Non-enzymatic glycation of proteins: A cause for complications in diabetes

R B Nawale, V K Mourya* and S B Bhise*

Govt. College of Pharmacy, Osmanpura, Aurangabad 431 005, India
*Govt. College of Pharmacy, Vidyanagar, Karad, Satara 415 124, India

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Diabetes mellitus is one of the most common non-communicable diseases, and is the fifth leading cause of death in most of the developed countries. It can affect nearly every organ and system in the body and may result in blindness, end stage renal disease, lower extremity amputation and increase risk of stroke, ischaemic heart diseases and peripheral vascular disease. Hyperglycemia in diabetes causes non-enzymatic glycation of free amino groups of proteins (of lysine residues) and leads to their structural and functional changes, resulting in complications of the diabetes. Glycation of proteins starts with formation of Shiff’s base, followed by intermolecular rearrangement and conversion into Amadori products. When large amounts of Amadori products are formed, they undergo cross linkage to form a heterogeneous group of protein-bound moieties, termed as advanced glycated end products (AGEs). Rate of these reactions are quite slow and only proteins with large amounts of lysine residues undergo glycation with significant amounts of AGEs. The formation of AGEs is a irreversible process, causing structural and functional changes in protein leading to various complications in diabetes like nephropathy, retinopathy, neuropathy and angiopathy. The present review discusses about role of glycation in various complications of diabetes.

Keywords: Diabetes, Glycated protein, Advanced glycated end products, Diabetic retinopathy, Diabetic neuropathy, Diabetic nephropathy, Diabetic angiopathy

Introduction

The WHO report suggests that the prevalence of diabetes in adults worldwide would increase to 300 million in the year 2025. It is one of the main threats to human health in the 21st century and is the fifth leading cause of death in most developed countries. There are an estimated 86 million persons with diabetes in Asia, of which a third i.e., around 25-30 million are in India. High prevalence is reported, particularly from urban areas. In adult population, its prevalence is increasing all over the world. By the year 2025, more than 75% of individuals with diabetes would reside in the developing countries. In India, its prevalence has been steadily increasing in urban areas (it increased from a low of 2.1% in early 1970, to a whopping 11.6% in 1996), as well as in rural areas. Moreover, there is an equally large pool of persons with impaired glucose tolerance, many of who would be prone to develop diabetes in the future.

High genetic predisposition, changing lifestyle, associated stress of rapid urbanization and intake of ill-balanced diet rich in refined carbohydrates and dietary fats are believed to play a role in the rising diabetic prevalence. It is estimated that the number of diabetic persons in India is expected to increase to 57.2 million by the year 2025 (from 33 million in 2000) and the prevalence only be 14.7% in subjects aged 20 years or more. According to WHO report, in India, the annual cost of care in year 2002 was Rs. 12,000 and Rs 2,400 for patients taking insulin and those treated with oral hypoglycemic agents, respectively. Thus, for all affected diabetic patients (17.3 million) in India, with an estimated 20% treated with insulin, Rs.75.2 billion would be needed to provide the standard treatment. This was about 3-times higher than the total health care cost spending for India. This indicates that the majority of
those affected must be receiving care of much lower standard. In the USA, 11% diabetic men and 6% diabetic women in age group 45 to 64 years reported to have a heart attack. The percentage was 2.5-times higher in men and 4.0-times higher in women than in non-diabetic population. Increased risk of atherosclerosis is found even in pre-diabetic individuals and in populations at high risk of coronary heart disease (CHD). Almost half of middle-aged men and women with type 2 diabetes have symptomatic CHD, at the moment when their disease is diagnosed. These findings indicate that atherosclerosis develops gradually, resulting in hyperinsulinemia and hyperglycemia, before the actual onset of type 2 diabetes.

Diabetes can affect nearly every organ system in the body. It can cause blindness, end stage renal disease, lower extremity amputation, increased risk of stroke, ischaemic heart diseases, peripheral vascular disease and neuropathy. The present review discusses about role of glycation in various complications of diabetes.

Glycation

Heating proteins in the presence of sugars results in the formation of new cross-linked chemical bonds and this reaction is the responsible for browning of toast and many baked or cooked foods. The formation of complexes between sugars and amino acids of proteins is termed as glycation and these complexes cause toughening and discoloration of food during cooking process and after prolonged storage.

Hyperglycemia is one of the most important causative factors for secondary complications in diabetes. Sustained hyperglycemia leads to glycation of the proteins, preferably at amino group of lysine residue, which adversely affects their function. Reducing sugars, such as glucose react non-enzymatically with the free amino groups of proteins to initiate advanced glycation, resulting in formation of a diverse group of moieties. This reaction initiates formation of reversible Schiff bases, which by intermolecular rearrangement are converted into stable, covalently-bonded Amadori products. When large amounts of Amadori products are accumulated, they undergo further rearrangement and cross-linkage to form a heterogenous group of protein-bound moieties called ‘advanced glycated end products’ (AGEs). Rates of these reactions are quite slow and only the proteins with long half-lives and containing lysine residues, such as collagen under high sugar concentrations undergo this glycation (Fig. 1). AGEs play an important role in the structural and functional alterations of proteins, which occur during aging and diabetes. Glycation is an unavoidable process of metabolism in physiological states. In hyperglycemic condition, rate of glycation is increased, renal clearance of AGEs is decreased and/or the expression

![Fig. 1—Simplified reaction pathway involved in the formation of AGEs (R-NH₂ is a amino group of protein)](image-url)
of AGEs receptors is increased, leading to the AGE-mediated cell activation and amyloidosis. Structural proteins such as collagen and elastin undergo continual non-enzymatic cross-linking during aging and in diabetic individuals. AGE-derived protein cross-linking of structural proteins contributes to the complications of long-term diabetes, such as nephropathy, retinopathy, and neuropathy. AGE-mediated cell activation and amyloidosis are involved in several disease processes, with lysine and arginine and has a cross-link structure, one of the major AGEs that is formed by the reaction of ribose with lysine and arginine and has a cross-link structure, is involved in several disease processes. Although the proteins contain many surface amino groups, only a few are preferentially glycated. Glycation preferably occurs at amino groups that are either close to an imidazole moiety or part of a lysine doublet. Proximity (~5 Å) of an amino group to an imidazole moiety is the strongest predictor of susceptibility to glycation.

The chemical reactions leading to the non-enzymatic glycation of the protein is termed as Maillard reaction or advanced glycation. The Maillard reaction, initiated by non-enzymatic glycation of amino groups by reducing sugars, has been studied for its potential role in aging and the complications of diabetes. One of the major consequences of advanced Maillard reaction in protein is the formation of covalently cross-linked aggregates. AGE receptors participate in the elimination and change of aged, reticular and denatured molecules of ECM, as well as other AGEs molecules. However, in diabetes, AGEs protein accumulation may exceed the ability of their elimination, due to chronic hyperglycemia and excessive glycation. Interactions between glucose and protein lead to formation of chemically reactive AGEs (RAGEs) called glycotoxins. A large number of pathological sequels of diabetes result from the accumulation of tissue macromolecules. Although the predominant pathogenic AGEs structure present in vivo remains unknown, tissues from diabetic patients show significantly elevated levels of AGEs.

AGEs induce irreversible cross-links between molecules and alter their chemical and biological properties. Most of the carbohydrate-derived products which accumulate in tissue proteins with age and accumulate at an accelerated rate in diabetes are products of both glycation and oxidation reactions. Cross-linking of collagen proteins contributes both to rigidity and loss of elasticity of tissues and to thickening of capillary walls observed in diabetes and during aging. Non-enzymatic glycation of body proteins and subsequent advanced glycation reactions have been implicated in aging process, while caloric restriction in rodents results in an increase in both mean and maximum life span.

Administration of vitamin E may reduce protein glycation in diabetic subjects, independent of changes in plasma glucose, an effect that may be due to the inhibition of labile glycation, the first step of the Maillard reaction. In addition to cross-linking, the AGEs are able to stick the rapidly renewable plasma molecules together, with albumin, antibodies or low-density lipoproteins (LDL) cholesterol. The cross-linking of proteins and trapping of various molecules by the AGEs contribute to developing atherosclerosis, kidney, vascular and neurological diseases both in diabetes and aging process. Also, glycated substances may be involved in the pathogenesis of Alzheimer's disease, since their accumulation has been observed at the sites of neuronal degeneration during the course of disease.

Retinopathy
More than 60% of diabetic patients would have some degree of retinopathy 20 years after the onset of diabetes. Diabetic retinopathy (DR) is a leading cause of blindness amongst the working class (<55 years old). Its prevalence varies with the age of onset of diabetes and its duration, blood pressure and serum lipid levels. In younger patients (below 30 years of age), its prevalence is minimal during the first 5 years, but increases to more than 95% after 25 years of disease. In contrast, in patients whose onset of diabetes occurs after the age of 30, up to 20% may have signs of retinopathy on presentation, which rises slowly to approach 60% after 15 years of disease.

The lens of eye is a thin homogeneous, refractile epithelial basement membrane in the form of a capsule and is made up of glycoprotein and collagen produced by the epithelial cells. The lens capsule collagen contains higher quantities of hydroxyllysine than the interstitial collagen. The amino group of amino-terminus, C-amino group of lysine residue and
C-hydroxyamino group of hydroxylysine residue are potential sites of glycation in proteins. A large number of hydroxylysine residues are present in the lens epithelial basement membrane (LEBM) and their prolonged exposure to the aqueous humour glucose can cause a substantial non-enzymatic glycation. Non-enzymatic glycation of LEBM in diabetics is twice than that of non-diabetics.

A hallmark of early DR is the change in structure and cellular composition of the microvasculature. Endothelial cells are responsible for maintaining the blood-retinal barrier, and damage to them results in increased vascular permeability. In early stages of diabetic macular edema (DME), breakdown of inner blood-retinal barrier may occur, resulting in accumulation of extra-cellular fluid in the macula. Pericytes are essential cellular components in the regulation of retinal capillary perfusion and damage to these cells in diabetes leads to altered retinal hemodynamics, including abnormal autoregulation of retinal blood flow. Loss of retinal pericytes represents another early feature of DR and correlates with microaneurysm formation.

Another common feature of DR is the thickening of capillary basement membrane and increased deposition of ECM components. This feature may contribute to the development of abnormal retinal hemodynamics. Retinal leukostasis may also play an important role in pathogenesis of DR. Leukocytes possess large cell volume, high cytoplasmic rigidity, a natural tendency to adhere to vascular endothelium and a capacity to generate toxic superoxide radicals and proteolytic enzymes. In diabetes, there is increased retinal leukostasis, which affects retinal endothelial function, retinal perfusion, angiogenesis and vascular permeability. In particular, leukocytes in diabetes are less deformable, higher proportions are activated and they may be involved in capillary non-perfusion, endothelial cell damage and vascular leakage in the retinal microcirculation. Diabetic vascular leakage and non-perfusion are temporally and spatially associated with retinal leukostasis. There are many capillary occlusions by leukocytes and capillary dropout or degeneration associated with leukocytes in the diabetic retina. The trapped leukocytes have been found to be directly associated with areas of down-stream non-perfusion in the diabetic retinal microcirculation. Whereas leukostasis probably plays a key role in pathogenesis of DR, platelets and erythrocytes are also involved in this process. Hemorrhaging of new vessels into the vitreous may also lead to tractional retinal detachment. DR is a progressive disease that includes no apparent or non-proliferative DR and PDR. Non-proliferative DR is characterized by the presence of venous dilatation, microaneurysms, retinal hemorrhag and edema and hard exudates. Some DR patients may develop vision loss from DME. Clinically significant macular edema occurs, if there is thickening of retina involving the center of retina (macula) or the area within 500 µm of it.

Incubation of lens proteins with reducing sugars results in formation of fluorescent yellow pigments and cross-links, similar to those reported in aging and cataractous human lenses called non-enzymatic browning. Level of early glycation products in the lenses is high in the α-crystallin fraction than with β and γ-crystallins. The total early glycation products level in α-crystallin increases with age, but remain relatively constant in the β and γ-crystallins. The glycation rate in α-crystallin increases as a result of aging and diabetes, but remains almost constant in β and γ-crystallins. This non-enzymatic glycation of proteins causes opacification of the lens, leading to cataract formation.

The AGEs have been shown to accumulate in diabetic and ageing organs, including ocular tissues like cornea, lens, vitreous and retina. Their accumulation elicit several changes in ECM, including decreased solubility, decreased susceptibility to the enzymes and changes in properties such as thermal stability, mechanical strength, and stiffness. These changes are believed to contribute, in part to the development of age-related changes and diabetic complications like retinopathy. Accumulation of AGEs in the ECM of optic nerve heads, in the elderly may decrease elasticity of lamina cribrosa and compromise the ability of cribiform plates to bear the strain caused by elevated intraocular pressure.

**Neuropathy**

Neurogenic reactions occur when the decrease in blood glucose is rapid with tachycardia, palpitations, diaphoresis, tremors, pallor and arousal anxiety. The response is probably generated when the hypothalamus senses decrease glucose levels. The neuron receives inadequate supplies of carbohydrates to metabolize and is thus unable to maintain normal function. Cellular malnutrition produces further...
syrnpms including headache, dizziness, irritability, fatigue, poor judgment, confusion, visual changes, hunger, seizures and coma. Cerebral infarction (stroke) is a potentially disastrous complication of diabetes mellitus.

The AGEs have been shown to accumulate in myelin and tubulin of peripheral nerves. Non-enzymatic glycation of such neural proteins is thought to impair axonal transport, which may induce diabetic neuropathy. In the peripheral nerve, persistent hyperglycaemia leads to metabolic and vascular disorders responsible for nerve fiber abnormalities. There are various possible mechanisms by which glycemia could have an adverse effect on the peripheral nerve system (PNS) and it is difficult to disentangle the importance of different insults. Not only do the nerve fibers degenerate, but also attempts at regeneration by the damaged fibres, although vigorous, are short-lived and the numerous regenerative sprouts produced fail to survive. Therefore, the neuropathy becomes progressively worse. This worsening occurs in a dying back pattern (distal proximal direction) that is characteristic of failure in fast axonal transport. Actin is a neuronal protein involved in axonal transport and nerve regeneration, both of which are known to be impaired in diabetes, due to glycation.

Cause for failure of growing of sprout neuron is deleterious effects of glycation on the ECM and glycation of collagen in the nerve trunk, which acts as a physical barrier to elongation of axonal sprouts. Increased transport of glycated serum albumin across the blood-nerve barrier may induce deleterious osmotic changes in the endoneurium. High glucose concentrations could damage sensory neurons preferentially, because of their location in the dorsal root ganglia, where the blood-nerve barrier is less complete. This is the result of fenestration of a proportion of the blood vessels within the capsule, making it easier for proteins to leak out of the blood vessels into the endoneurium. The AGEs are localized in the perineurium, endothelial cells and pericytes of endoneurial microvessels, as well as myelinated and unmyelinated fibers. At the sub-microscopic level, the AGEs deposition appear focally as irregular aggregates in the cytoplasm of endothelial cells, pericytes, axoplasm and Schwann cells of both myelinated and unmyelinated fibers.

Diabetic polyneuropathy is a complication that affects most patients with long-standing diabetes mellitus, deteriorating their quality of life. In the last few years, new therapeutic approaches have been developed that can improve symptoms and neurologic function and which may prevent and in some cases stop nerve damage and even promote nerve fiber regeneration. These treatments are supported by: a) tight glycemic control (insulin), b) aldose reductase inhibition (tolrestat), c) prevention of protein glycation (amino-guanidine), d) improvement of nerve ischemia (vaso-dilators, L-linolenic acid), and e) administration of neurotrophic factors (gangliosides).

Nephropathy

Diabetic nephropathy is a vascular complication that affects the tiny glomerular capillaries, thus reducing the kidney’s filtration ability. This is first indicated by appearance of protein in the urine. In the presence of high amount of glucose, aldose reductase catalyzes the production of sorbitol. The amount of sorbitol can create problem. A patient with elevated serum glucose levels would produce more sorbitol. This causes a fluid imbalance within these cells and the disruption of cellular osmo-regulation might corrupt sufficient cell function to cause organ impairment and failure. This process is known as the polyol pathway. Other manifestations associated with the polyol pathway and nephropathies are increased permeability and thickening of glomerular basement membranes that eventually decrease the renal function. There are number of physiological changes at the onset of diabetes and during the course of disease. These changes begin with an enlargement of the kidneys. The major feature in development of renal dysfunction in type 2 diabetes is tubular injury. Tubular cells are primary targets for pathological influences in diabetes. Typical glomerulopathy is present in only one-third of type 2 diabetic patients with microalbuminuria. Renal function correlates better with tubular and interstitial changes than with glomerular changes. This indicates that renal pathology in diabetes is only, in part explained by glomerulopathy. It has been shown that renal tubular damage can even precede microalbuminuria in the absence of glomerular proteinuria. Tubular cells are direct targets for enhanced glucose levels present in diabetes and their glucose uptake is independent of insulin, resulting in a direct relation of the plasma glucose concentration to the intracellular glucose level. In addition, excess glucose in the glomerular
Glucose-dependent metabolic pathways and vasoactive hormones may directly influence tubular and interstitial cells, leading to renal dysfunction caused by non-glomerular mechanisms\textsuperscript{71,76}. High intracellular glucose levels lead to the enhanced formation of AGEs, in particular CML modified proteins. The AGEs such as CML have potential to directly target the renal tubular system. The renal tubule, particularly its proximal segment is exposed to theglomerular effluent containing large quantities of AGEs, particularly in diabetes. The effects of AGEs proteins such as CML are mediated by their binding to various distinct cellular receptors, found on different cell types. One of these receptors is the RAGE\textsuperscript{30,32}. Increased RAGE expression is demonstrated in tubular cells in diabetic nephropathy\textsuperscript{77}.

During early phases of diabetes, prior to microalbuminuria, a number of alterations occur in the kidney, including an increase in glomerular filtration rate, glomerular hypertrophy and hyperplasia and changes in the ECM. Modifications of structural as well as circulating proteins by glycation have much importance, because of their potential role in the etiopathogenesis of diabetes\textsuperscript{21,78}. Albumin, a major serum constituent involved in osmotic regulation, undergoes glycation when exposed to increased concentrations of glucose. In fact, the initial compounds generated during albumin glycation, the Amadori adducts, are abundant in plasma and urine of hyperglycemic subjects. This glycated albumin penetrates normal glomerular wall deeper than the non-glycated one and increases infiltration of non-glycated tracer through the normal glomerular wall. Circulating glycated serum proteins appear to play an important role in onset of glomerular dysfunction and proteinuria, which take place in long-term hyperglycemic states. Amadori proteins participate in diabetic glomerulosclerosis. Glycated albumin has also been found to stimulate mRNA expression, as well as protein synthesis of fibronectin and type IV collagen in glomerular endothelial cells\textsuperscript{79}.

During glycation, glucose reacts covalently with basic amino acids\textsuperscript{80}. This reaction induces a decrease in the isoelectric point, leading to a more anionic molecule. However, enhanced filtration of glycated albumin through glomerular basement membrane cannot be explained by changes in its physicochemical properties\textsuperscript{81}, because the modified molecule is heavier and more anionic than the non-modified one. Structural changes in the protein have thus been proposed to explain its altered handling by glomerular basement membrane\textsuperscript{38}. Presence of glycated albumin in circulation allows non-glycated albumin to traverse glomerular basement membrane more easily. In normal subjects, non-glycated albumin is rapidly reabsorbed by the proximal tubules, whereas the filtered glycated albumin seems to be excluded from tubular reabsorption. At very early stages of diabetes, efficient tubular reabsorption prevents the excretion of albumin. However, early tubular dysfunction\textsuperscript{32} or tubular saturation in prediabetic subjects, combined to the glomerular protein hyperfiltration that could be induced by glycated albumin certainly contributes to pathogenesis of diabetic nephropathy. Therefore, the presence of glycated albumin in circulation may contribute to pathogenesis of diabetic nephropathy\textsuperscript{37,83}.

**Angiopathy**

Evidences suggest linking of both glucose intolerance and insulin resistance to an increased risk of coronary artery disease (CAD). One of the potential mechanisms by which hyperglycemia may contribute to CAD is through the formation of AGEs\textsuperscript{21}. The AGEs formation may lead to oxidative stress, which might be responsible for a decline in insulin-mediated glucose uptake, leading to insulin resistance. Moreover, AGEs deposits have been found in the atherosclerotic plaques of patients with atherosclerosis and diabetes\textsuperscript{24,31}. Serum concentration of AGEs is increased in patients with type 2 diabetes and CAD\textsuperscript{84}.

Serum concentration of AGEs is an important determinant of the degree of impairment of endothelium-dependent vasodilation, independent of other risk factors. The underlying mechanism by which AGEs cause endothelial dysfunction, is not fully understood. Deposits of AGEs have been observed in the fatty streaks and atherosclerotic lesions. Accumulation of AGEs in the ECM of atheromatous arteries might result in nitric oxide (NO) depletion, enhanced subintimal protein and
lipoprotein deposition and increased smooth muscle cell proliferation. The vasoactive properties of AGEs may not be mainly attributable to the NO quenching effect of AGEs, but that additional mechanisms might also be involved.

In vascular wall, formation of AGEs adducts may interfere with vasodilation either by the reduced elasticity of AGE-modified structural proteins or by interference of AGEs with the vascular response to NO. AGEs formation may trap LDL or immunoglobulins in the intima by cross-linking to structural proteins. In turn, the prolonged intimal residence time of LDL may enhance generation of oxidized LDL, which may contribute to atherogenesis by multiple mechanisms, including rapid uptake of oxidized LDL via scavenger receptors. Macrophages also possess specific receptors that recognize AGEs proteins and uptake of AGE-modified lipoproteins via these receptors could contribute to foam cell formation. The AGEs could also enhance the intimal accumulation of macrophages, either by binding to specific receptors on endothelial cells or by a direct chemotactic effect.

Conclusion

Increased non-enzymatic protein glycation, formation of AGEs and their accumulation in tissue and serum have an important role in the pathogenesis of diabetes complications. Long-lived extra-cellular proteins have highlighted the importance of intracellular glycation. The chemical nature of AGEs, their synthesis in vivo and their precise role in the pathogenesis of diabetes complication is under investigation. The diabetic complication can be reduced by reducing glycation synthesis, cross-link formation and tissue accumulation of AGEs by blocking AGEs receptors. Numerous natural and synthetic compounds are being investigated for their possible therapeutic potential. The compounds like aminoguanidine and pyridoxamine prevent formation of AGEs and have proved promising in the prevention of diabetic complications in animal models. However, they could not be developed into an effective marketable drug, due to safety considerations. Better understanding of the molecular mechanism, responsible for diabetic complications is necessary for development of the inhibitors of AGEs.

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