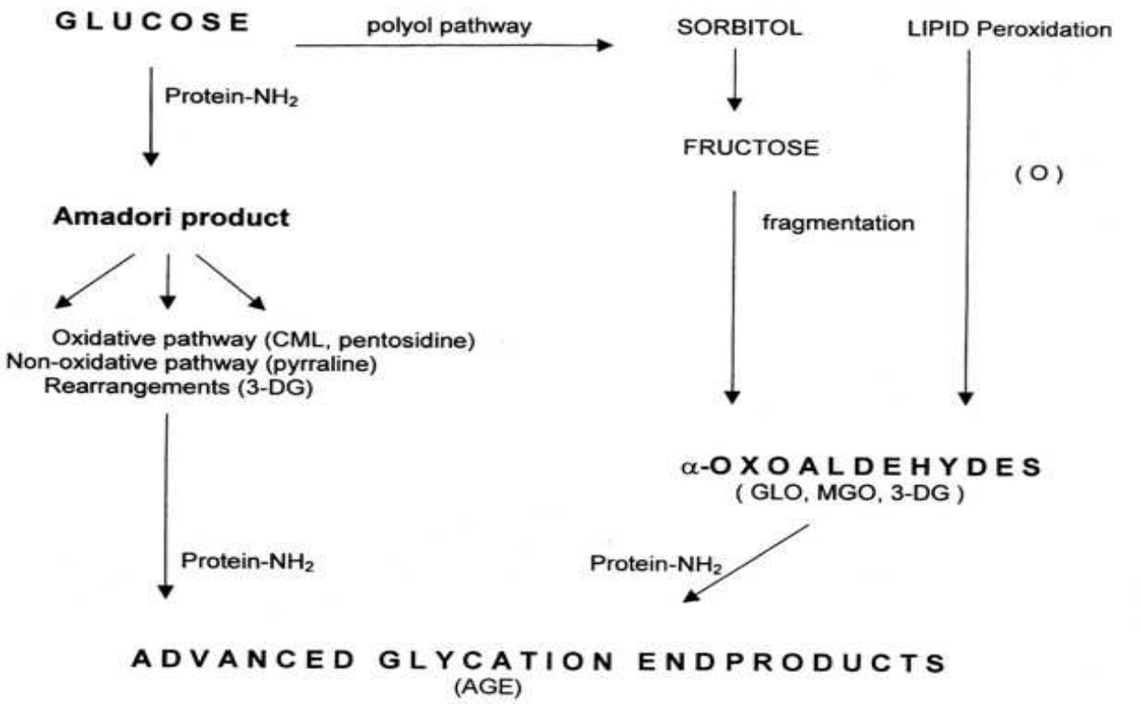

Fig. 1. Schematic presentation of potential pathway leading to AGE formation. The abbreviations given above are represented as, GLO=glyoxal; MG=methylglyoxal; 3-DG=3-deoxyglucosone; CML=carboxymethyl-lysine (Turk, 2001).

AGE constitute a heterogenous group of molecules (Peppa & Vlassara 2005) and its formation takes place continuously within the body during ageing, however it is extremely accelerated in diabetes (Vlassara & Palace 2002; Fu et al., 1996; Thorpe & Baynes 1996; Peppa et al., 2004). Some of the major AGEs are carboxymethyl lysine (CML) and pentosidine and also include many reactive intermediates or AGE-precursors such as 1- or 3-deoxyglucosone, methylglyoxal (MG) and their derivatives. AGE can cause tissue damage by two main pathways: they either form cross-links that disrupts the structure and function of short and long-lived proteins and lipids or they bind with specific and nonspecific cell

surface receptors inducing deleterious consequences, leading to altered intracellular events that induce oxidative stress and inflammation (Vlassara & Palace 2002; Peppa et al., 2002; Peppa et al., 2004; Vlassara 2001). AGE induced pathogenesis of diabetic retinopathy occurs via alteration of small vessel wall integrity and structure, by inducing cytokines, growth factors and increased oxidative stress (Sheetz & King 2002; Vlassara & Palace 2002; Peppa et al., 2002; Peppa et al., 2004; Vlassara 2001; Stitt et al., 1997; Stitt 2001; Yamagishi et al., 2002). *Ex vivo*, retinal endothelial cells exposed to AGE overproduce vascular endothelial growth factor (VEGF) through oxidative stress induction, protein kinase-C pathway activation and abnormal endothelial nitric oxide synthase (eNOS) expression (Mamputu & Renier 2002; Chakravarthy et al., 1998). Retinal organ cultures show an increased glyoxal induced CML formation in association with increased apoptosis and cell death, restored by anti-AGE agents and antioxidants (Mamputu & Renier 2002). Increased AGE accumulation was found in the retinal pericytes of diabetic rats after 8 months of diabetes (Stitt et al., 1997). In addition, exogenous AGE-albumin administration in non-diabetic animals accumulated around and within the pericytes, colocalized with AGE receptors inducing retinal vessel wall thickening and loss of retinal pericytes (Xu et al., 2003; Clements et al., 1998). In humans, it has been found that with the increasing severity of retinopathy there is a proportional increase in AGE accumulation around retinal blood vessels (koya et al., 2002). Glycation of vitreous collagen was also observed in human donor eyeballs (Sulochana et al, 2003). In addition, studies using anti-AGE agents further support the role of AGE in diabetic retinopathy (Yamagishi et al., 2002; Chappey etal., 1997; Reber et al., 2003). AGE have also been linked to the changes associated with diabetic keratopathy through their effect in reducing corneal epithelial cell adhesion (Matsumoto et al., 1997). Furthermore, glycation of the vitreal collagen fibrils leading to dissociation from hyaluronan and resultant destabilization of the gel structure has been associated with vitreous liquefaction and posterior vitreous detachment in diabetes (Stevens 1998; Sebag et al., 1992; Stitt et al., 1998).

3. Pathophysiological mechanism of AGEs formation in diabetic retinopathy

The knowledge of gluco-oxidation of proteins and AGEs has considerably expanded over the years, and a large body of evidence has documented their implication in diabetes-related complications (Singh et al., 2001; Turk et al., 2001; Brownlee 2001; Monnier et al., 2005; Huebschmann et al., 2006). In the process of glycation, AGE peptides that are released as degradation products, which partly occur through proteolysis of the matrix component are commonly named as glycotoxins. Glycated proteins are toxic for neuronal cells, retinal capillary cells, leukocytes, pericytes, and endothelial cells (Takeuchi et al., 2000; Yamagishi et al., 2002; Lyons et al., 2000). Toxicity of glycated polypeptides may be due to the AGE modification or due to the aggregation state of the polypeptides. Glycotoxins are very reactive on entering blood circulation. Non elimination of these proteins through the kidneys leads to recirculating AGE peptides which can generate new AGE products that react with other plasma or tissue components. At this stage, glycation becomes an autonomic process, which significantly accelerates the progress of the complication (Turk 2001).

Immunoglobins are glycated differently according to their class. The glycation of immunoglobulin-M is twofold greater than that of immunoglobulin G, and can be related to

the difference in amino acid composition. Albumin can be glycated at multiple sites. In diabetic patients, excessive glycation of fibrinogen and fibrin has been reported (Chappey et al., 1997). Hemoglobin is glycated at two sites: on the valine residue of the N-terminal β-chains at the ε-amino group of the α and β-chains, and at the N-termini of the α-chains (McDonald et al., 1978). Other intracellular and membrane proteins of red blood cells (RBC) are also glycated, for example Spectrin, a major RBC membrane protein, band 3 transmembrane protein, and band 4-1 (Retnaikes et al., 1987; Bryszewska & Szosland 1988). Hence glycation results in RBC deformability and an increased adherence to endothelium. Membrane proteins of platelets can also be glycated. Increased binding of fibrinogen and platelet aggregation observed in diabetic patients can be related to the glycation of adenosine diphosfate receptors. Lipids can also contribute to the modifications of platelet functions in diabetes (Chappey et al., 1997). Hyperglycemia can also induce protein aggregation which is associated with diabetes and its complications. Gluco-oxidation of proteins induces refolding of globular proteins, accompanied by the formation of cross β-structure (Bouma et al., 2003).

Gluco-oxidation of proteins forms complex and irreversible molecules, which accumulate in the retinal vasculature of patients with diabetes and streptozotocin-induced diabetic rats (Hammes et al., 1999; Stitt et al., 1997) and have been implicated in the development of diabetic retinopathy (Boehm et al., 2004 Genuth et al., 2005). Chronic exposure of the endothelium to AGEs has been shown to increase retinal vascular permeability in vivo (Stitt et al., 2000) and *ex vivo* (Leto et al., 2001). AGEs, however, have also been shown to increase capillary permeability acutely (Sampietro et al., 1987). Activation of AGE recepter (RAGE) and production of oxygen free radicals have been shown to mediate cellular responses to AGEs; however, the signalling pathways involved in the early permeability response are unknown (Kislinger et al., 1999; Bonnardel-Phu et al., 1999).

It has been suggested that, in diabetes, oxidative stress plays a key role in the pathogenesis of vascular complications, both microvascular and macrovascular, and an early marker of such damage is the development of an endothelial dysfunction (Giugliano et al., 1996; Cai & Harrison 2000). Evidence implicates hyperglycemia-derived oxygen free radicals as mediators of diabetic complications. Recently recognized relationship between α-oxoaldehydes and biologically important macromolecules highlights the intermediate step of advanced glycation cascade (Beisswenger et al., 2003a; Beisswenger et al., 2003b; Thornalley 2005). Diabetic individuals may exhibit elevated levels of iron and free copper ions (Cutler 1978; Mateo et al., 1978), which in the presence of glycated proteins *ex vitro* have been shown to generate free radicals (Hunt 1994). The accumulation of glycated material in tissues that contain free copper ion contribute to the generation of free radical mediated damage. These highly reactive species are capable of causing oxidative degradation of protein *ex vivo* (Hunt 1994). The formation of α-dicarbonyl compounds is known to be an essential step for the cross-linking of proteins and subsequent free radical generation (Rahbar & Figarola 2003). Methylglyoxal is increased 5-6 fold; in adult onset, non-insulin dependent diabetes mellitus as compared to healthy individuals. In the presence of oxidative stress, glycation of proteins by methylglyoxal is enhanced. This may underlie the link of glycation and oxidative stress with diabetic complications, and may also contribute to pathological processes of ageing.

Structural and functional modification of host-protein is a common feature of all AGEs irrespective of their generating precursors. Through their effects on the functional properties

of extracellular matrix, intracellular signal transduction and protein function, AGEs may contribute to the pathogenesis of diabetic retinopathy (Poukupec et al., 2003). A mechanism by which AGE-modified proteins may exert their effect is binding to RAGE identified on a variety of cells including endothelial and smooth muscle cells, and by internalization and degradation *via* monocyte/macrophage AGE-receptors. Using radiolabeled AGE proteins it has been shown that several cells, such as human and mouse monocyte, macro-phage and lymphocyte, bind these types of glycated compounds in a relatively selective way (Gilcrease & Hoover 1990; Imani et al., 1993). Gluco-oxidative modified proteins bind to these cells in a saturable manner with a dissociation constant in the range of 50–200 mmol/l^{-1}. The putative receptors for AGE have been isolated from cell membranes and purified, and were reported to have different molecular weights: 30–50 KD for renal tissue, 36–83 KD for a macrophage cell line, 60–90 KD for liver cells (Yang et al., 1991; Skolnik et al., 1991). A carbohydrate-binding protein of 35 KD named Galectin 3 is present on lymphocytes, macrophages, endothelial, mesangial, smooth muscle cells, and fibroblasts, and binds AGE with a higher affinity than other carbohydrates (Vlassara et al., 1995).

Increased hyperglycemia caused protein gluco-oxidation and/or glucose auto-oxidation enhanced formation of AGEs, stimulate the expression of RAGE and hence NADPH oxidase activation. Activation of NADPH oxidase increased the production of free reactive oxygen radicals can up-regulate vascular endothelial growth factor (VEGF) in retinal cells via nuclear transcription factors (eg. NF-kappaB) potentially promoting retinal neovascularisation and increasing permeability to proteins across the retinal barrier. Increased RAGE expression has been found on endothelial cells, vascular smooth muscle cells and cardiac myocytes of diabetic patients (Schmidt et al., 1999). It has been reported that ligation of AGE with RAGE causes activation of intracellular signaling, gene expression, and production of proinflammatory cytokines and free radicals, thus playing an important role in the development and progression of diabetic micro- and macroangiopathy (Kim et al., 2005).

4. Oxidative stress and diabetic retinopathy

Diabetic retinopathy pathogenesis is multifactorial, and the precise mechanisms are unclear. Several mechanisms have been proposed, including enhanced free radical production ROS (Brownlee et al., 1998; Koya & King 1998). Oxidative stress is increased in the retina in diabetes, and it is considered to play an important role in the development of retinopathy (Manikanth et al., 2010; Armstrong et al., 1998). It has been already proved that oxidative stress and hyperglycemia are central to chronic pathogenesis of diabetic retinopathy (Turk 2010). Increased levels of free radicals have a direct effect on *in vivo* protein. Oxidative stress induced modification of proteins is initiated mainly by reactions with hydroxyl radical; however, the course of the oxidation process is determined by the availability of oxygen and superoxide radical or its protonated form ($HO_2^{\cdot}$). Collectively, these ROS can lead to oxidation of amino acid residue side chains, cross-linking of soluble and/or membrane-bound proteins, oxidation of the protein backbone resulting in protein damage and yielding larger aggregates fragmentation. In the meantime, it has been shown that other forms of ROS may yield similar products and that transition metal ions can substitute for hydroxyl and superoxide radicals in some of the reactions (Berlett & Stadtman 1997). Even peptide bonds are subject to oxidative modification by ROS (Adams et al., 1999; Dhalla et al., 2000; Schoonover 2001).

Animal studies have demonstrated that oxidative stress contributes not only to the development of diabetic retinopathy but also to the resistance of retinopathy to reverse after good glycemic control is reinstituted—the metabolic memory phenomenon (Berg et al., 1997). Various mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals. Glucose auto-oxidation is one of the major sources of ROS that is generated by oxidative pathways of glycation. Glucose exists in equilibrium with its enediol, which can undergo auto-oxidation to form an enediol radical. This radical reduces molecular oxygen to generate the superoxide radical and becomes oxidized itself to a dicarbonyl ketoaldehyde that reacts with protein amino groups forming a ketoamine, **Fig. 2** (Wolff and Dean 1987a). Ketoamine are similar to, although more reactive, than Amadori products and participate in AGE formation (Ahmed et al., 2005). The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Wolff and Dean 1987; Jiang et al., 1990).

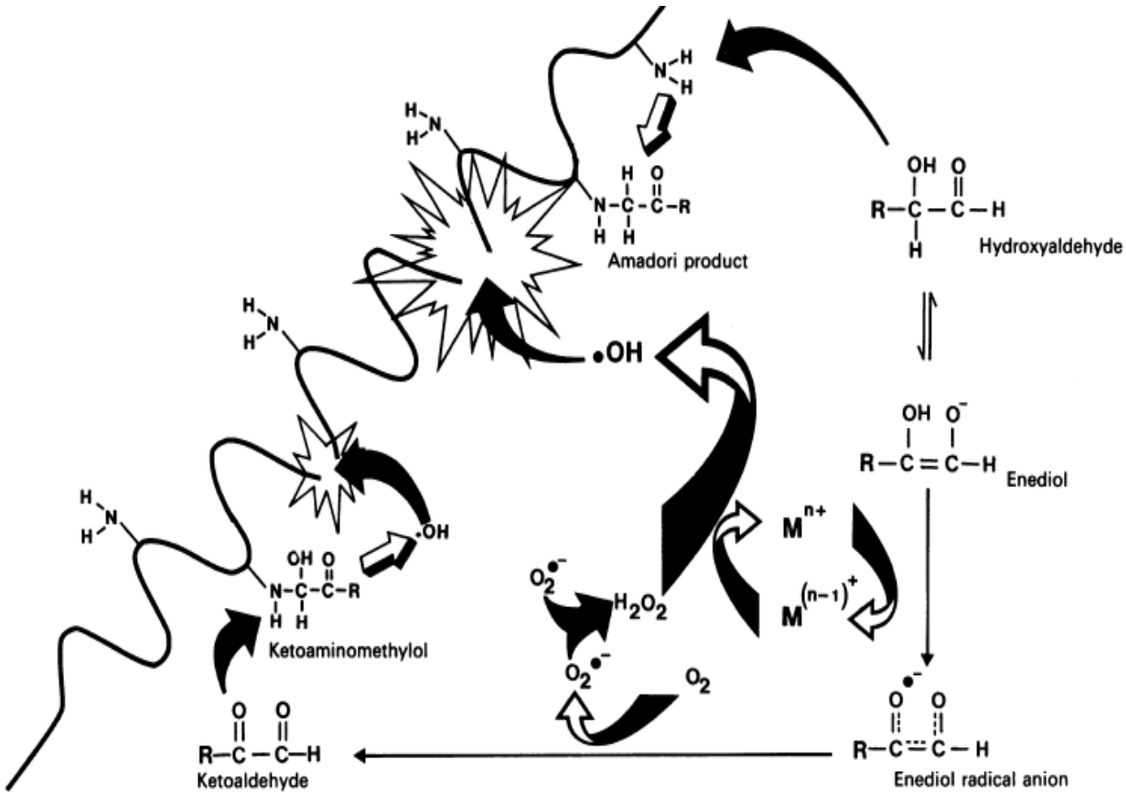

Fig. 2. Role of glucose auto-oxidation in the formation of reactive oxygen species induced protein damage (Wolff and Dean 1987a).

In hyperglycemic conditions, most of the carbonyl compounds generated by glycation need oxidative steps in their formation. The protein dicarbonyl compounds can participate in AGE formation and are referred to as glyco-oxidative products (Liggins & Furth 1997). Studies in diabetic rats showed elevated levels of superoxide in retinal cells with high glucose levels (Du et al., 2003; Cui et al., 2006), as well as increased levels of hydrogen peroxide (Ellis et al., 2000). Normally retinal blood vessels have tight junctions that protect

them from leaking. Prolonged hyperglycemia damages the tight junctions by oxidative stress and the vessels become leaky allowing fluid or blood to seep into the retina, thus resulting in the swelling of the retina (Harhaj & Antonetti 2004). Recently the etiology behind the production of superoxide in endothelial cells in diabetic complications has been elucidated (Brownlee 2001). There are four pathways suggested to be involved in the pathogenesis of diabetic complications due to increased production of free radical (increased polyol pathway flux, increased advanced glycosylation end product formation, activation of protein kinase C, and increased hexosamine pathway flux (Nishikawa et al., 2000; Du et al., 2002). In diabetes, the activities of antioxidant defense enzymes responsible for scavenging free radicals and maintaining redox homeostasis such as SOD, glutathione reductase, glutathione peroxidase, and catalase are diminished in the retina (Kowluru et al., 2001; Haskins et al., 2003). The intracellular antioxidant GSH is probably the most important antioxidant in the cell and acts as an ROS scavenger and modulates intracellular redox state (Meister 1988). The levels of this intracellular antioxidant are decreased in the retina in diabetes (Kern et al., 1994), and enzymes responsible for its metabolism are compromised (Lou 2003). Some nonenzymic antioxidants such as vitamin C, vitamin E, and β-carotene are also depressed during hyperglycemia induced oxidative stress (Ford et al., 2003).

5. Autoantibodies in diabetes complications

The lack of an immune response to self when responses to environmental antigens are retained is due to immunological tolerance. The role of tolerance, or lack of tolerance, is important to the understanding of autoimmune diseases and transplantation immunobiology (Mackay 2000). A loss of natural tolerance (to self) underlies all autoimmune diseases. Many more individuals develop autoimmune phenomena than autoimmune disease. Immune-mediated (Type I) diabetes results from an organ-specific autoimmune-mediated loss of insulin-secreting β cells. This chronic destruction process involves both cellular and hormonal components detectable in the peripheral blood, months or even years, before the onset of clinical diabetes (Kukreja & Maclaren 1999). In order to elicit an immune response, a molecule must be recognized as non-self by the biological system.

Proteins containing AGE are highly immunogenic and anti-AGE antibodies have been found in the sera of patients with diabetes (Reddy et al., 1995; Shibayama et al, 1999; Baydanoff et al., 1996). Several AGE structures have been identified including pyrraline, pentosidine (Sell & Monnier 1989), (carboxymethyl)lysine (Ahmed et al., 1986), and crosslines (Nakamura et al., 1992). Immunological studies using antibodies specific for these compounds have confirmed their presence in vivo (Ienaga et al., 1995). However, it is still not known whether one of these compounds contributes, as a major AGE structure, to the pathogenesis of these diseases, or whether other structures may involve in this process. Immunological approaches have been attempted to determine the major AGE structures expressed in vivo. Using AGE-BSA as an antigen, researchers prepared a monoclonal anti-AGE antibody (6D12) in mice as well as a polyclonal anti-AGE antibody in rabbits (Hoeiuchi et al., 1986). Immunoreactivity studies of these antibodies have demonstrated an interesting observation: both antibodies react with AGE samples obtained from proteins, peptides, lysine derivatives, and monoaminocarboxylic acids, suggesting the presence of a common AGE structures in these AGE preparations. Immunologic studies using 6D12 monoclonal antibodies have disclosed the presence of AGE in several tissues and their potential

involvement in disease processes. Anti-AGE antibodies use as a potential biomarker of AGE depositions during diabetes and its associated secondary complications.

There is increasing evidence of the presence of anti-AGE antibodies in diabetes and its complications. The role of these antibodies and specifically which particular anti-AGE antibodies are involved in the aetiology of diabetic micro- and macrovascular complications is, however, yet to be established. The possibility of effective therapeutic intervention stresses the importance of detecting anti-AGE antibodies, and advancements in measuring anti-AGE antibodies using reliable methods will help determine the role they have in the pathogenesis of many diseases, especially diabetes and its complications.

Antibodies against AGE structures led to the discovery that only a minor proportion of AGE are detectable by autoflourescence and they form to a greater extent in intracellularly than extracellularly because several glucose fragmentation products which occur during the metabolism of glucose in the cell are more reactive than glucose itself (Giardino et al., 1994). It was also found that the non-flourescent CML is the major epitope against which AGE-antibodies are directed (Reddy et al., 1996). Some AGE-antibodies used so far have not been characterized at all. To circumvent this problem researchers applied antibodies directed against the proteins that are abundantly available in blood and more exposed to blood glucose levels in diabetes mellitus as representative markers for the gluco-oxidative pathway. Anti-HSA antibodies have been observed in diabetes (Eilat *et al*., 1981), a fivefold greater occurrence than in nondiabetic persons (Gregor *et al*., 1986). Proteins containing AGEs are highly immunogenic and CML is one of the major epitopes recognised by anti-AGE antibodies (Reddy *et al*., 1995; Ikeda *et al*., 1996). The presence of AGE-antibodies in the serum of streptozotocin-diabetic rats as well as in a small number of diabetic patients have been reported (Shibayama *et al*., 1999) AGE can exert their immunogenicity, demonstrate that presence of AGEs-immune complexes (AGE-IC) in the diabetic patients that may play a role in the artherogenesis (Turk *et al*., 2001). Interactions of AGE autoantibodies with AGEs as a continuously produced antigen result in the formation of AGE-ICs that may play role in diabetic complications (Jakus and Rietbrock, 2004). The analysis of the frequency distribution profile shows that 14% of the diabetic subjects display significant antibody binding to AGE-HSA than the control subjects (Vay *et al*., 2000).

6. Autoantibodies against gluco-oxidative modified proteins in diabetic retinopathy

The autoantibodies have always been important for clinical interest due to their potential role in screening, diagnosis, monitoring treatment of effectiveness and prognosis. Non-enzymatic glycation of proteins can lead to the formation of reactive AGEs, which are thought to be implicated in the formation of micro- and macrovascular complications in diabetes mellitus. Proteins such as serum albumin, collagen, elastin, lens crystalline, are particularly susceptible to glucose modification (Festa et al., 1998). Elevated serum levels of these glycated proteins were detected in diabetic subjects moreover, higher levels of glycated form of proteins or AGEs were found in diabetic patients with secondary complications such as retinopathy, nephropathy and artherosclerosis (Nicoloff et al., 2000; Nicoloff 2001; Ahmed 2005). Previous studies showed reactive AGE can directly alter the physical and structural properties of the extracellular matrix, for instance, by inducing

collagen cross-linking, basement membrane thickening, and covalent trapping of plasma proteins such as LDL and IgG (Bouma et al., 2003). *Ex vivo* HSA was incubated with glucose at the concentration of 50 mM for 5 weeks at 37°C under aerobic conditions (Khan et al., 2007). Biochemical, spectral, electrophoretic, circular dichroism spectropolarimetric, and thermodynamic analyses confirmed that the structure and stability of HSA is significantly affected by glucose induced modification. Recently we showed that gluco-oxidation of proteins alter the structural complexity of the molecule and make them highly immunogenic (Khan et al., 2010). Ex vivo designed gluco-oxidative modified human serum albumin (RG-HSA) were used as an antigen and the titres of antibodies against (RG-HSA-Abs) it were screened in both types of diabetic patients, as well as screening was also done in diabetic patients with complications like retinopathy, nephropathy and artherosclerosis (Table 1). Interestingly, diabetic patients with associated complications (retinopathy, nephropathy and atherosclerosis) generated higher autoantibodies against gluco-oxidative modified HSA than controls and diabetic subjects without secondary complications. This above contention supports that gluco-oxidative proteins are toxic and highly immunogenic. From overall cohort of diabetic patients, the highest recognition of RG-HSA as an antigen by circulatory autoantibodies from diabetic retinopathy as compared to diabetic nephropathy and atherosclerosis (Table 1).

Groups	Sera positive for RG-HSA[1]	Sera positive for N-HSA[2]	Sera positive for both antigens	Carbonyl content (nmol/mg protein)
Type 1 diabetes (n = 30)	21 (52 ± 5.5)	-	1 (43 ± 4.7[1]; 51 ± 5.2[2])	2.9 ± 0.35
Type 2 diabetes (n = 30)	16 (48 ± 4.7)	-	2 (47 ± 4.7[1]; 43 ± 5.2[2], 45 ± 3.3[1]; 41 ± 4.7[2])	2.8 ± 0.4
Diabetes retinopathy (n = 12)	8 (76 ± 4.5)	-	-	3.9 ± 0.5
Diabetes nephropathy (n = 12)	7 (69 ± 3.1)	1 (41 ± 4.7)	-	3.5 ± 0.35
Diabetes atherosclerosis (n = 14)	9 (67 ± 4.0)	-	1 (55 ± 4.1[1]; 55 ± 2.8[2])	3.3 ± 0.55
Controls NH (n = 60)	-	-	-	2.3 ± 0.42

Table 1. Detection of N-HSA-Abs and RG-HSA-Abs and the estimation of carbonyl contents as oxidative stress in the sera of various diabetic groups and control. ELISA plate coated with the respective antigen (20 µg/ml). Sera positive means serum samples which gave inhibition greater than 30%, as less than that may be due to non specific bindings. n denotes the number of sera tested. Values in parentheses are mean ± SD of maximum percent inhibition of positive serum samples at 20 µg/ml of competitor. [1]ROS-glycated and [2]native HSA were used as inhibitor.

Diabetic retinopathic patients also exhibited maximum amount of carbonyl content, which showed a significant correlation between high oxidative stress and presence of anti-RG-HSA antibodies. Clinical and Laboratory examination was also done for 96 diabetic patients (66 males and 30 females) and 60 normal human (41 males and 19 females) serve as controls. According to the data given in Table 2, oxidative stress with chronic hyperglycemia and

advanced age has been considered a potential risk factor in the development of autoantibodies in retinopathy and other diabetic complications as well. AGEs are products of oxidative modifications of glycated proteins, which damage blood proteins. High oxidative stress and toxic blood glucose levels are found to be the common factors behind the generation of high autoantibodies and the progression of disease complications.

Subjects	Number of subjects	Age Years	Duration of disease Years	Fasting blood glucose (mg/dL)	HbA_{1C} (%)
Type 1 diabetic	30	36 ± 14	9 ± 3	254 ± 32	7.4 ± 0.6
Type 2 diabetic	30	44 ± 11	7 ± 3.6	263 ± 28	7.1 ± 0.4
Diabetic retinopathy	12	68 ± 2.9	21 ± 5	434 ± 11	9.0 ± 0.4
Diabetic nephropathy	12	62 ± 4.3	18 ± 3.3	394 ± 13	9.2 ±0.6
Diabetic atherosclerosis	12	59 ± 3.6	17 ± 3.6	390 ± 15	8.8 ± 0.3
Control NH	60	32 ± 9.5	–	88 ± 9.8	5.8 ± 0.4

Table 2. Clinical characterization of the patients and normal control subjects. For the blood glucose estimations, blood was collected in oxalated fluoride containers and the assays were performed immediately. Values are in mean ± SD. NH represents normal human subjects.

Gluco-oxidative modified HSA was immunized in the white New-Zealand rabbit that exhibited high titre of anti-glycated albumin antibodies in the serum of experimental animals (Khan et al., 2010). High titre showed immunogenicity of the gluco-oxidative modified proteins. These antibodies were proven to be a potential probe for the detection of protein lesion in blood proteins during diabetes mellitus. ELISA experiments of these antibodies with the isolated blood proteins such as albumin, IgG and RBC membrane from the diabetic patients showed high recognition. It means that during hyperglycemia there is damage of blood proteins that modifies the normal conformation of and hence generate new-epitopes that share binding specificity with the ex vivo designed gluco-oxidative modified albumin. Moreover, anti-gluco-oxidative modified HSA antibodies showed cross reaction with proteins such as BSA, poly L-lysine, immunoglobulins that were incubated ex vivo with 25 µM of glucose or fructose for 20 days. These findings suggests that paratopes of anti-gluco-oxidative albumin antibodies recognise common epitopes that are present in most gluco-oxidative modified proteins.

During diabetes, persistent hyperglycemia causes increased production of free radicals, especially ROS in all tissues by glucose auto-oxidation, protein glycation and due to decreased destruction by nonenzymic and enzymic catalase , glutathione peroxidase, and superoxide dismutase activity (Kowluru et al., 2001; Baynes & Thorpe 1999; Haskins et al., 2003). The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes. Also this is particularly relevant and dangerous for the beta islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defences (West 2000; Robertson 2004). In diabetes mellitus, alterations in the endogenous free radical scavenging

defence mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury.

Antibodies against glutamic acid decarboxylase-65 (GAD65Abs) are often considered to be an epiphenomenon resulting from the autoimmune destruction of the pancreatic beta cells in type 1 diabetes. Previous studies suggest that they are involved in antigen processing and presentation and thus modulate the immune response (Banga et al., 2004). Because of the high diagnostic sensitivity for autoimmune diabetes, the presence of GAD65Ab is currently used to identify subjects at high risk for the disease. GAD65Abs are detected in about 60% of new-onset cases of type 1 diabetes, and high levels of these autoantibodies were also reported in diabetic patients with secondary complications (such as retinopathy and nephropathy), the leading cause of blindness and renal failure (Falorini et al., 1998; Bonifecio et al., 1995; Jakuc & Reitbrock 2004). The exact aetiology behind these complications is not completely clear. In our recent study; ROS modified GAD65 was found to be more immunogenic in T1D than its native form (Khan et al., 2009). GAD65Abs in T1D are predominantly directed at conformational epitopes located in the middle region of the molecule, whereas they also recognize linear epitopes and epitopes located in the middle, COOH- and NH2-terminuses (Hampe et al., 2000). Shifts in GAD65 epitopes were detected in a subgroup of newly diagnosed children within the first 12 months after disease onset (Hampe et al., 1999). Moreover, epitope spreading has gained credence as a major driver underlying autoimmunity (Cheung & Wong 2007). Growing evidence suggests that ROS plays an important role in the initiation and progression of diabetes and its associated complications. These increased levels of free radicals pose a direct toxic effect on GAD65 and increase its immunogenicity. Specificity of autoantibodies for epitopes on GAD65 and their levels may be a better indicator of impending or actual destruction of islet beta-cells and increasing complications associated with diabetes.

In our 2009 study (Khan et al., 2009), while searching for a potential epitopes, high titre autoantibodies were detected in type 1 diabetes patients. GAD65 was considered a potential marker for type 1 diabetes. GAD65 was exposed to hydroxyl radical (ROS-GAD65), induced structural and conformational alterations were observed and investigated. Presence of autoantibodies against them were found in diabetes patients. Higher titres of autoantibodies were detected against ROS modified GAD65 (ROS-GAD65-Abs) in type 1 diabetic patients as compared to unmodified native GAD65. Increased levels of ROS in type 1 diabetes by molecular pathways or over produced metal catalyzed reactions modified GAD65 and induced biophysical structural alterations that would probably alter immunogenicity leading to induction and elevated levels of autoantibodies in type 1 diabetes. The data demonstrates possible role of ROS in presenting neo-epitopes that may be one of the factors in antigen-driven autoimmune response. Specificity of autoantibodies for epitopes on GAD65 and their levels may be a better indicator of impending or actual destruction of islet beta-cells and increasing complications associated with diabetes. The etiology of ROS-GAD65 in the pathogenesis of diabetic complications was further investigated in patients' diabetic complications in our new study (Khan et al., 2011). In this finding, significantly high levels of circulating ROS-GAD65Abs were detected in complicated diabetic patients especially in retinopathy as compared to uncomplicated type1 diabetic patients (Table 3).

Subjects	Age years	Gender (M:F)	Smoking duration Years	Duration of disease Years	Fasting blood glucose (mg/dl)	HbA_{1C} (%)	ROS-GAD65-Abs (MMPI)	Hyper-tension 140/90 (%)	Carbonyl Content (nmol/mg protein)
Uncomplicated T1D (n=60)	30 ± 09	37:23	*8(5±3.4)	09 ± 5.6	238 ± 27	7.9 ± 0.7	50.6 ± 7.2*	36(60)	3.0 ± 0.22
Complicated T1D Nephropathy (n=20)	37 ± 11	12:8	*14(6±3.8)	14 ± 4.9	311 ± 21	8.8 ± 0.6	70.3 ± 8.2	17(85)	3.4 ± 0.28
Complicated T1D Retinopathy (n=20)	42 ± 14	11:9	*17(8 ± 3.6)	17 ± 4.3	335 ± 17	9.3 ± 0.7	74.5 ± 6.5	16(80)	3.9 ± 0.31
Control (n=50)	32 ± 8	28:22	—	—	96 ± 11.2	5.8 ± 0.4	7.2 ± 3.7	4(8)	2.1 ± 0.17

Table 3. Clinical and laboratory data from complicated and uncomplicated T1D patients; normal human subjects serve as controls. Data are means ± SD. The sign "* "represents number of smokers from given total respective subjects. For blood glucose estimations, blood was collected in oxalated fluoride containers and the assays were performed immediately. Hypertension is defined as sitting systolic blood pressure ≥140mmHg and/or diastolic blood pressure ≥90 mmHg or the use of antihypertensive medication. Signs * represents 20 number of sera from different patients in the respective group. R-GAD65-Abs (Antibodies against ROS modified GAD65).

This risk of the disease may be enhanced due to acceleration in the formation of free radicals with gradual increase in duration of disease. Smoking and hypertension were also associated with increased antibody production in diabetic retinopathy. Gluco-oxidative stress leads to conformational alterations in native GAD65 protein which could increase or expose cryptic epitopes. Dynamic changes in the GAD65Abs binding pattern suggest subsequent epitopes spreading with disease progression. Concomitantly, these two studies on GAD65 provide us the evidences that hyperglycemia, age, oxidative stress, smoking, and as well as extent of blood protein glycation (HbA1C) participate in etiology of increased GAD65Ab immunogenicity implicated in diabetic retinopathy.

The exact mechanism for the formation of these autoantibodies and progression of diabetic retinopathy is still not well explained. We hypothesized that anti-gluco-oxidative protein autoantibodies bind to soluble glycated proteins and form an intermediary immune complex in the bloodstream that can bind to the basement membranes of the retinal blood vessels. At these sites they can activate complement cascade, resulting in damage to the walls of microvascular cappillaries associated with diabetic retinopathy. This phenomenon results in local necrosis of the vessels. If there is no continuous source of antigen, under conditions of controlled hyperglycemia then gluco-oxidative modified proteins are cleared and the disease can be controlled. However, if there is chronic hyperglycemia that enhances a continuing modification of blood protein, formation of increased immune complexes cause chronic autoimmune pathogenesis of diabetic retinopathy.

Gluco-oxidation associated damage of proteins due to hyperglycemia can be enhanced due to multiple factors such as duration of disease, age, smoking and hypertension and hence

can accelerate production of autoantibodies. This suggests that it is perhaps the rate of accumulation rather than the absolute concentration of gluco-oxidative proteins that is important. The exact mechanism behind the production of these autoantibodies is yet to be elucidated. However it stands to reason that the measurement of serum levels of gluco-oxidative proteins or anti-gluco-oxidative modified protein antibodies is important for estimation of an increased risk for development of diabetic retinopathy.

7. Conclusion

Gluco-oxidation is considered to be an important pathophysiological mechanism in the development of diabetic retinopathy. Gluco-oxidation leads to toxicity of blood proteins in diabetic retinopathic patients. Considerable amounts of AGEs are formed from blood proteins that subsequently develop into immune complexes with anti-AGE antibodies in retinopathic subjects. The ROS and gluco-oxidative modified protein autoantibodies were detectable in high titers in patients suffering from diabetic retinopathy. Chronic hyperglycemia and increased age, that are often seen in such cases, have proven to cause abnormally high production of free radicals with decreased antioxidant defence system. Proteins are damaged by the concomitant effect of glycation and oxidative stress leading to conformational alterations in native structure which could induce neo-epitopes or may increase exposed cryptic epitopes. Dynamic changes in the autoantibody binding patterns suggest subsequent epitope spreading with disease progression. Immune complexes of gluco-oxidative proteins and antibodies against them possibly activate complement cascade system and hence destroy capillaries within the retina. Measurement of these autoantibodies could be useful in assisting the prediction of the development of disease even before non-proliferative diabetic retinopathy. Reduction in the levels of glycation and ROS may lead to decrease in *in vivo* protein modifications, thus delaying the progression of diabetic retinopahy.

8. References

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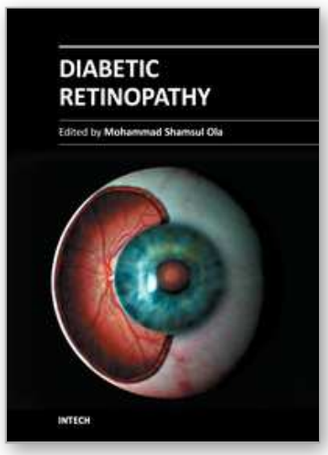

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The aim of this book is to provide a comprehensive overview of current concepts in pathogenesis, diagnosis and treatments of diabetic retinopathy. It provides a collection of topics written by excellent authors, covering discussions on advances in understanding of pathophysiology, immunological factors and emerging concepts, relating to clinical aspects and treatment strategies. The contents of the book will not only provide a resource for our knowledge but also improve diagnosis and treatment options for those patients who suffer vision loss due to diabetic retinopathy.

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