Introduction
The term "PROTEIN" was derived from Greek word (prota) which means of primary importance. Proteins were first described by the Dutch chemist Gerardus Johannes Mulder and named by the Swedish chemist Jöns Jacob Berzelius in 1838. Early nutritional scientists such as the German Carl von Voit believed that protein was the most important nutrient for maintaining the structure of the body, because it was generally believed that "flesh makes flesh". Most proteins consist of linear polymers built from series of up to 20 different L-α-amino acids. All proteinogenic amino acids possess common structural features, including an α-carbon to which an amino group, a carboxyl group, a hydrogen and a variable side chain are bonded. The sequence of amino acids, or primary structure of the protein, is dictated by the nucleotide sequence of the gene coding for that particular protein. Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essential parts of organisms and participate in virtually every process within cells. Many proteins catalyze biochemical reactions which are vital to metabolism. Proteins also have structural or mechanical functions, such as actin and myosin in muscle and the proteins in the cytoskeleton, which form a system of scaffolding that maintains cell shape. Other proteins are important in cell signaling, immune responses, cell adhesion and the cell cycle.

**Protein antigenicity**

Oxidative damage to proteins *in vivo* may affect the function of receptors, enzymes, transport proteins, etc. and perhaps generate new antigens that provoke immune responses. Antigens are non-self substances, that are recognized by receptors on lymphocytes, thereby eliciting the immune response. The receptor molecules located on the membrane of lymphocytes interact with small portions of those foreign cells or proteins, designated as antigenic determinants or epitopes. The antigenicity of a protein refers to its capacity to bind specifically to the binding sites or paratopes of certain immunoglobulin molecules. Immunological properties of proteins have been widely used to study their structure. Experimental studies on a number of proteins, like sperm whale myoglobin (Atassi, 1975), hemoglobin (Kazim and Atassi, 1980, 1982) and lysozyme (Atassi, 1978) have also pointed out that antigenic sites on proteins may be formed either by sequential continuous regions or by bringing together several antigenic determinants to form antigenic sites. Thus, the antigenic
epitopes of proteins are composed of continuous or discontinuous portions of the polypeptide chain. The discontinuous epitopes are formed by two or more non-contiguous segments brought into proximity by the folding of the protein.

**Human serum albumin**

Albumin is the most abundant protein in human plasma, comprising 60% of the total proteins in blood (Shaklai et al., 1984). It is a 66 kDa, single-chain protein with 585 amino acid residues, three homologous domains (I–III), and a helical heart-shaped globule structure (He and Carter, 1992). Each domain is composed of two subdomains that are stabilized by internal disulfide bridges. The three domains, I, II and III, are subdivided into two subdomains A and B (Peters, 1996). It possess 28 α-helical segments which accounts for 67% of the protein secondary structure. The remaining secondary structure consists of 10% β-turns and 23% extended peptide chain. The sole tryptophan in HSA, Trp-214, is located in domain II (Fig.1).

After being synthesized in the liver, HSA circulates in the body for 2–3 weeks before being degraded and replaced (Brownlee, 1995). It is known as a multipurpose transport protein, as it has a set of quite diverse functions including oncotic pressure regulation, binding and transport capacities (Peters, 1996) and antioxidant properties (Coussons et al., 1997). HSA also plays an important role in normal physiology by transporting a remarkably broad spectrum of molecules throughout the body. These include inorganic cationic metals such as Cu$^{2+}$ and Zn$^{2+}$, organic anions, amino acids, metabolites, many drug compounds, and most importantly hydrophobic molecules like bilirubin, hemin, free fatty acids and a spectrum of therapeutic compounds.

**Antigenicity of human serum albumin**

Analysis of the antigenic structure of human serum albumin, after partial enzymatic hydrolysis, has demonstrated that the albumin molecule possesses several antigenic sites with distinct specificities. Fragments carrying different antigenic sites have been obtained by both enzymatic digestion and cyanogen bromide (CNBr) degradation (Lapresle and Doyen, 1975; Doyen and Lapresle, 1979).
Fig. 1 (A) Structure of human serum albumin with domains and subdomain.
(B) Structure of human serum albumin around Trp-214 with three closest lysine residues.

Albumin is a potent antigen. With the advent of monoclonal antibodies and monovalent Fab fragments, it became possible to identify particular sites by inhibiting or blocking techniques that eliminate the steric problems of aggregation with precipitation. Nineteen monoclonal antibodies have recognised thirteen epitopes on HSA (Doyen et al., 1985). This is consistent with the no. of sites produced by precipitation and suggest that the surface of an albumin is antigenic.

**Glycation**

The first effect of high glucose concentration in biological fluids and tissues is an important multi-step process involving chemical modification of reactive amino groups in biological macromolecules (proteins, lipids and DNA) by reactive carbonyls (Bucala, 1985; Suarez, 1989; Sengupta and Swenson, 2005). The free carbonyl groups (-C=O) of the sugars and related moieties react with the free amino groups (-NH₂) of the macromolecules in a series of chemical processes. This reaction was first described by the French biochemist Louis Maillard at the beginning of the 20th century, and is known as Maillard reaction. The intermediate products formed are known, variously, as Amadori, Schiff Base and Maillard products, named after the researchers who first described them (Dalle-Donne et al., 2005). Maillard first described the non-enzymatic reaction of glycine with glucose in 1912. In principle, all reducing sugars whether aldoses or ketoses (Yaylayan and Huyghues, 1994) and even molecules related to sugars, such as ascorbic acid, can initiate the reaction in vivo. However, most studies to date have been focused on glycation, the nonenzymatic reaction with glucose, because glucose is the most abundant sugar in blood and is elevated in diabetes.

Glycation is initiated by a nucleophilic attack of the aldehyde group of the reducing carbohydrate on the free amine to form a Schiff base (Cohen, 2003). The reversible N-glucosylamine (Schiff base) reaches an equilibrium over a period of several hours. Through acid-base catalysis, the N-glucosylamine undergoes a characteristic intramolecular re-arrangement reaction to form chemically stabilised 1-amino-1-deoxy-2-ketose (ketoamine) (Horvat and Jakas, 2004; Lapolla et al., 2005), which is named as Amadori product after the Italian scientist Mario Amadori (Amadori, 1925). The Amadori product subsequently cyclizes, and forms a pyranose
or furanose carbohydrate adduct (Cohen, 2003). Cyclization of the ketoamine to the hemiketal structure probably contributes to the stability of the glucosyl-protein adduct under physiological conditions (Armbuster, 1987) (Fig. 2a) This product undergoes cycles of condensations with additional amines, dehydrations, and oxidative fragmentations to yield a class of heterogeneous chemical compounds collectively referred to as advanced glycation end products (AGEs) (Fig. 2b).

The Schiff base and Amadori adduct also undergo facile oxidation, especially in the presence of transition metal ions, and fragment to yield shorter chain sugars and reactive intermediates, such as glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone. These reactive carbonyl intermediates, which are much more reactive than the sugars from which they are derived, again react with the amino groups of proteins and act as propagators of non-enzymatic glycation reactions. Such intermediary products (methylglyoxal) are also generated during glycolysis, lipid peroxidation (Thornalley et al., 2003) or along the polyolic pathway (Turk, 2001) and can also be formed by auto-oxidation of carbohydrates (glyoxal) (Thornalley et al., 1999; Cervantes-Laurean et al., 2005). These reactions have gained significant attention in recent days because of their association with free radicals, which have been implicated in the development of cancer, diabetes, heart disease, cataract, atherosclerosis, neurodegenerative disorders (e.g., Parkinson’s disease, Alzheimer’s disease) etc.

**Adducts of glycation and their pathological fates**

Excessive formation of both early and advanced glycation products appears to be the common biochemical link between chronic hyperglycemia and a number of pathophysiologic processes potentially involved in the development of long-term diabetic complications. The major biological effects of excessive nonenzymatic glycosylation include: inactivation of enzymes; inhibition of regulatory molecule binding; crosslinking of glycosylated proteins and trapping of soluble proteins by glycosylated extracellular matrix; decreased susceptibility to proteolysis; abnormalities of nucleic acid function; altered macromolecular recognition and
Fig. 2a Scheme of the reaction of glucose and protein to form Ketoamine (Amadori product)


Fig. 2b Formation of Amadori product and its subsequent conversion into AGE

endocytosis; and increased immunogenicity.

**Early glycation products**

Early glycation products are direct chemical precursors of a group of more slowly forming advanced glycation end products. Schiff's base and fructosamines (Amadori product) are called as early glycation adducts (Thornalley et al., 1999). They are formed both inside and outside the cells, as glucose rapidly attaches to the amino groups of the proteins through the non enzymatic process of nucleophilic addition, to form schiff base adducts. Within hours, these adducts reach equilibrium levels that are proportional to the blood glucose concentration and subsequently undergoes rearrangement to form more stable early glycation products (Amadori product), which reach equilibrium over a period of several weeks (Kumar and Kumar, 2012). During the lifetime of most cellular and plasma proteins, Amadori-products are in equilibrium with glucose and, therefore, the levels of Amadori-products tend to rise and fall depending on the glucose concentration. High level of Amadori products are formed through glucose mediated glycation of collagen, HSA and glutathione (Fu et al., 1994; Nagai et al., 1997; Linetsky et al., 2005). Among the substances involved in tissue damage, Amadori adducts have been shown to play a significant role (Angulo et al., 1996; Amore et al., 1997, 2004; Mandl-Weber et al., 2001; Hattori et al., 2002; Rodriguez-Manas et al., 2003). Excessive formation of early glycation products may adversely affect several functions relevant to diabetic complications. In blood vessels, uptake of LDL may be enhanced resulting in atherogenesis (Witzum et al., 1982). Free radical mediated damage is also increased (Gillery et al., 1988). These reversible biochemical abnormalities probably play a role in the pathogenesis of the early functional changes in the diabetic microvasculature.

**Advanced glycation end products**

Irreversible chemical reactions in Amadori products generate AGEs which may be fluorescent with cross-linking properties (such as pentosidine and crosslines) or non-fluorescent and non-crosslinking (such as Ne- (carboxymethyl) lysine (CML) or non-fluorescent crosslinked product such as pyrrline (Ahmed, 2005). AGEs may be generated rapidly or over long times stimulated by a range of distinct triggering mechanisms, thereby accounting for their roles in multiple settings and disease states.
AGEs contribute to pro-inflammatory and pro-oxidative processes in diabetes. Several mechanisms have been proposed by which AGEs lead to diabetic complications. For example:-

- accumulation of AGEs in the extracellular matrix causing aberrant crosslinking, resulting in a decreased elasticity of vessels.
- binding of AGEs to AGE-receptors on different cell types and activation of key cell signalling pathways such as NF-κB activation with subsequent modulation of gene expression in vascular cells such as endothelial cells, smooth muscle cells and macrophages (Stern et al., 2002; Miyazaki et al., 2002).
- intracellular AGE formation leading to quenching of nitric oxide and impaired function of growth factors (Shinohara et al., 1998).
- in endothelial cells, basic fibroblast growth factor is one of the main cellular AGE-modified proteins accompanied by markedly decreased mitogenic activity (Giardino et al., 1994).

Some of the biological effects of AGEs are modulated through their interaction with the receptor for advanced glycation end-products (RAGE) (Schmidt et al., 1996). AGEs bind to specific receptors on cells such as macrophages and induces receptor-mediated ROS production, thereby causing intracellular oxidative stress as well as the synthesis of growth factors and cytokines (Yan et al., 1994) resulting in damage to the affected tissues (Westwood and Thornalley, 1996). AGE produce cellular injury by a cascade of receptor-dependent (e.g., RAGE) and independent events that include intracellular generation of ROS which activates signaling pathways involving activation of protein kinase C (Mene, 1999) tyrosine phosphorylation of Janus kinase (JAK)/signal transducers and activators of transcription (STAT) (Huang, 2001), transcriptional activation (Lander, 1997) and induction of oxidative stress cascades involving NF κB and AP-1 which lead to proinflammatory (e.g., NF κB, monocyte chemoattractant protein-1, TNF-α) and profibrotic effects (e.g., TGF- α, connective tissue growth factor, PDGF) (Bierhaus et al., 2001; Twigg et al., 2002; Zhou et al., 2004).
Protein glycation

The process of protein glycation is now understood to be both a marker for the progress of diabetes complications and an underlying cause of many of the serious complications. Excess glucose binds to proteins throughout the body, changing their shape and properties in ways that have been shown to cause damage to both the structure and function of body organs. Protein glycation is a complex cascade of condensations, rearrangements, fragmentations and oxidative modifications which lead to heterogeneous products often with considerable structural alterations at the secondary and tertiary levels and consequently functional properties of the proteins (Luthra and Balasubramanian, 1993; Liang and Chylack, 1986). Glycation-induced damage to proteins associated with complications of diabetes have been investigated in the recent years. The damage caused by glycation to long lived structural proteins has been studied extensively. However, the proteins that turn over rapidly (like insulin and enzymes) are also damaged by glycation (Ganea, 2004; Harding and Elena, 2006). A number of studies have been carried out the glycation of proteins exposed to high glucose concentration, e.g. human serum albumin (Shaklai et al., 1984), lens crystalline protein (Stevens et al., 1978), collagen (Fu et al., 1994), IgM and IgG (Menini et al., 1993, Newkirk et al., 2003) and haemoglobin (Watkins et al., 1985).

Glycation primarily occurs at intrachain lysine residues of proteins (Watkins et al., 1985). The amino groups of arginine, histidine, tryptophan and cysteine residues (Munch et al., 1999) are also prone to glycation. The extent of glycation is proportional to glucose concentration in the protein’s environment and the duration of the exposure. There is growing evidence that under physiological conditions glucose reacts nonenzymatically with a wide variety of proteins to form stable adducts (Bunn et al., 1978; Schleicher and Wieland, 1981). Accelerated non-enzymatic glycation of cellular and extracellular proteins appears as an interesting mechanism in the pathogenesis of diabetic vascular disease (Bucala et al., 1995).

Impact of glycation on albumin structure

Albumin is the most abundant protein in human serum, prone to glycation (Carter and Ho, 1994). Glycation induced structural alterations has profound impact on the functional properties of albumin (Rondeau and Bourdon, 2011). Changes in
HSA structure and conformation by Amadori product formation strongly affects its anti-oxidant properties and foster acquisition of pro-oxidant activity in presence of copper (Coussons et al., 1997; Howard, 2005). It has been reported that the drug binding property of HSA may be altered at various stages of diabetes as the extent of glycation and type of modifications are varied in this protein (Barnaby et al., 2011; Lautenslager et al., 2011).

Non-enzymatic glycation of HSA occurs at multiple sites (Shaklai et al., 1984). Evidences suggest that ε-amino groups of lysine residues in HSA are preferred locus for glycation (Wa et al., 2007; Barnaby et al., 2011). Previous studies have indicated that the most significant sites in HSA for early glycation are the N-terminus and lysine 199, 281, 439 and 525 (Iberg and Fluckiger, 1986; Robb et al., 1989). In several studies, lysine-525 is depicted as the predominant site of the non-enzymatic glycosylation of HSA in vivo (Shaklai et al., 1984; Garlick and Mazer, 1983). Nonenzymatic adduction of glucose at this residue accounts for 33% of the overall glycation (Iberg and Fluckiger, 1986). Decrease in the affinity of in vivo and in vitro glycated albumin for different ligands suggests that this principal glycation site may play a key role in binding (Rondeau and Bourdon, 2011). Glycation of HSA with methylglyoxal has identified certain regions that are prone to advanced glycation end product modification (Ahmed et al., 2005b; Wa et al., 2007; Barnaby et al., 2011) including arginine 410 as a major region of modification and arginine 114, 186, 218, and 428 as regions of minor modification.

**Hemoglobin and albumin as indices of protein glycation**

Glycation of human proteins is currently of much experimental and clinical interest, and formation of fructosamine (Amadori adduct) has attracted much attention in the context of diabetes. This is because the glycation rate is of first order with respect to glucose concentration. Furthermore, fructosamines are thought to participate in the pathogenesis of long-term diabetes complications by acting either as such (Cohen et al., 1996) or after conversion to advanced glycation end products (Baynes, 1991; Brownlee, 2001).

The chemistry of early glycation products has been well described, with HbA1c as the best-studied example (Kennedy, 1992). HbA1c, the Amadori product of
HbA, formed by the non-enzymatic reaction between glucose and the amino groups of valine and lysine of β-globin, was the first early glycation product to be detected in vivo (Koenig et al., 1976). Most HbA1c reflects the presence of the fructosamine residues \( \text{Na-fructosylvaline} \) and FL (Ne-fructosyl-lysine) (Zhang et al., 2001). Measurement of early glycation products (Amadori product) is routinely used to evaluate metabolic control in diabetic patients. HbA1c has been firmly established as an index of long term glucose concentrations and as a measure of the risk for the development of complications in patients with diabetes mellitus. The degree of glycation of hemoglobin provides information about the glucose level (quality of diabetes control) over previous 6-8 weeks (Gugliucci, 2000, Cohen et al., 2002).

Glycated HSA is another important short time (2–3 weeks) indicator of glycemic control and diabetes that is more sensitive to changes in blood glucose level than HbA1c (Ulrich and Cerami, 2001; Kim and Lee, 2012). In fact, the initial compounds generated during albumin glycation, the Amadori adducts, are abundant in plasma and urine of hyperglycemic subjects (Guthrow et al., 1979). The glycated albumin level also provides useful information on short term glycemic control when monitoring the efficacy of therapy (Kisugi et al., 2007). The level of glycated albumin might also be of value as an indicator of the degree of hyperglycemia in diabetics (Nakajou et al., 2003). This process also occurs in individuals having normal blood glucose level, but HSA is three times more glycated in conditions of hyperglycemia (Guthrow, 1979; Bourdon et al., 1999; Nakajou et al., 2003).

**Importance of early glycation products**

Elevated levels of glycated proteins may lead to long-term tissue damage associated with the pathogenic effects observed in diabetics, including poor circulation to extremities, retinopathy, nephropathy, neuropathy, and coronary artery disease (Brownlee, 1995). Glycated HSA accounts for 80% of the circulating glycated protein (Cohen, 2003) and has been implicated in several complications associated with diabetes (Cohen et al., 1997, 2005). Amadori albumin has been reported in glomeruli of patients with diabetic nephropathy (Sakai et al., 1996). Early glycation reactions lead to the formation of Amadori products, fructosyl-lysine (FL) and related fructosamine residues, which degrade slowly to form AGEs. The FL residues have
been detected in protein extracts of rat tissues and plasma proteins. The FL residue concentration has been found to be markedly increased in renal glomeruli (6-fold), retina (3-fold), sciatic nerve (7-fold) and plasma protein (3-fold) of diabetic rats compared to normal controls (Karachalias et al., 2003). A specific and sensitive method for the quantification of early glycation products (fructose-lysine) can be achieved by determination of furosine (Scheilder and Wieland, 1981). Furosine is formed during acid hydrolysis of the fructosyl-lysine (1-deoxy-fructosyl-lysine) and is a widely used marker for evaluation of early stages of glycation in a wide variety of foods (Resmini et al., 1990) and biological samples (Forster et al., 2005).

**Diabetes mellitus**

Diabetes mellitus (DM) is not a single disease entity. It is a group of metabolic disorders sharing the common underlying feature of hyperglycemia. Advances in clinical science during the second half of the 20th century have led to improvements in understanding the causes and complications of diabetes, together with alleviation of suffering to an extraordinary degree. DM is characterised by chronic hyperglycemia resulting either from a deficiency of insulin, or decreased ability to transduce the insulin signal (insulin insensitivity), or both, eventually affecting all systems in the body and ultimately leading to a cluster of disorders. It is a systemic metabolic disease characterised by alterations in the metabolism of carbohydrates, proteins and lipids (Gleisner et al., 2006). Decreased insulin secretion affects glucose metabolism causing significant disturbance of water and electrolyte homeostasis. Low level of insulin results in hyperglycemia. While exogenous insulin and other medications can control many aspects of diabetes, numerous complications affecting the vascular system, kidney, retina, lens, peripheral nerves and skin are common and are extremely costly in terms of longevity and quality of life. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications (Ceriello, 2000; Rains and Jain, 2011). Diabetes is usually accompanied by increased free radical production (Baynes and Thorpe, 1999, 2000) or impaired antioxidant defences (Halliwell and Gutteridge, 1990; McLennan et al., 1991; Saxena et al., 1993). The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with diabetes. Many other metabolic
abnormalities occur, notably an increase in ketone bodies in the blood when there is a severe lack of insulin. The classic symptoms of diabetes include polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger) and unexplained weight loss. When the glucose concentration in the blood is raised beyond its renal threshold (about 10 mmol/L), reabsorption of glucose in the proximal renal tubuli is incomplete and part of the glucose remains in the urine (glycosuria). This increases the osmotic pressure of the urine and inhibits reabsorption of water by the kidney, resulting in increased urine production (polyuria) and increased fluid loss. Lost blood volume is replaced osmotically from water held in body cells and other body compartments, causing dehydration and increased thirst.

**Classification of diabetes mellitus**

Although all forms of diabetes mellitus share hyperglycemia as a common feature, the pathogenic processes involved in the development of hyperglycemia vary widely. Thus DM is classified on the basis of the pathogenic processes that leads to hyperglycemia (American Diabetes Association, 2012). The two broad categories are designated as type 1 and type 2.

* Type 1 diabetes

This form of diabetes results from a severe lack of insulin caused by immune mediated destruction of β cells. Type 1 diabetes most commonly occurs in childhood, becomes manifested in puberty and progresses with age. It is thought to be caused by a combination of environmental factors and viral infection, superimposed on a genetic susceptibility. The main cause of the beta cell loss is a T-cell mediated immune attack (Rother, 2007). Hyperglycemia accompanied by the classical symptoms of diabetes occurs only when 70-90% of β-cells have been destroyed. The rate of β cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Sensitivity and responsiveness to insulin are usually normal, especially in the early stages.

Type 1 diabetes can be further sub classified into type1A if autoimmune markers can be identified (Diabetes care, 1997) and type 1B-an absolute insulin deficiency in which no autoimmune markers can be identified. Type 1B may be more
common in people of Asian heritage (Abiru et al., 2002). A number of autoantibodies have been detected as the markers of autoimmune diabetes (Winter and Schatz, 2011). These include islet cell autoantibodies, autoantibodies to insulin (Atkinson et al., 1992), autoantibodies to glutamic acid decarboxylase (GAD 65) (Jun et al., 2002) and autoantibodies to the tyrosine phosphatases IA-2 and IA-2 β (Lan et al., 1996; Lu et al., 1996). Type 1 diabetes is partly inherited and there is a strong association of type 1 diabetes with individuals who possess particular haplotypes. HLA DR4-DQ8 and DR3-DQ2 are present in more than 90% of children with type 1A diabetes (Powers, 2008). Moreover, 30-50% of patients with type 1A diabetes are heterozygotes for HLA DR4-DQ8 and DR3-DQ2, whereas this combination of alleles is only present in 2.4% of the general population (Devendra and Eisenbarth, 2003). Type 1 diabetes is also triggered by certain infections with some evidence pointing at Coxsackie B4 virus, enteroviruses and rubella but have not been definitely shown to induce type 1 diabetes (Lammi et al., 2005). There is a genetic element in individual susceptibility to some of these triggers which has been traced to particular HLA genotypes.

Type 2 diabetes

Type 2 diabetes mellitus, or adult-onset diabetes, is characterized by insulin resistance which may be combined with relatively reduced insulin secretion. The defective responsiveness of body tissues to insulin is believed to involve the insulin receptor. The risk of developing type 2 diabetes increases with age, obesity and lack of physical activity. Overweight and obesity (especially central obesity or central adiposity) has been related to insulin resistance, hyperinsulinemia, glucose intolerance, and with subsequent development of type 2 DM (Wannamethee and Shaper, 1999). Furthermore, weight loss is strongly associated in prospective studies with decreased progression from impaired glucose tolerance (IGT) to type 2 diabetes (Knowler et al., 2002). A metabolic consequence of obesity, especially central adiposity, is the development of insulin resistance, which induces an increase in the secretion of insulin from the pancreas. Increased insulin production leads to compensatory hyperinsulinemia. In addition, many genetic polymorphisms may play a part in insulin resistance, possibly through post-insulin receptor signal transduction mechanisms (Powers, 2008).
There are probably many different causes of type 2 diabetes. Insulin resistance alone does not cause diabetes. Many obese people do not develop type 2 diabetes despite increased insulin resistance (Polansky, 2000). The development of insulin resistance is one of the earliest negative effects of obesity and is associated with the early altered glucose metabolism, chronic inflammation and oxidative stress. In the early stage of type 2 diabetes, the predominant abnormality is reduced insulin sensitivity. At this stage hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver. This form of diabetes frequently goes undiagnosed for many years because hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes.

It has been proposed that there is measurable beta cell hypertrophy in obese subjects who do not have diabetes. For unclear reasons, β cell secretory capacity gradually declines in some individuals leading to the development of type 2 diabetes. Several studies have shown that 40% of β-cell mass may be lost in individuals who have glucose tolerance and 60% may be lost when clinical type 2 diabetes develops (Butler et al., 2003). The cause of β-cell failure in type 2 diabetes is not known. The pathogenesis of insulin resistance is currently focussed on PI-3 kinase signalling defect which reduces translocation of GLUT 4 to the plasma membrane. Another emerging theory proposes that increased level of free fatty acids, a common feature of obesity, may contribute to pathogenesis. Free fatty acids can impair glucose utilization in skeletal muscles, promote glucose production by the liver and impairs β-cell function (Powers, 2008).

Asian Indians are more prone to type 2 diabetes and premature coronary disease due to Asian Indian Phenotye. The phenotype refers to certain unique clinical and biochemical abnormalities in Indians which include increased insulin resistance, greater abdominal adiposity (i.e higher waist circumference despite lower body mass index), lower adiponectin and higher C-reactive protein levels (Mohan et al., 2007). It also occurs more frequently in women with prior GDM and in individuals with hypertension or dyslipidemia, and its frequency varies in different racial ethnic subgroups. It is often associated with a strong genetic predisposition, than the autoimmune form of type 1 diabetes. However, the genetics of this form of diabetes
are complex and not clearly defined. The autoantibodies of type 1 diabetes mellitus especially GAD may also occur in up to 10% of adults initially classified as type 2 diabetes in a condition known as Latent Autoimmune Diabetes in Adults (LADA) or type 1.5 diabetes. The disease process in LADA patients is similar to that in type 1 diabetes as they share some HLA genetic susceptibility and some type 1 associated autoantibodies (Agardh et al., 2005).

Epidemiology and prevalence of DM

Diabetes mellitus has reached epidemic proportions worldwide. The prevalence of diabetes in the United States of America is estimated to be 23.6 million, or 8% of the population, with type 2 diabetes accounting for 90% to 95% of all cases of diagnosed diabetes (American Diabetes Association, 2009). Type 1 diabetes patients are estimated to be 5–10% of all diabetes cases (Daneman, 2006). The incidence of type 1 diabetes has been increasing by about 3% per year (Aanstoot et al., 2007). In 2006 it affected 440 thousand children under 14 years of age and was the primary cause of diabetes in those who were less than 10 years of age (Aanstoot et al., 2007). Over the past two decades the worldwide prevalence of diabetes mellitus has risen dramatically, from an estimated 30 million cases in 1985 to 177 million in 2000 (Wild et al., 2004). The incidence of diabetes mellitus is rapidly increasing and based on the current trends it is estimated that by the year 2030 more than 360 million individuals will be diabetic (Wild et al., 2004). Estimates which have been produced by the International Diabetes Federation (IDF) is that there will be 333 million diabetics by the year 2025 (International Diabetes Federation, Diabetes Atlas, 2006). Evidence has also accumulated that diabetes is also a risk factor for other age-related diseases such as Alzheimer's disease and a variety of cancers (Coughlin et al., 2004).

Recently, it has been estimated that diabetes is the fifth leading cause of death worldwide and is responsible for almost 3 million deaths annually, which is a 1.7-5.2% of deaths worldwide. For developing countries, adult diabetes numbers are likely to increase by 69% from 2010 to 2030, compared to 20% for developed countries, whereas total adult populations are expected to increase by 36% and 2% respectively (Shaw et al., 2010).
India now being termed the "diabetes capital of the world" as it leads the world with largest number of diabetic subjects. WHO report shows that 32 million people in India had diabetes in the year 2000 and is expected to increase to about 80 million people by 2030. IDF estimated around 36 million diabetics in India in 2003, and this is further set to rise to 73.4 million by the year 2025. A recent report, estimated that from 50.8 million by the end of year 2010 the number of diabetics will shoot up to 87.0 million by the end of year 2030 (Shaw et al., 2010).

Diagnosis of DM

Diabetes mellitus is characterized by recurrent or persistent hyperglycemia and is diagnosed by demonstrating any one of the following:-

- Fasting plasma glucose level at or above 7.0 mmol/L (126 mg/dL).
- Plasma glucose at or above 11.1 mmol/L (200 mg/dL) two hours after a 75 g oral glucose load as in a glucose tolerance test
- Symptoms of hyperglycemia and casual plasma glucose at or above 11.1 mmol/L (200 mg/dL).
- Glycated hemoglobin (HbA1c) at or above 6.5 (This criterion was recommended by the American Diabetes Association in 2010, although it is yet to be adopted by the WHO).

Pathophysiologic features of various diabetic complications

**Diabetic nephropathy**

During hyperglycemia, glycation of structural and circulating serum proteins is a critical event associated with the glomerular dysfunction accompanying diabetic nephropathy (Cohen and Ziyadeh, 1996). It encompasses discrete structural alterations, including renal hypertrophy, thickening of basement membranes, proteinuria progressing to renal insufficiency and progressive expansion of extracellular matrix components, key events in the genesis of diabetic renal damage.

The earliest detectable change in the course of diabetic nephropathy is thickening in the glomerular basement membrane. Various mechanisms that are
operative in diabetic milieu (i.e., high glucose concentration, nonenzymatic glycation products, and glomerular hypertension) can stimulate the synthesis and release of a number of growth factors, cytokines, chemokines and vasoactive agents. These factors are thought to stimulate either proliferation or hypertrophy of various renal cells, as well as increased extracellular matrix production. Formation of early Amadori-glucose adduct and albumin AGEs have been shown to stimulate renal expression of various growth factors, including transforming growth factor (TGF-β) which are important mediators of diabetic nephropathy (Pugliese et al., 1997). The Amadori-glucose adduct, increases TGF-β type II receptor mRNA and protein expression in mesangial cell cultures thereby contributing towards the thickening of basement membrane, altered function and ultimately loss of glomelular function (Ziyadeh et al., 1998). The concentration of the initial Amadori products of glycation on collagen (measured as fructosyllysine) are increased about threefold in type 1 diabetic patients (McCane et al., 1993) and were independently associated with early nephropathy. In addition, in patients with long-standing type 1 diabetes with nephropathy, Amadori-glycated albumin levels were increased as compared with type 1 diabetic patients without nephropathy (Schalkwijk et al., 1999).

**Diabetic retinopathy**

Retinopathy is the most common microvascular complication of diabetes, and it remains a major cause of visual impairment worldwide (Marshall and Flyvbjerg, 2006). Microvascular lesions such as microaneurysms, blood barrier dysfunction, haemorrhages and infraction affecting retina of the eye accompanied by thickening of basement membrane and capillary dropout are key features of diabetic retinopathy (Klein et al., 1992). Early and the advanced glycation products differently affect retinal microvascular cell growth (Amin et al., 1997). Increased amounts of AGEs as well as early glycation products have been detected in the vitreous humor of diabetic patients with proliferative diabetic retinopathy (Sebag et al., 1992). In retinal pigment epithelial cells, Amadori-modified glycated albumin increases the production of the inflammatory cytokines interleukin (IL-8) and monocyte chemotactic protein (MCP-1) and activates the mitogen activated protein kinase (MAPK) pathway, protein kinases, JAK, and the transcription factor nuclear factor (NF)-κB (Bian et al., 1998; Ibrahim et al., 2011). Fructoselysine, an initial Amadori adduct in collagen in type 1
diabetic patients has been shown to be independently associated with retinopathy (McCane et al., 1993). AGEs contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage (Chibber et al., 1997).

**Diabetic atherosclerosis**

Both diabetes and atherosclerosis, the diseases that are intimately linked to one another, may be described as diseases resulting from chronically elevated levels of plasma glucose and/or lipids. It is characterized by the deposition of atherosclerotic plaques in arterial walls and myocardial infraction. Nonenzymatic glycation of Apolipoprotein B (Apo B) in low density lipoproteins (LDL) is considered to be a proatherogenic modification contributory to the increased susceptibility of patients with diabetes to atherosclerotic disease. Plasma concentrations of glycated LDL measured by several different methods are increased in diabetic patients (Jack et al., 1998; Tames et al., 1992; Cohen et al., 1993; Akanji et al., 2002) and show positive associations with other markers of cardiovascular disease such as serum cholesterol and triglyceride levels (Cohen et al., 2004). Amount of Amadori-modified LDL correlates positively with microalbuminuria (Cohen et al., 2004), a marker of inflammation and an independent risk factor for cardiovascular mortality (Agewall et al., 1997) that has been found to be associated with increased cardiovascular mortality in type 1 and type 2 diabetes (Borch-Johnsen et al., 1985; Jarrett et al., 1984; Nelson et al., 1988; Schmitz and Vaeth, 1988). Approximately 2-5% of apo B in the plasma of diabetic patients are glycated, compared to about 1% in the plasma of non-diabetic control subjects (Turk, 2001). Evidence for the participation of glycated LDL in atherogenesis in vivo can be found in results from animal experiments and from studies of human atherosclerotic plaques. Syrian hamsters made diabetic with streptozotocin developed atheromatous lesions in the aortic arch and exhibited an elevated concentration of glycated LDL that is more susceptible to oxidation, and glycoxidative products are immunochemically detectable in the foam cells of fatty streaks (Yamanouchi et al., 2000).
Role of Amadori-modified albumin in the pathophysiology of diabetic complications

Evidence suggest that early glycated albumin is not just an index of glycemia or the precursor of AGEs. By itself, it may have important direct impacts on cellular functions and thus may play a pathophysiological role in microvascular complications of diabetes. It has been observed that Amadori-modified albumin is an independent and potent trigger of molecular mediators contributory to complications of diabetes (Cohen et al., 1994, 2005). The pathophysiological role of early glycated albumin is further evidenced by the finding of existence of specific receptors for early glycated albumin (Krantz et al., 1993; Salazar et al., 1995; Verbeke et al., 1996). These receptors differentially bind Amadori-modified glycated albumin, but not AGEs, suggesting that the functional role of early glycated albumin may differ from that of AGEs. Specific receptors for early glycated albumin have also been described on murine aortic endothelial cells (Wu and Cohen, 1994). Binding of glycated albumin to these receptors inhibits aortic endothelial cell replication and basement membrane collagen production (Cohen et al., 1995). It has been demonstrated that the infusion of Amadori products in the circulation of normal rats induces glomerular vasodilatation and hyperfiltration, similar to those found in streptozotocin-induced diabetic animals (Sabbatani et al., 1992).

Endothelial dysfunction is closely associated with both micro- and macrovascular complications of diabetes (Raptis and Viberti, 2001). Endothelial cells preferentially take up circulating glycated proteins which may lead to endothelial dysfunction and contribute to diabetic microangiopathy (Williams et al., 1981; Schalkwijk et al., 2002). In vitro studies indicate that physiologically relevant concentrations of Amadori-modified albumin possesses multiple proatherogenic effects that include adhesion of macrophages (foam cells precursor), promoting oxidative stress, production of inflammatory mediators, endothelial damage and vessel wall hypertrophy, phosphorylation of extracellular signal regulated kinase (ERK), increase Transforming Growth Factor (TGF)-β1 production and nuclear translocation of the transcription factors (NF-κB) (Fig. 3). Interaction of the Amadori-glucose epitope with specific cell-associated binding proteins is believed to trigger the above and other effects (Salazar et al., 2000, 2001). That glycated albumin augments oxidative stress and free radical impairment that contribute to atherogenesis in
diabetes is supported by the finding that conformational shifts in the albumin molecule attendant to the formation of Amadori adducts, without the extensive damage to the folded structure that is typically induced by AGE, can affect antioxidant properties of the protein and foster acquisition of pro-oxidant activity (Coussons et al., 1997; Howard and Smales, 2005). Data from experiments with glomerular mesangial and endothelial cells have established that an increased level of Amadori-modified albumin is accompanied by alterations in key cellular mediators that modulate cell signaling pathways which are important in regulating extracellular matrix production (ECM) and are known to participate in the development of diabetic nephropathy. Glycated albumin-receptor binding elicits a signal transduction pathway leading to the generation of oxygen free radicals. These reactive oxygen species activate the redox-sensitive transcription factor NF-κB (Hattori et al., 2002), a pleiotropic regulator of many genes. Amadori albumin has been shown to elicit the microglial activation in retina and secretion of TNF-α (Ibrahim et al., 2011) (Fig. 4), activate PKC-β1 and stimulate the expression of TGF-β1, α 1 (IV) collagen and fibronectin, prominent constituents of the expanded ECM that is seen in diabetic glomeruli (Howard, 2005; Cohen et al., 1997), (Chen et al., 2001) (Fig. 5). Pharmacological approaches to mitigate the deleterious effects of Amadori-albumin may be therapeutic approaches to adverse cardiovascular consequences of diabetes.

**Autoimmunity**

It is a physiological inherent feature of the immune system defined by the presence of auto-reactive lymphocytes and antibodies (Wardemann and Yurasov, 2003). An autoantigen is usually a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognized by the immune system as non-self leading to autoimmune reactions which causes serious damage to cells and organs. Sometimes the damage to self-cells or organs is caused by antibodies; in other cases, T cells are the culprit. Many autoimmune diseases such as type 1 diabetes are characterised by tissue destruction mediated directly by T-cells. Additionally, diseases such as rheumatoid arthritis and multiple sclerosis are primarily due to the action of self
Fig. 3 Glycated albumin promotes oxidative stress, produces inflammatory mediators and may cause endothelial damage and vessel wall hypertrophy.

Source: Adapted from Epinex diagnostics, 2008 “Glycated albumin and diabetes monitoring.”
Fig. 4 Retinal microglial activation and inflammation induced by Amadori-glycated albumin in a rat model of diabetes.

**Fig. 5** Interaction of glycated albumin with mesangial cells causes amplification of cell signaling cascade, involving protein kinase C and secretion of potent cytokines like TGF beta leading to glomerular dysfunction.

**Source:** Adapted from Epinex diagnostics, 2008 “Glycated albumin and diabetes monitoring.”
reactive T-cells.

In order to elicit an immune response, a molecule must be recognised as non-self by the biological system. Studies with experimental auto-immune animal models have revealed a central role for CD4 T\(_H\) cells in the development of autoimmunity. Numerous mechanisms have been proposed for induction of autoimmunity including:

- Release of sequestered antigens
- Molecular mimicry
- Inappropriate class II MHC expression on cells
- Polyclonal B-cell activation

It has been suggested that self proteins are rendered immunogenic if chemically or post-translationally modified under physiologic or pathologic conditions (Doyle and Mamula, 2001; Utz and Anderson, 1998). Protein modifications may lead to the generation or unmasking of new epitopes that will serve to activate B-cells and/or T-cells, thereby impassing immunological tolerance (Ohmori and Kanayama, 2005). Amadori products of HSA are immunogenic and type 1 diabetic patients with retinopathy and nephropathy have higher Amadori albumin levels than those without it (Cohen and Ziyadeh, 1994; Schalkwijk et al., 1999). AGE modified proteins are highly immunogenic and CML is one of the major epitopes recognised by anti-AGE antibodies (Reddy et al., 1995; Ikeda et al., 1996).

**Sources of ROS in diabetes**

At high concentrations, ROS may be deleterious to cell structures, nucleic acids, lipids and proteins (Valko et al., 2006). Diabetes is one of the pathological processes known to be related to an unbalanced production of ROS, such as hydroxyl radicals (OH\(^-\)), superoxide anions (O\(_2^-\)) and H\(_2\)O\(_2\). Under normal conditions, the key sites of superoxide formation in the mitochondrial membrane are complex I and the ubiquinone–complex III interface (Kwong and Sohal, 1998). However, diabetes alters the primary sites of superoxide generation so that complex II becomes the primary source of electrons that contribute to superoxide formation under diabetic conditions (Nishikawa et al., 2000). Another source of ROS in diabetes is NAD(P)H. Several lines of evidence support that NAD(P)H oxidases are a major source of glucose
induced ROS in the vasculature and kidney cells, confirming thus NAD(P)H as a mediator of diabetic complications (Li and Shah, 2003). Involvement of other cells has not been satisfactorily confirmed. Since hypertension is a common complication of diabetes, it is possible that expression of NAD(P)H oxidase is regulated similarly in both disease states. This arises from increased angiotensin II labelling in cardiac myocytes and endothelial cells from human diabetic patients (Li and Shah, 2003). High glucose-induced formation of ROS and p47phox [cytosolic component of activated NAD(P)H] can be blocked with Ang II type 1 receptor antagonists, confirming thus a link between the two pathways of NAD(P)H oxidase activation. The NAD(P)H oxidase-mediated production of ROS in diabetes can be suppressed by a variety of PKC inhibitors, implicating this family of kinases in the regulation of hyperglycemia-induced NAD(P)H oxidase activity.

**ROS and beta cell damage**

More than three decades ago, it was demonstrated that pancreatic islets contain relatively small amounts of the antioxidant enzymes CuZn-SOD, Mn-SOD, catalase, and glutathione peroxidase (GPx) (Grankvist et al., 1981). Further work demonstrated that β cells in rats were sensitive to peroxide and that the activity of GPx was low (Gorus et al., 1982). These and many other observations have reinforced the notion that the intrinsically low levels of antioxidant activity of islets render them particularly at risk for ROS-induced damage. Due to the low level of antioxidant enzyme expression and activity, the beta cells are at greater risk of oxidative damage than tissues with higher levels of antioxidant protection. For protection from the highly toxic hydroxyl radical and other ROS, the beta cells must metabolize hydrogen peroxide via catalase and GPx. However, a potentially major problem for the beta cells is their unusually low complement of SOD, catalase and GPx. This unusual situation sets up the beta cell as an easy target for ROS, whether generated by interactions with cytokines or elevated level of glucose.

Recent studies suggest that oxidative and nitrosative stress play major role in the onset of diabetic complications. Nitric oxide synthase oxidizes arginine to citrulline in the presence of biopterin, NADPH, and oxygen. Generally, nitric oxide at physiological levels produces many benefits to the vascular system. However,
increased oxidative stress and subsequent activation of the transcription factor NF-κB have been linked to the development of late diabetic complications. The NF-κB enhances nitric oxide production which is believed to be a mediator of islet beta-cell damage.

**Mechanism of action of alloxan**

Alloxan (2,4,5,6-tetraoxypyrimidine; 5,6-dioxypyrimidine) was first described by Brugnatelli in 1818. The diabetogenic properties of this drug were reported in the year 1943 by Dunn and McLetchie, who studied its effect in rabbits and reported specific necrosis of pancreatic islets. Since then, alloxan diabetes has been commonly utilized as an animal model of insulin dependent diabetes mellitus (IDDM). Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing beta cells in the pancreas when administered to rodents and many other animal species. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals with characteristics similar to type 1 diabetes in humans. Alloxan is selectively toxic to insulin-producing pancreatic beta cells because it preferentially accumulates in beta cells through uptake via the GLUT2 transporter. Alloxan, in the presence of intracellular thiols, generates ROS in a cyclic reaction with its reduction product, dialuric acid. It is then re-oxidized back to alloxan establishing a redox cycle generating superoxide radicals (Munday, 1988). Moreover, superoxide radicals undergo dismutation to hydrogen peroxide. In presence of Fe$^{2+}$ and hydrogen peroxide, highly reactive hydroxyl radicals are then formed by Fenton reaction. The action of hydroxyl radicals following alloxan treatment has been reported *in vitro* (Grankvist *et al.*, 1981; Munday, 1988) and *in vivo* (Kurahashi *et al.*, 1993). Alloxan mediated toxicity of beta cells is initiated by free radicals formed in this redox reaction.

Alloxan has two distinct pathological effects; it selectively inhibits glucose-induced insulin secretion through specific inhibition of glucokinase, the glucose sensor of the beta cell, and it causes a state of insulin-dependent diabetes through its ability to induce ROS formation, resulting in the selective necrosis of beta cells. These two effects can be assigned to the specific chemical properties of alloxan, the
common denominator being selective cellular uptake and accumulation of alloxan by the beta cells.

**Beta cell selectivity of alloxan**

The alloxan molecule is structurally so similar to glucose that the GLUT2 glucose transporter in the beta cell plasma membrane accepts this glucomimetic and transports it into the cytosol (Gorus *et al.*, 1982). Alloxan does not inhibit the function of the transporter (Elsner *et al.*, 2002), and can therefore selectively enter beta cells in an unrestricted manner. Alloxan has a central 5-carbonyl group that reacts very avidly with thiol groups. Glucokinase (hexokinase IV) is the most sensitive thiol enzyme in the beta cell (Tiedge *et al.*, 2000). Alloxan reacts with two -SH groups in the sugar binding site of glucokinase resulting in the formation of the disulfide bond and inactivation of the enzyme. Inhibition of glucokinase reduces glucose oxidation and ATP generation (Gunnarsson and Hellerstrom, 1973) thereby suppressing the ATP signal that triggers insulin secretion (Lenzen and Panten, 1988).

**Beta cell toxicity and diabetogenicity of alloxan**

Alloxan exerts its diabetogenic action when administered intravenously, intraperitoneally or subcutaneously. It exhibits a high affinity to the SH-containing cellular compounds, reduced glutathione (GSH), cysteine and protein-bound sulphydryl groups (including SH containing enzymes) (Lenzen and Munday, 1991). The reduction of alloxan to dialuric acid in the cell requires a suitable thiol, typically the tripeptide glutathione (GSH) to generate the redox cycling partner, dialuric acid, and oxidised glutathione (Bromme *et al.*, 2000) The triketone structure of alloxan is vitally important for this two-step reaction with glutathione (Elsner *et al.*, 2008) which generates the alloxan radical as an intermediate product. Thiols such as cysteine (which are present at lower concentrations in the cell), dithiols and ascorbic acid are also suitable reducing agents and may therefore contribute to alloxan reduction (Elsner *et al.*, 2006). Alloxan can also generate ROS by reaction with thiol groups on enzymes (Lenzen and Mirzaie-Petri, 1992) and albumin (Sakurai and Miura, 1989). During each typical redox cycle a small amount of ‘Compound 305’, an alloxan–GSH adduct, is formed (Bromme *et al.*, 2002). The intracellular concentration of compound 305 increases in a time-dependent manner which gradually decreases
the amount of reduced GSH available in the cell for redox cycling, thus producing a lower pro-oxidative ratio between alloxan and GSH, rather than a higher antioxidative ratio.

**Hyperglycemia and free radical link**

Pathogenesis of diabetic complications continues to be a central issue in current diabetes research. Hyperglycemia, a key clinical manifestation of diabetes mellitus causes enhanced non-enzymatic glycosylation of proteins that leads to secondary complications in diabetes (Brownlee et al., 1984; Kirschenbaum, 1984). In diabetes, sugar concentration, extent of glycation and the degree of damage increases in parallel. Free radicals are formed disproportionately by glucose auto-oxidation, non-enzymatic glycation and due to subsequent degradation of glycated proteins. Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death. Various mechanisms have been suggested for the formation of reactive oxygen free radicals. Glucose oxidation is believed to be the main source of free radicals (Robertson et al., 2003). In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical. This radical reduces molecular oxygen to generate superoxide radical and becomes oxidized itself to a dicarbonyl ketoaldehyde that is converted into reactive ketoaldehydes that reacts with protein amino groups forming a ketoamine. Ketoamines are similar to, although more reactive, than Amadori products and participate in AGE formation (Ahmed, 2005). The superoxide 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; Robertson et al., 2003). Superoxide radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Zou et al., 2002). Hyperglycemia is also found to promote lipid peroxidation of low density lipoproteins (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Kawamura et al., 1994). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then AGEs (Mullarkey et al., 1990; Thomas et al., 2005).
**Superoxide radical**

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems, by autooxidation reactions, and by nonenzymatic electron transfers that univalently reduce molecular oxygen. In aqueous solution, O$_2^-$ can oxidize ascorbic acid. It can also reduce certain iron complexes such as cytochrome c and ferric ethylenediaminetetraacetic acid (Fe$^{3+}$-EDTA). Superoxide dismutase (SOD) accelerates the dismutation of O$_2^-$, converting it to H$_2$O$_2$ and O$_2$. During glycation, Amadori products generate O$_2^-$ which is converted to H$_2$O$_2$ by non-enzymatic dismutation reaction (Nagai *et al.*, 1997).

**Hydroxyl radical**

It is a highly reactive oxygen-centered radical with an estimated in vivo half-life only 10$^{-9}$s. One feature of OH is that it begets another radical i.e. when it reacts with a molecule, the result is the formation of another radical species. The resulting species usually has lower reactivity than OH. Hydroxyl radical attacks proteins, DNA, PUFA in membranes and almost any biological molecule it touches. During glycation, hydroxyl radical generated by Fenton reaction between Fe$^{3+}$ and Amadori derived endogeneous H$_2$O$_2$ plays an important role in oxidative cleavage of Amadori compounds into CML (Nagai *et al.*, 1997).

**Protein damage by free radicals**

Protein damage by free radicals is critical in many biological processes (Cabiscol *et al.*, 2000). It causes cross-linking, backbone fragmentation, as well as amino acid modification leading to functional inactivation of proteins (Dean *et al.*, 1997). Amino acids can undergo oxidative damage if they interact with oxygen free radicals. As free radicals are capable of initiating chain reactions, cross-linking of soluble and membrane/or membrane bound proteins yield larger aggregates. The direct consequence is a change in higher order structure of the protein, resulting in loss of biological function (Dhalla *et al.*, 2000; Schoonover, 2001). Usually the inactivated proteins are degraded, so that proteolysis forms a secondary defence after anti-oxidants. However, when the target proteins are critical for rapid homeostatic
mechanisms (e.g., transport proteins) or when the proteolytic defence and/or other anti-oxidant defences are overwhelmed, toxic events may ensue. The physiologically damaged proteins are degraded and replaced by the cells (Thornalley et al., 2003). Cellular proteolysis liberates the glycated and oxidized amino acids as free adducts. The plasma concentration and urinary secretion of the free adducts are increased excessively in type 1 diabetes (Ahmed et al., 2005a).

**Protection against glycation**

Having understood the damaging effects of glycation, scientists are exploring the methods of protection against glycation. The underlying inhibitory mechanisms of anti-glycating agents is mainly through the blocking of sugar attachment to proteins, attenuating glycoxidation and oxidative stress through trapping or scavenging some intermediates including reactive dicarbonyls, free radicals and nitrogen species produced in the process of glycation. Recent studies on AGE inhibition by synthetic compounds and natural products have led to the development of various AGE inhibitors and AGE-cross-link breakers.

**Anti-glycating agents**

**Synthetic inhibitors**

Synthetic AGE inhibitors are generally strong nucleophiles capable of reacting with reducing monosaccharides and other carbonyl metabolites and hence prevent modifications of protein amino groups (Khatami et al., 1988; Lewis and Harding, 1990). A few of them exert their effect at the early stage of glycation (Type a and b inhibitor) by interfering with the initial attachment between reducing sugars and amino groups, thus inhibiting the further formation of Schiff base and AGEs (Harding and Ganea, 2006). Several potential drug candidates have been examined in vitro or in diabetic animal models (Blakytny and Harding, 1992; Shyadehi and Harding, 2002). A competitive mechanism of action of these drug candidates is mainly ascribed to their scavenging abilities on both reactive carbonyls (Type c inhibitor) and radicals (Type c inhibitor) formed during glycation, or blocking the formation of intermediate Amadori products (Type e inhibitor) (Harding and Elena, 2006).
Among the pharmacological agents tested, aminoguanidine (AG) has been reported to be one of the most effective inhibitors of AGE formation in lens proteins (Lewis and Harding, 1990; Matsumoto et al., 1997). Other guanidine derivatives like arginine (Menzel et al., 1991) and metformin (Beisswenger et al., 1999) have shown similar effects on protein glycation. It has been found that free amino acids (lysine, glycine, glutamic acid and aspartic acid) reduce the incorporation of radioactive labeled glucose (Ramakrishnan et al., 1996) and galactose (Ramakrishnan et al., 1997) into lens proteins. Potential beneficial effects on the prevention of protein glycation have been reported for vitamin B1 and vitamin B6 (Booth et al., 1996), carnosine (Hipkiss, 2005; Argirova and Argirov, 2003) and taurine (Devamanoharan et al., 1997).

**Natural inhibitors**

Natural products as anti-glycating agents are relatively safe for human consumption as compared to synthetic compounds. The anti-glycation activities of both plant materials and naturally occurring phenolic compounds with antioxidant properties have been investigated (Salem, 2005; Ismail et al., 2010; Liu et al., 2011). As oxidative stress accompanies and accelerates the formation of AGEs, antioxidant compounds appear to be promising agents for the prevention of AGE formation.

**Anti-glycating agents as scavengers of free radical in hyperglycemia**

Several groups have reported that Schiff base or Amadori product generate reactive oxygen species (Mullarkey et al., 1990). Therefore, scavenging of free radicals by preventing radical formation and intercepting radical from further activity are done by antioxidants (Cotgreave and Gerdes, 1998). Any compound (natural or synthetic) with antioxidant and antiglycating activity might totally or partially alleviate this damage. Among the synthetic compounds, aminoguanidine is quiet interesting as it has both antioxidant and antiglycating properties. Aminoguanidine, a nucleophilic hydrazine has been proposed to react with the Amadori product and block further reactions (Januel et al., 2003; Bucala et al., 1994). Hydrazine compounds are capable of interaction with carbonyl groups of different biological constituents, including glucose. The hydrazine moiety of aminoguanidine can mop up with the carbonyl group of glucose and thus can prevent the attachment of the sugar.
residue with the amino groups of the proteins. It also prevents the loss of antioxidant enzyme activities as well as cellular damage (Kedziora-Kornatowska et al., 1998). Another compound thymoquinone (TQ) [2-Isopropyl-5-methyl-1, 4-benzoquinone] is one of the most active ingredients of *Nigella Sativa* seeds. Thymoquinone is a powerful antioxidant (Nagi et al., 1999; Ismail et al., 2010) and a potent superoxide radical scavenger (Badary., *et al.*, 2003; Kruk *et al.*, 2000). Moreover, animal studies have also shown that thymoquinone has anti-diabetic effect (Kanter *et al.*, 2003; 2004).

**Antioxidant classification**

Endogenous compounds in cells can be classified as enzymatic antioxidants and non-enzymatic antioxidants. The major antioxidant enzymes directly involved in the neutralization of reactive oxygen species are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) (Halliwell, 2007). Superoxide dismutase, the first line of defense against free radicals, catalyzes the dismutation of superoxide anion radical (O$_2^-$) into hydrogen peroxide by reduction. The hydrogen peroxide is transformed into water and oxygen by catalase or glutathione peroxidase. The selenoprotein Gpx enzyme consumes hydrogen peroxide for oxidizing reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, Gpx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH) (Bahorun *et al.*, 2006; Genestra, 2007). Non-enzymatic antioxidants include lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoids, antioxidant minerals (copper, zinc, manganese and selenium) and cofactors (folic acid, vitamins B1, B2, B6, B12).

**Antioxidants defence status in diabetic patients**

Aerobic metabolism is always accompanied by the production of ROS, consequently all aerobic organisms possess some sort of anti-oxidant defence system with enzymatic and non-enzymatic constituents capable of preventing excess radical production, neutralizing free radical and repairing the damage caused by them (Sies, 1993). Under diabetic conditions, ROS increases in various tissues and are involved in the development of diabetic complications (Dandona *et al.*, 1996; Brownlee, 2001;
Antioxidant defence mechanism are overwhelmed in diabetic patients and an altered balance between ROS production and antioxidant levels have been reported (Ceriello, 2000). The overproduction of ROS lowers antioxidant defence and alters enzymatic pathways in humans with poorly controlled diabetes mellitus (Hoghkinson et al., 2003).

**Oxidative stress under hyperglycemic conditions**

Pancreatic β-cells have emerged as a target of oxidative stress-mediated tissue damage (Kaneto et al., 1996; Evans et al., 2003). The β-cells express abundant levels of the high-Km glucose transporter GLUT2 and thereby display highly efficient glucose uptake when exposed to high glucose concentrations. Accordingly, extracellular hyperglycemia causes intracellular hyperglycemia in β-cells leading to the induction of ROS in pancreatic islets of diabetics. Sources of ROS production in cells are (1) the non-enzymatic glycosylation reaction (Matsouka et al., 1997), (2) the electron transport chain in mitochondria (Nishikawa et al., 2000) and (3) the hexosamine pathway (Kaneto et al., 2001). Glycation suppresses the insulin gene transcription in β-cells by provoking oxidative stress (Matsouka et al., 1997). Chronic hyperglycemia suppresses insulin biosynthesis and secretion by provoking oxidative stress through various pathways which play a role in maintaining normal β-cell function by regulating many important β-cell genes including insulin, GLUT2 and glucokinase (Petersen et al., 1994). Also, the electron transport chain in mitochondria is likely to be an important pathway triggering the production of ROS. Indeed, it has been suggested that mitochondrial overwork, which causes the emergence of ROS, is a potential mechanism causing impaired first-phase glucose-stimulated insulin secretion found in the early stage of diabetes (Sakai et al., 2003) as well as diabetic complications.

**Role of antioxidants in ameliorating oxidative stress**

Human body is equipped with several mechanisms to counteract oxidative stress. Both endogenous antioxidants and exogeneous antioxidants are involved in handling oxidants. The roles of antioxidants are to neutralize the excess of free radicals, to protect cells against their toxic effects and to contribute to disease prevention. An antioxidant functions in one of the two ways:
• chain-breaking
• or prevention

When a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin C, E, carotenoids, etc), or it simply disintegrates into an inoffensive product. The classic example of such a chain reaction is lipid peroxidation. Antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase can prevent oxidation by reducing the rate of chain initiation (e.g. either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and iron) (Young and Woodside, 2001).

**Beneficial effects of antioxidant therapy**

Beta cells are particularly sensitive to ROS because they are low in free-radical quenching enzymes such as catalase, glutathione peroxidase and superoxide dismutase. Oxidative stress is implicated in apoptosis of pancreatic beta cells and impaired insulin signalling in diabetes (Newsholme et al., 2007). Antioxidant treatment may exert beneficial effects in diabetes. Antioxidant treatment can suppress apoptosis in cells without changing the rate of cell proliferation, supporting the hypothesis that in chronic hyperglycemia apoptosis induced by oxidative stress causes reduction of cell mass. In a previous study, antioxidant treatment preserved the amounts of insulin content and insulin mRNA (Kaneto et al., 1999).

**Objectives of the present study**

Recent studies have clearly pointed out that nonenzymatic glycation products (early and advanced) are involved in hyperglycemia-induced diabetic complications. Efforts are continuing to develop physiologically active substances which can delay or prevent protein modifications by glycation. Accumulated Amadori products may lead not only to AGE deposition in tissues but also to other pathophysiological consequences, such as activation of oxidative stress by excessive free radical generation. These products and free radicals are known to cause severe protein damage resulting in major structural alterations which is implicated either through their functional disability or these being highly immunogenic. It has been found that
in diabetic state, serum proteins containing Amadori glucose adducts acquire biologic properties that are strongly linked to the pathogenesis of vascular complications of diabetes. The in vitro exposure of protein to glucose results in the non-enzymatic covalent attachment of glucose to lysine side chains in a manner that is observed in vivo. As lysine residues are potential target for early glycation, lysine rich proteins like HSA can be used as marker for early glycation induced changes and antibody detection against it might emerge as an important parameter for early diagnosis of diabetes related complications. Moreover, deciphering the mechanism of protein damage caused by glucose is fundamental in understanding the biochemical basis of diabetic complications and in the development of new therapeutic strategies.

The major objectives of the study were; i) to study biophysical and immunological changes in HSA after modification with glucose, ii) antiglycation effect of aminoguanidine and thymoquinone both in vitro and in vivo and iii) evaluation of autoantibodies against glycated-HSA in sera of type 1 diabetes patients. To achieve the objectives, the work was carried out according to the following plan:- Commercially available human serum albumin was modified by D-glucose to obtain high yield of Amadori products (an early glycation product). The Amadori product was assayed by NBT assay and authenticated by boronate affinity chromatography. Structural changes were studied by ultraviolet, fluorescence, circular dichroic and FT-IR spectroscopy, heat induced thermal denaturation, HPLC, LC-MS and ESI-MS. The inhibition of glycation and free radical production by aminoguanidine and thymoquinone have also been studied. Antigenicity of native and glycated-HSA was probed by inducing antibodies in rabbits and assessed by direct binding ELISA. Specificity of the induced antibodies was evaluated by competition ELISA and gel retardation assay. Binding profile of autoantibodies against glycated-HSA in sera of type 1 diabetic patients have been studied to assess the correlation between progressive rise in autoantibodies and secondary complications in diabetes. The polyclonal anti-glycated-HSA antibodies were used as immunochemical probe to detect glycation induced changes in albumin isolated from diabetic patients.