Discussion
Hyperglycemia plays a critical role in the pathogenesis of diabetes related complications by enhanced protein glycation. Glycation is a major cause of spontaneous damage to cellular and extracellular proteins in physiological systems, affecting 0.1-0.2% of lysine and arginine residues (Thoralley, 1999; Thoralley et al., 2003). Diabetic state promotes the Amadori-modification of many proteins in circulation (Jaleel et al., 2005). Such modifications on proteins may lead to generation of neo-antigens which could in turn initiate autoimmunity. The predominant form of glycated proteins in plasma exist as Amadori products rather than the more labile Schiff base form or AGEs (Curtiss and Witzman, 1983). Furthermore, concentration of Amadori proteins is at least 2% of serum proteins, whereas the concentrations of AGE-equivalents is less than 0.01% (Makita et al., 1992). Numerous studies have indicated that serum proteins containing Amadori glucose adducts play a substantive role in various diabetic complications (Hansson, 2005; Cohen et al., 2006). Physiologically relevant concentrations of Amadori-modified albumin possesses multiple proatherogenic effects that include promotion of oxidative stress, production of inflammatory mediators, endothelial damage and vessel wall hypertrophy. Free radicals are formed disproportionately in diabetes by glucose auto-oxidation, non-enzymatic glycation and subsequent degradation of glycated proteins (Hunt, 1988). Massive increase in ROS generation during acute and chronic hyperglycemia is attributed to non-enzymatic glycation, which might alter the structure and function of antioxidant enzymes such that they are unable to detoxify free radicals, exacerbating oxidative stress. Glycation not only causes an increase in free radical production but it also reduces free scavenging defences (Marra et al., 2002). This may lead to cellular damage. The effect of increased glycation on ROS generation and cellular damage, therefore, can be additive or synergistic. Therefore, preventing the non-enzymatic early glycation of relevant proteins or blocking their subsequent effects may be helpful in ameliorating the vascular complications of diabetes.

In the present study, HSA modified with varying concentration of glucose for a week resulted in high content of Amadori product as detected by NBT reagent. The reagent is specific for Amadori products and not for AGEs (Johnson et al., 1982; Lapolla et al., 2005). However, the Amadori yield decreased when glycated samples were treated with NaBH₄. The treatment causes elimination of keto group of Amadori products and consequent decrease in the fructosamine. DTPA added to the mixture of
HSA and glucose did not interfere with early glycation (i.e. Amadori formation) but prevented AGE formation by inhibiting glycoxidation and cross-linking (Graf et al., 1987; Fu et al., 1994; Lapolla et al., 2005). The occurrence of AGEs in our preparation was further ruled out by negligible absorbance of the glycated samples in 300-400 nm wavelength range. Additional evidence of HSA glycation was provided by the thiobarbituric acid (TBA) assay (Ney et al., 1981) which was originally developed for providing evidence of hexose adduction to haemoglobin (Fluckiger and Winterhalter, 1976). The method is now widely used for quantification of glycated proteins as it detects ketoamine linkage in glycated proteins. Increase in TBA reactive HMF in glycated samples was observed. This observation is supported by an earlier study (Ney et al., 1981).

SDS-PAGE of glycated-HSA samples showed gradual decrease in electrophoretic mobility with concomitant increase in band intensity. The observed shift in mobility may be due to the adduction of glucose moieties with the amino groups of the protein. The extent of Amadori adduct in glycated-HSA samples was evaluated on boronate affinity matrix. The glycated species retained on the boronate matrix speaks of Amadori product. The glycated species was retained on the boronate whereas, the non-glycated portion was washed away. On the basis of results it may be plausible to think that at high glucose concentration adduct formation is facilitated in a short period of time.

Early glycation primarily occurs on the ε-amino groups of lysine residues (Barnaby et al., 2011). Glycation of lysine residues might alter the tryptophan micro-environment. Since HSA contains only one tryptophan residue (Tryp-214), the observed loss in the fluorescence intensity in glycated-HSA samples could be ascribed to the alteration of tryptophan-214 micro-environment as a result of glucose adduction.

Since Amadori products are stable ketoamines they have a definite effect on protein structure. The results of far-UV CD studies on glycated-HSA indicated increase in alpha helix content as compared to native HSA. This is in agreement with an earlier report (Nejad et al., 2002), where incubation of HSA with low concentrations of glucose for 7 days destabilized protein’s structure but caused increase in alpha helix at high glucose concentration. Furthermore, the substantial
increase in the Tm of glycated-HSA may be linked to increase in alpha helical content as suggested earlier (Nejad et al., 2002).

FT-IR spectral analysis helped us locate the position and intensity of characteristic bands in native and glycated-HSA. It is well known that an IR spectrum of protein exhibits a number of amide bands, which represent different vibrations of peptide moieties (Rahmelow and Hubner, 1996). Amide I and II constitute two major bands of the protein infrared spectrum. Amide I band is mainly associated with the C=O stretching vibration of the peptide linkage and corresponds mainly to α-helix. The amide II band, in contrast, derives mainly from N-H in-plane bending and from C–N stretching vibrations (Rahmelow and Hubner, 1996). The shifting of amide I and II bands and decrease in intensity indicate changes in intramolecular bonding and in present case may arise from the impact on overall protein conformation due to early glycation. Appearance of a new peak at 1711.2 cm⁻¹ corresponding to the carbonyl group of glycated-HSA ketones suggested Amadori product (Wnorowsky and Yaylayan, 2003). The vibrational mode due to OH stretching is observed in the region 3100–3600 cm⁻¹ (Lertsiri et al., 1998). An increase in absorption in the stretching vibration region of OH groups observed in glycated-HSA sample could be due to the attachment of glucose moieties with HSA. Furthermore, the IR profile of glycated-HSA exhibited a low intensity absorption peak at 1082.7 cm⁻¹. This might be due to C-C/C-O stretching of glucose adducted to HSA, because carbohydrates (glucose) have strong absorption bands in this region (Mazdak and Jennifer, 2006).

Furosine, formed during acid hydrolysis of fructosyl-lysine (Amadori product) (Schleicher and Wieland, 1981; Wu et al., 1995; Krause et al., 2003) is currently the most specific and important indicator of early Maillard reactions. HPLC analysis of standard furosine showed a retention time of 28.3 min. A matching peak with a retention time of 28.9 min was observed in glycated-HSA. Native and NaBH₄ reduced glycated-HSA did not show any peak at the above retention time. Furosine presence in the hydrolysate of glycated-HSA was re-confirmed by LC-MS.

For a typical non-enzymatic glycation reaction to occur, the carbonyl group of reducing sugars (like glucose) must first react with the free amino groups of a protein to form Schiff’s base and the intermediate then undergoes Amadori rearrangement to
generate stable ketoamine product. Our ESI-MS data on glycated-HSA is in conformity with the above events and the ions at m/z 309, 273 and 225 suggests Amadori product, oxonium ion, and furylium ion respectively.

It is known that ROS are produced at many steps of the sequential glycation cascade. Both Schiff’s base and Amadori product generate free radicals. Superoxide radical is generated when the Amadori product takes on an enamino structure. It has been reported that glycated proteins in vitro produce 50 times more superoxide radicals and cause a twofold increase in peroxidation of lipid vesicles (Mullarkey et al., 1990). Formation of superoxide radicals under our experimental conditions was confirmed by using superoxide dismutase (known quencher of superoxide), AG and TQ. The results suggest that AG and TQ are potent quenchers of superoxide generated during glycation process. Our results are in agreement with previous reports showing that Amadori product generate ROS, mainly superoxide anions (Vallejo et al., 2000; Yoo et al., 2004; Ortwerth et al., 1988).

The antiglycating/antioxidant effect of AG and TQ was also studied in diabetic rabbits. The alloxan-induced type 1 diabetes model is well-established in rabbits. It is also known that alloxan causes severe necrosis of pancreatic β-cells (Dun and McLetchie, 1943; Gumieniczek et al., 2007). Both alloxan and its metabolite, dialuric acid, establishes a redox cycle and superoxide radicals are formed. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed. Thus, the cytotoxic action of alloxan on beta cells is mediated by the generation of superoxide and hydroxyl radicals (Munday, 1988; Kurahashi et al., 1993). High blood glucose level confirmed the efficacy of alloxan in producing experimental diabetes. However, the alloxan effect on pancreas of the rabbits was reduced in presence of AG and TQ. The effect may be attributed to the potent scavenging action of AG and TQ for superoxide and hydroxyl radicals.

Previous studies have shown that the concentration of Amadori-product exceeds that of AGEs not only in serum, but also in in vitro protein preparations that contain AGEs (Makita, 1992; Schalkwijk, 1999). Therefore, it may be argued that the effects that have been ascribed to AGEs may be in fact due to Amadori-proteins (fructosamines). Diabetics tend to have elevated concentration of sugars, therefore, the degree of glycation of Hb has been considered as an index of glycation (Jain et al., 133...
Glycation of Hb probably reflects the level of glycation of other proteins as well (Valeri et al., 2004). The most encouraging result of our in vivo study was the significant decrease in HbA1c and serum fructosamine in diabetic animals treated with AG and TQ as compared to diabetic group. Here, it is important to mention that although AG is a potent inhibitor of AGEs, but under our experimental conditions AG prevented the formation of early glycation products (Amadori product). However, the mechanism by which AG prevents the formation of early glycation products remain to be fully elucidated. Previous studies suggest that AG inhibits the original glycation by limiting the attachment of glucose with the amino groups of the proteins (Lewis and Harding, 1990; Hirsch et al., 1995; Khatami et al., 1988). It has been further suggested that AG probably interacts with the carbonyl groups of early glycation products and forms stable products that do not react further (Edelstein and Brownlee, 1992; Chen and Cerami, 1993). Against this backdrop, the reduction in the indices of early glycation products in our study may be attributed to fast binding of AG (compared to glucose) with proteins or due to the interaction of AG with unstable earliest glycation products preventing their accumulation.

It has been widely held that oxidative stress plays vital role in the pathogenesis of various complications of diabetes. In healthy subjects both enzymatic and non-enzymatic antioxidants play important role in scavenging free radicals. During hyperglycemia increased glycation promotes free radical generation. Therefore, inactivation of enzymes (especially antioxidant ones) by glycation or free radical attack appears to be inevitable during hyperglycemia. SOD is inactivated by glycation at specific lysine residues (Arai et al., 1987). Glycation also induces random fragmentation of the enzyme (Ookawara et al., 1992; Park and Lee, 1995). The decreased serum level of SOD, catalase, GPx and GSH observed in diabetic rabbits reiterates that hyperglycemia promotes free radical generation. Restoration of the parameters of oxidative stress in AG and TQ treated group support their role as antioxidant/antiglycating agents. Hydroxyl radical attack on unsaturated fatty acids of phospholipid components of membranes produce malondialdehyde (MDA), a lipid peroxidation product. The serum MDA level in AG and TQ treated diabetic rabbits was found to be significantly dropped as compared to diabetic rabbits. This finding is consistent with the results of El-Missiry and El-Gindy (2000), where increased lipid peroxidation and decrease in antioxidant enzymes level in diabetes mellitus has been
reported. Carbonyl content is the most common marker of protein oxidation (Shacter, 2000) and provides a significant clue to the magnitude of oxidative stress under disease conditions, such as diabetes (Beal, 2002). The decrease in carbonyl content in AG and TQ treated diabetic rabbits may be attributed to the antioxidant action of both AG and TQ.

Increased nitric oxide (NO) production in early diabetes has been reported (Lindsay et al., 2009), which can damage pancreatic beta cell DNA, effect insulin secretion and cell viability. NO as a free radical is relatively unstable in oxygenated solutions where it rapidly and spontaneously reacts with molecular oxygen to yield a variety of nitrogen oxides. The only stable product of NO in oxygenated medium are nitrites and nitrates, which are considered to be indicators of NO production (Guevara et al., 1998). Nitrite level in AG treated diabetic rabbits sharply decreased in comparison to diabetic group. This shows that AG is a potent inhibitor of iNOS (Corbett and McDaniel, 1996). Moreover, TQ might have exhibited its protective effect by down regulating the immunological inflammatory effects mediated by nitric oxide (El-Mahmoudy et al., 2005).

Proteins modified by glycation and glycoxidation processes are immunologically active and can elicit strong antibody response. Native human serum albumin per se is immunogenic (Doyen et al., 1985). However, glycation and free radical modification of HSA may result in the generation of highly immunogenic new epitopes (Rasheed and Ali, 2006; Khan et al., 2010). Lysine residues play an important role in the enhanced immunogenicity of HSA (Arif et al., 2012). In vivo, direct role of glycated lysine residues in the immunogenicity has been advocated on the basis of reports of autoantibodies against glycated proteins in diabetes (Turk 2001; Araki et al., 2001; Ansari et al., 2009). The in vivo generation of autoantibodies appear to be the result of hyperglycemia mediated modifications in protein structure leading to induction of antibodies.

Antigenicity of native and glycated-HSA was evaluated by inducing antibodies in rabbits. Glycated-HSA was found to be a potent immunogen and induced high titre antibodies compared to native HSA which induced low titre antibodies. The results suggest glycation-induced generation of immunologically active neo-epitopes. Specificity studies on affinity purified anti-glycated-HSA IgG reiterated that glycated-
HSA was preferentially recognized. Notable feature of the anti-glycated HSA IgG was that the maximum inhibition in antibody binding was caused by glycated-HSA followed by NaBH₄-reduced glycated HSA and least with the native HSA. Reduction of Amadori product by NaBH₄ leads to the elimination of keto group which causes shift in the existing equilibrium between the linear configuration and the furanose or pyranose ring structures more towards the linear configuration (Schalkwijk et al., 1999). Therefore, anti-glycated-HSA antibodies showed decreased binding with NaBH₄ treated glycated-HSA. This is in agreement with the earlier report that the ring structure of Amadori-HSA is essential for antibody recognition (Schalkwijk et al., 1999). Furthermore, anti-glycated-HSA antibodies showed binding with glycated BSA, IgG, histone and poly-L-lysine, most likely due to epitope sharing. Furthermore, glucose-modified lysine was effectively recognized by anti-glycated-HSA antibodies. The importance of lysine residues in Amadori generation has been demonstrated in HSA and poly-L-lysine (Ansari et al., 2009; Arif et al., 2012).

Accumulation of Amadori-proteins and protein-AGE play important role in the pathogenesis of various diabetes related complications. More recent studies have highlighted the role of Amadori-configured proteins in various complications of diabetes (Schalkwijk et al., 2000). Amadori products have been found in various tissues of diabetic rats as well as in diabetic subjects (Myint et al., 1995; Schalkwijk et al., 1999; Jaleel et al., 2005). That Amadori-albumin is an independent and potent trigger of complications of diabetes has been demonstrated in experimental animals. The results pointed out that neutralizing the biologic effects or inhibiting the formation of glycated albumin can ameliorate structural, functional and cell biology abnormalities in the kidney and retinal microvasculature of diabetic rodents (Cohen et al., 1994, 2005).

Much of the information regarding the possible role of Amadori-HSA in the pathogenesis and natural history of diabetes has been obtained from type 1 diabetic subjects with and without secondary complications. The likely involvement of glycated-HSA in diabetes related complications was derived from binding of autoantibodies in sera of diabetic subjects with and without secondary complications. Autoantibodies in type 1 diabetic subjects with secondary complications showed higher recognition of glycated-HSA, indicating a causal role of Amadori-glycated
proteins in the pathogenesis of diabetes related complications. Amadori-HSA is found to be immunogenic and type 1 diabetic subjects with retinopathy and nephropathy have higher Amadori albumin (Schalkwijk et al., 1999; Cohen and Ziyadeh, 1994). Furthermore, animal studies have shown that elevated concentrations of Amadori albumin promote a generalized vasculopathy (Cohen et al., 1996) and have been implicated in the development of diabetic nephropathy (Cohen et al., 1994; Cohen and Ziyadeh, 1996) and retinopathy (Clements et al., 1998). Likely presence of in vivo Amadori-epitopes on HSA purified from diabetic subjects was probed with experimentally produced anti-glycated HSA antibodies. The data suggests that a significant population of diabetic albumin bears the epitopes typical of Amadori-HSA.

**Based on the above studies following conclusions can be drawn:-**

- Good amount of Amadori product can be formed if HSA is kept under high glucose concentration for one week.

- Early glycation of HSA results in hyperchromicity and decreased fluorescence intensity.

- Glycation confers stability on HSA.

- Under our experimental conditions Amadori product formation was confirmed by FT-IR, HPLC, LC-MS and ESI-MS

- Free radicals are generated during Amadori product formation, and both AG and TQ are effective quenchers of free radicals.

- Amadori formation during hyperglycemia may be ameliorated by AG and TQ.

- During hyperglycemia immunogenicity of HSA may be augmented due to generation of neo-epitopes.

- The induced antibodies against glycated-HSA were specific for the immunogen but exhibited polyspecificity and recognise glycated form BSA, poly-L-lysine, IgG and histone.
- Decreased recognition of NaBH₄ reduced glycated-HSA by anti-glycated-HSA antibodies indicate immunogenic role of Amadori product.

- Autoantibodies derived from the sera of diabetic subjects, with and without secondary complications, showed higher recognition of glycated-HSA. This indicates that the major population of autoantibodies in diabetes patients are directed against Amadori component of the glycated-HSA.