Discussion
Glycation is a type of post translational modification that occurs when the aldehyde/keto group of sugar or its derivative dicarbonyl compounds undergo an association with amine group of biological macromolecules resulting in the formation of Schiff base which readily forms amadori/early glycation products. These early glycation products following a series of dehydration, oxidation, cyclisation reactions get transformed into stable advanced glycation end products (AGEs) (Goldin et al., 2006). Sugar derived dicarbonyl compounds (methylglyoxal, glyoxal, 3-deoxyglucosone) are known to have much more reactivity than the parent sugar and amongst all methylglyoxal (MG) deserves special attention (Rabbani and Thornalley, 2014). It is a yellow coloured pungent smelling compound formed during glycolysis as well as fatty acid and amino acid metabolism (https://pubchem.ncbi.nlm.nih.gov/compound/methylglyoxal, Allaman et al., 2015). The major source however is glycolysis, in particular from glyceraldehyde-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) (Beisswenger et al., 2003). As per estimates 0.1 - 0.4 % of the glycolytic flux generates MG. Rat tissues have a MG production rate of 0.1 mM/day (Richard, 1991; Kalapos, 2008a). Kuhla et al., reported CSF level of MG between 10-20 µM, its cellular level are in micro molar (µM) range (Kuhla et al., 2005; Rabbani and Thornalley, 2010) with significantly higher level at the site of production. Because of its short half-life, it is able to target molecules close to its site of production (Kalapos, 2008b). It reacts with arginine and lysine amino acids of protein and form AGEs (Degenhardt et al., 1998). They are very harmful and their long term accumulation results in multiple abnormalities and disease states. On the basis of sugar and macromolecule type involved, different types of AGEs are found in the tissues. They can be further categorized into fluorescent and non-fluorescent ones. Most abundant of these include: pentosidine (Dyer et al., 1991), Nε-(carboxymethyl) lysine (Ahmed et al., 1986), pyrraline (Njoroge et al., 1987), imidazolone (Niwa et al., 1997), crossline (Obayashi et al., 1996), etc., with MG as the reactant sugar, the main AGEs formed are: argpyrimidine, imidazolone derivatives, Nε-(carboxyethyl)lysine etc. (Nowotny et al., 2015). It has to be noted that there is an extricable linkage between glycation and oxidation with reactive oxygen species (ROS) release seen at various stages of glycation (Kaneto et al., 2010). It may be the protein bound sugar that undergoes auto-oxidation releasing ROS. The sugar in free form can also release ROS, a process termed as auto-oxidative glycosylation. Besides, the release of ROS is also seen from glycolytic intermediates, Schiff base and amadori products (Hunt et al., 1990; Nishikawa and Araki, 2013). MG in particular is also a source of free radical and it is both during formation and
degradation of MG that free radicals are released. Thus it can be said that oxidative stress plays an important role in MG toxicity (Kalapos., 2008; Desai et al., 2010). ROS is a collective term for free radicals as well as non-radical species. Amongst these, hydroxyl radical (OH•), the neutral form of hydroxide ion the is most potent; its short in-vivo half-life limits its action to the site of release (Valko et al., 2007). It is formed by the reaction between superoxide anion (O2•−) and ferrous ion (Fe2+), the process called Fenton’s reaction. Normally Fe2+ is not available in free form and is bound to [4Fe-4S] cluster of dehydratase lyase family. During stress, O2•− triggers the release of bound iron (Lloyd et al., 1997; Jang and Imlay., 2010). Besides, OH• is also released in hyperglycemic conditions. Both the formation and action of AGEs is linked to oxidative stress. AGE’s when interact with their receptors activate NF-κB, cytokines, growth factors, adhesive molecules, increases vascular permeability and other toxic effects, all inducing oxidative stress (Yan et al., 1994; Bierhaus et al., 1998; Kislinger et al., 1999). AGEs target proteins and cause a number of structural alterations. These structural changes give rise to neo-epitopes that triggers autoimmune diseases like diabetes and rheumatoid arthritis (RA) (Wenzlau and Hutton., 2013; Burska et al., 2014). Amongst proteins, IgG being abundant and stable with a half life of 24 days is an attractive glycoxidation target (Correia., 2010). It is a 150 KDa glycoprotein having 2 each of heavy and light chain types linked together by inter and intrachain disulfide bonds (Vidarsson et al., 2014). Its long half-life, stable nature and high serum concentration makes it vulnerable to glycoxidation. This tight junction between glycation associated oxygen toxicity is a causative factor of many diseases like diabetes, chronic renal failure, atherosclerosis, cataract and Alzheimer’s disease (Well-knecht et al., 1996; Newkirk et al., 1998; Kalousova et al., 2004). Diabetes mellitus is a multifactorial complex metabolic disease in which the body become resistant to insulin secretion or action (Ozougwu et al., 2013). The disturbed insulin level in turn causes abnormality in glucose metabolism leading to hyperglycemia (ADA., 2009). Broadly, it is characterized into 2 major types i.e. type 1 diabetes mellitus (T1DM) (10 %) and type 2 diabetes mellitus (T2DM) (90 %). Besides, there are other categories also. Owing to its rapidly rising rate, it is a disease of great concern (Atkinson., 2012). Hyperglycemic conditions lead to free radical production via several mechanisms like polyol pathway, AGE pathway, protein kinase C pathway (Giacco and Brownlee., 2010). So, diabetes and its associated co-morbidities are a result of cumulative glycative and oxidative damage.
RA is a complex disease whose exact cause has been not elucidated yet. It is characterized by persistent inflammation of joints that ultimately lead to damage of cartilage, bone and eventual disability (Picerno et al., 2015). It is evident from numerous studies that oxidative stress plays a key role in destruction associated with RA (Wruck et al., 2010). RA synovium consumes 20 times more oxygen than normal tissue that in turn causes free radical generation (Edmonds et al., 1995). Oxidative stress in turn contributes to the formation of AGEs, which damage proteins and generate neoepitopes resulting in autoimmune response (Takahashi et al., 1997; Miyata et al., 1998). The major autoantibodies in the sera of RA patients is rheumatoid factor (RF), a group of antibodies directed against the Fc portion of IgG. They can be either IgM, IgE or IgG itself, however, the majority are of IgM types. Thus it can be concluded that IgG plays a fundamental role in chronic synovial inflammation, a characteristic feature of RA (Abruzzo and Heimer., 1974).

Keeping in mind a vicious interdependency between MG, ROS, diabetes, RA and IgG, this study has aimed to mimic this situation in vitro. IgG, the most abundant and stable serum protein, was modified with MG followed by OH’. Structural and immunological characterization of modified IgG indicated that MG in combination with OH’ is able to cause extensive damage. UV spectral analysis revealed marked increase in absorbance of hydroxyl radical modified glycated IgG (OH’-MG-IgG) compared to methylglyoxal modified IgG (MG-IgG), hydroxyl radical modified IgG (OH’-IgG) and native IgG (N-IgG). The significant hyperchromicity in UV region is suggestive of conformational alterations such as exposure of chromophoric aromatic amino acid residues. These structural changes are due to unfolding and/or fragmentation of molecule (Jairajpuri et al., 2007). This ligand mediated increase in absorbance have been studied earlier with many different proteins (Ahmad et al., 2013; Neelofar et al., 2015). The aromatic amino acid tyrosine and tryptophan offer intrinsic fluorescence, a meaningful parameter to monitor properties of unfolded state (Duy and Fitter., 2006). It is the indole chromophore in tryptophan that is most popular factor offering intrinsic fluorescence (Sarkisyan et al., 2012). We report quenching in fluorescence intensity upon modification. The change observed points to alteration in the structure and/or conformation around aromatic amino acid residues.

The quenching at 275 nm indicates microenvironment change in and around tyrosine residues whereas at 295 nm, the affect is around tryptophan microenvironment.
(Gorinstein et al., 2000). Maximum quenching in OH⁻-MG-IgG followed by MG-IgG and OH⁻-IgG indicates high 3-D structural alteration post glycoxidation. Another fluorescence spectrum characteristic of AGE formation was observed in MG-IgG and OH⁻-MG-IgG when the samples were excited at 370 nm with emission at 435 nm. Thus, AGE formation after MG treatment is evident from the above results. Furthermore, the structural changes in MG-IgG, OH⁻-IgG and OH⁻-MG-IgG were analyzed by SDS-PAGE. The detergent masks the charge on protein so that they move according to their molecular weight (Suhail et al., 2011). A noticeable decrease in band intensity along with retention of sample at the origin of the well with increasing concentration of OH⁻ and MG may be due to cross linking (dityrosine and cysteine formation) and aggregation (Guptasarma et al., 1992). Maximum decrease in band intensity compared to native counterpart was found in OH⁻-MG-IgG. The results give clear indication that maximum crosslinks and aggregates are formed when MG and OH⁻ are used in combination. MG and OH⁻ mediated formation of crosslinks and aggregates (dimers, trimers and even tetramers) have been reported in literature. 90 % of these linkages are non-disulfide covalent cross links whereas rest are due to non-covalent interactions and disulphide bonds (Davies., 1987, Ansari and Dash., 2013). The SDS-PAGE results are in full agreement with spectral findings. Circular dichroism (CD) spectroscopy provides a convenient means to detect secondary and tertiary structural changes that may arise as a result of modifications (Kelly et al., 2005). It is being increasingly recognised as a valuable technique for examining the structure of proteins in solutions. The signal arise as a result of differences in absorption of left handed and right handed polarized light (Pelton and McLean., 2000). In proteins peptide bonds, aromatic amino acid residues and disulfide bonds are chromophores of interest. Amide chromophore has an absorption in the range of 190 – 250 nm, the so called far-UV CD. Our far-UV CD results suggest glycoxidised samples (OH⁻-MG-IgG) to have maximum decrease in the shape factor value S²₀₀nm/²₁₁₇nm (i.e. ellipticity at 200 nm/ ellipticity at 217 nm) followed by MG-IgG and OH⁻-IgG in comparison to native counterpart. These findings points that loss in β sheet conformation and increase in α helix and random coil is maximum when the samples are glycoxidised (Kelly et al., 2005; Arfat et al., 2014 ). Thus the combined effect is highly potent in causing structural alterations in IgG (Li et al., 2005). The near-UV CD spectra gives the fingerprint of proteins tertiary structure. Signals in the near-UV region i.e. from 260 – 320 nm arise from aromatic amino acid residues. Each
of these residues has a characteristic wavelength profile. The number and type of
aromatic amino acid residues, their mobility and nature of their environment, their
spatial distribution in the protein together arise signals that give the shape and
magnitude of near-UV CD spectrum (Kelly and Price., 2000; Gasymov et al., 2014).
Reports indicate that IgG exhibit a CD spectrum having positive band at 295 nm,
attributed to phenyl alanine and sulphhydryl groups, the negative band fall in the range
of 250 – 290 nm and is attributed to tyrosine and tryptophan residues (Jiskoot et al.,
1991; Demeule et al., 2007; Aghaie et al., 2008; Hawe et al., 2009). Our results
indicate that modification resulting in exposure of aromatic amino acid residues as
well as oxidation of sulphhydryl groups is maximum post glycoxidation. The
secondary structural changes were further substantiated by fourier transform infrared
spectroscopy (FTIR), a useful tool for quantitative determination of changes in
secondary protein structure. This technique is more sensitive than CD and has an
ability to analyse protein in various conditions (Maruydama et al., 2001). IR spectrum
of protein has most prominent bands between 1700 to 1450 cm\(^{-1}\). Amide I bands
arises mainly from vibrations due to carbonyl stretching of amide group (Schule et al.,
2007). The amide II bands arises due to C-H stretching coupled with N-H bending
(Sirotkin et al., 2001). Peak shift in FTIR document that secondary structure of IgG is
altered following modification. A maximum shift of 16 nm and 20 nm in amide I and
amide II peak positions respectively compared to N-IgG is observed in OH\(^{-}\)-MG-IgG.
It is 6 nm, 8 nm and 12 nm, 10 nm more than MG-IgG and OH\(^{-}\)-IgG. The structural
perturbations induced by OH\(^{-}\) and MG were further confirmed by extrinsic
fluorescence studies using 8-Anilinonaphthalene-1-sulfonic acid (ANS). The dye
binds to hydrophobic clusters of protein, if accessible (Welfle et al., 1999). The more
the hydrophobic residues exposed, more the dye binds. An increase in fluorescence of
ANS in modified IgG compared to native counterpart suggest more of hydrophobic
patches are exposed as a consequence of modification of protein. Our ANS
fluorescence results suggest that glycoxidation (OH\(^{-}\) and MG together) causes
maximum alteration in protein conformation leading to high exposure of hydrophobic
surfaces. The protein unfolding is also caused by MG and OH\(^{-}\) although to a lesser
extent compared to glycooxidised samples. Protein carbonyl content is the most widely
used marker of protein oxidation (Shacter., 2000). Oxidative modification of side
chain of lysine, arginine, proline forms carbonyl groups (Berlett and Stadtman.,
1998). Increased carbonyl content has been attributed with potential role in various
pathological conditions. It was found that compared to N-IgG, a 3.75, 4.4 and 7.4 times increase in carbonyl content was found in OH’-IgG, MG-IgG and OH’-MG-IgG respectively again pointing glycoxidation to be highly damaging, similar increase in carbonyl content post modification is also reported earlier. Yadav et al., reported a 55% increase in carbonyl content of HSA post PON treatment compared to native analogue (Yadav et al., 2015). Another study reported the oxidative potential of heparin glucose system with a 35% increment observed post treatment (Finotti and Pagetta., 1997). Similar pattern was found on thiol content (a reactive functional group in cysteine side chains and an oxidative stress marker). A 1.87, 2.55 and 4.78 fold decrease in thiol content was seen in OH’-IgG, MG-IgG and OH’-MG-IgG compared to native counterpart. These estimations again suggest oxidative potential of glycoxidation to be much more than either alone. Various earlier studies have reported MG and OH’ to be potent aggregate forming agents (Biswas et al., 2008; Bento et al., 2010; Lipinski., 2011). A wide variety of diseases are known to occur due to aggregation of proteins. Factors such as temperature, pH, organic and inorganic solvents, mechanical stress, mutations are known to disturb structure of protein ultimately resulting in aggregation (Tatar et al., 2013). Modifications like oxidation, deamidation, truncation are known to cause aggregation in eye lens resulting in cataract formation (Moreau and King., 2012). Protein aggregation and inclusion body formation has been reported to be the leading cause of neurodegenerative diseases like Alzheimer’s disease, Parkinson’s disease, prion disease, Hungtinton’s disease and Amytrophic lateral sclerosis (Ross and Porier., 2004). Aggregation of IgG results in the formation of rheumatoid factor, the leading cause of RA (Pisco et al., 1985). MG in combination with OH’ are highly capable of aggregate formation as detected by several techniques. Thioflavin T (ThT), a benzothiazole dye is used to visualize aggregates. It has a unique property of binding to amyloid and amyloid like fibrils forming fluorescent complexes. This property is used to test ligand mediated formation of amyloid like fibrils in proteins (Levine., 1993; Levine., 1999). The dye incorporated into fibrils gets excited at 435 nm resulting in hyperchromicity in fluorescence spectra. Our results give a clear picture of amyloid type aggregate formation in IgG after treatment with MG, OH’ and the two together. The fluorescence emission is maximum in case of OH’-MG-IgG indicating maximum disturbance in its structure. It is this in vivo disturbance in protein folding that leads to diseases such as Alzheimer’s disease, Parkinson’s disease, type 2 diabetes and prion
diseases (Kuznetsova et al., 2012). In another study glucose oxidase showed ThT binding property when incubated with ribose and arabinose for 15 days indicating aggregation (Khan et al., 2012). Similar studies on ovalbumin report aggregation with denaturant as temperature (Naeem et al., 2011). Furthermore, congo red (CR) staining technique was performed to confirm our ThT results. This histologic dye is used extensively for amyloid detection. Like ThT, this dye also bind to amyloid type aggregates detected as a red shift in absorbance spectra (Hameetha et al., 2012). We report similar red shift in the absorbance spectra of IgG modified by MG, OH⁻ and OH⁻-MG. The shift is maximum in case of OH⁻-MG-IgG. Thus, it can be interpreted that glycoxidation is highly damaging inducing amyloid type aggregates in IgG. Slide pictorial analysis of CR stained samples under polarized light microscopy justify the above results. N-IgG appeared like uniformly distributed granules. MG-IgG and OH⁻-IgG samples showed red colored rough and irregular spots of variable sizes spread non-uniformly over the background. However, highly cross-linked and entangled aggregates were seen in OH⁻-MG-IgG. Earlier, congo red binding to amyloid in tissue sections have been performed (Lorenzo and Yankner., 1994; Seilheimer et al., 1997). The dye has shown to emit yellow green birefringence (Khurana et al., 2001). In addition, congo red binding with fibrillar β sheet insulin fibres is extensively studied (Klunk et al., 1999). Besides, many other proteins like ovalbumin, hemoglobin, hen egg white lysozyme and human serum albumin have shown a red shift in congo red absorbance spectra indicative of aggregation (Hu and Du., 2000; Cao et al., 2004; Juarez et al., 2009; Iram et al., 2013). The samples were then taken for higher order analysis by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The techniques reveal morphological changes and microarchitectural details of IgG following modification. The images for SEM were visualized at a resolution of 1000X and a scale bar of 10 µm. Micrograph of N-IgG appeared to have granular structures distributed uniformly over the surface. Unlike N-IgG, MG and OH⁻ modified samples were found to have irregular rod shaped structures of variable length aligned parallel and perpendicular to each other. Micrograph of OH⁻-MG-IgG is highly dense with more of such fibrils. Furthermore, the condensation of IgG into amyloid or amorphous aggregates is demonstrated by TEM. TEM images analysed at a scale bar of 100 nm with 15000X resolution shows aggregates in MG-IgG, OH⁻-IgG and OH⁻-MG-IgG. Native counterpart however is devoid of any such structures. The aggregates look more like disordered or
amorphous ones but in the light of previous results it can be said that it is probably the seeded polymerization stage that ends with amyloid type aggregates (Naeem and Fazli., 2011). Accumulation of these adducts/crosslinks/aggregates form the basis of a multitude of pathologies such as renal damage, cataract, Alzheimer’s disease, hypertension, diabetes (Kume et al., 1995; Chibber et al., 1997; Loske et al., 1998; Kikuchi et al., 1999; Min et al., 1999; Sakata et al., 1999; Schmidt et al., 1999; Takeuchi et al., 2000; Lyons et al., 2000; Raj et al., 2000; Cohen et al., 2001; Gasic-Milenkovic et al., 2001; Kirpichnikov and Sowers., 2001; Yamagishi et al., 2002). Several proteins like fibrinogen, hemoglobin, LDL, collagen, serum albumin, β-2 microglobulin have been reported to form aggregates after modification (Raj et al., 2000; Trueb et al., 1984; Cribbs et al., 2000). These aggregates in protein are actually the end result of a molecular cascade of several steps. Glycation induced formation of amyloid cross β structures have been earlier studied in albumin (Bouma et al., 2003). Hemoglobin is also reported to proceed into aggregates when incubated with 70 % v/v glyoxal for 20 days (Iram et al., 2013). 80 % of acetonitrile has been found to cause aggregation in IgG leading to its denaturation and functional disability (Amani and Naeem., 2011). Our observations are in line with earlier reports and it can be concluded that aggregation has serious effect on function and stability of protein. Our results are in full agreement with ThT and CR assays for aggregate formation. Dynamic light scattering (DLS) is a sensitive technique that measures the time dependent fluctuations in the scattering intensity to determine hydrodynamic size (Zheng et al., 2016). DLS result showing increase in hydrodynamic radius, is also due to cross linking and aggregation, thus supporting the above results. Differential scanning calorimetry (DSC) is a direct technique to determine thermal stability and enthalpy change. Unfolding of a part of protein molecule is responsible for endothermic enthalpy change. The thermogram of IgG showed two transitions one for Fab and second for Fe fragment (Gill et al., 2010). The increase in melting temperature and enthalpy as evident from DSC results indicate enhanced thermodynamic stability which may be due to crosslinking. The appearance of marker of hydroxylation (DOPA) in OH-IgG and OH-MG-IgG, marker of glycation (CEL) in MG-IgG and OH-MG-IgG was clearly evident by LCMS analysis. Our LCMS results confirm the entire earlier biophysical and biochemical characterization.
Mass spectrometry is an accurate and robust tool that provide absolute and fast analysis of molecular mass of sample. The technique has a variety of applications ranging from characterization of post translational modification, protein-protein interactions besides others (Hutanu and Darie., 2014). In contrast to other spectroscopic techniques it has low sample requirement, easy sample preparation and short analysis time making it highly suitable and useful for analysis (Pasch and Alpheus., 2011). Thus, to validate the attachment of MG and OH- in terms of accuracy and increment of molecular weight MALDI-TOF analysis was performed. All 4 MALDI chromatograms observed had 2 peaks each corresponding to signals obtained from single and double charges on protein molecule owing to ionization. N-IgG showed peaks at m/z of 73949.4 and 147596.7. Hydroxylation, glycation and glycoxidation is directly evident in the form of signature 194.6 Da, 921.8 Da and 1110.1 Da mass shift in spectra respectively. There is an approximate addition of 11 OH- residues, 13 MG residues post hydroxylation and glycoxidation as inferred from above results. Glycoxidated samples have simultaneous addition of both MG and OH- however, the number of each type of molecule can’t be said accurately. It can be approximated that same number of OH- and MG residues will be attached as in former case as the mass increase is summation of respective mass increase when the two are used separately. The molecular mass of OH- and MG as evident from literature is 17 Da (Xiaoxiao et al., 2011) and 72 Da respectively (Chumsae et al., 2013). OH- attaches to many amino acids and its attachment to aromatic amino acid residues form products like DOPA, o- and m-tyrosine, dimers of aromatic amino acids. Besides His, Glu, Leu, Val, Lys, Pro, Arg, Ile are reported to be a target of OH- forming hydroxyl derivatives (Dean et al., 1997; Fu et al., 1998). MG treatment results in formation of AGEs. It is mainly the arginine and lysine residues that are involved in the process. Important AGEs are hydroimidizolones of arginine like MG-H1 and lysine derivatives such as CEL, MOLD, GOLD etc. Besides there are some minor products also (Thornalley., 2008). The technique has been used earlier to analyse glycoxidative, oxidative and lipoxidative damage to protein in human brain cortex (Pamplona et al., 2005). It has also helped to understand the pathogenetic role of oxidation products post glycation of human globulins from nephritic patients (Lapolla et al., 2007). Besides, in vitro attachment of glucose molecules on Hb is also demonstrated earlier (Lapolla et al., 2013). MALDI analysis for glucose attachment on BSA showed clear increase in molecular mass with increase in incubation time. Thus the data collected
in the study clearly demonstrate attachment of MG and OH’ on IgG. It is this linkage that following a cascade of events caused abnormality in structure and function of proteins.

Amongst a variety of post translational modifications; glycation, oxidation and glycoxidation are of special interest. Their interaction with protein generate fluorogenic and non fluorogenic AGEs such as CML, CEL, argpyrimidine, pentosidine, crossline etc (Jakus and Reitbrock., 2004). It is these adducts that serve as neoepitopes and trigger autoimmune reaction. Ahmad et al., reported glycoxidative damage to DNA as highly immunogenic in experimental animals (Ahmad et al., 2014). In another study, glycated crystallins is also reported to generate polyclonal antibodies in rabbits (Ranjan et al., 2008). Besides, a study conducted by Virella et al., on glycated LDL report AGE-LDL to be immunogenic (Lopes-Virella et al., 2011). In this study, it was found that immune response is generated by both native and modified IgG. However, the titre of antibodies varied as per modifications. N-IgG was least immunogenic and induced low titre antibodies. However, modification by MG and OH’ resulted in a significant elevation in antibody titre. It was the glycoxidated (OH’-MG treated) IgG that was found to be the most potent inducer of antibodies with a titre reaching to $1 \geq 25600$. The enhanced immunogenicity of glucose modified IgG have been reported earlier also (Ahmad et al., 2012).

Furthermore, rabbit sera were assessed for its binding characteristics to respective immunogen on solid phase immunoassay. Serum antibodies against glycoxidised IgG (OH’-MG-IgG) showed maximum percent inhibition followed by glycated and hydroxylated samples. The data indicate maximum induction of antibodies with glycoxidised IgG. Along with it antibodies against OH’-MG-IgG were found to be highly specific as they require very less inhibitor concentration for 50 % reduction in antibody activity compared to antibodies generated against MG-IgG and OH’-IgG. N-IgG is least specific amongst all with $> 20\mu g/ml$ of antigen needed for 50 % inhibition. Thus it can be said that generation of neoepitopes on IgG post modification with MG and OH’ make it immunogenic and specific. The immunogenicity and specificity of induced antibodies was further confirmed by studying binding pattern of affinity purified IgG from rabbit sera. The amount of IgG required for antigen saturation was calculated by direct binding ELISA of isolated IgG. IgG from the sera of rabbit injected with glycoxidised IgG (OH’-MG-IgG) was needed least for antigen
saturation indicating its high and specific binding than others. Later competitive inhibition ELISA was also done on isolated IgG. The results were similar and repeated the trend of competitive ELISA performed on rabbit sera. The percent inhibition was however even higher than that observed earlier on respective sera samples. Thus our results confirm generation of antibodies in response to neoeptopes formed on modified IgG samples. The antibody titre was proportional to degree of modification. The results were further strengthened by gel retardation assay. Glycoxidated IgG showed preferential high binding to antibodies raised against them compared to native counterpart. It was found that with increasing concentration of Ab, more of Ag-Ab complex is formed and less of unbound antigen is left. The findings were clearly evident from the pattern observed. A similar trend in pattern of band was seen in case of native counterpart however, the band intensity of Ag-Ab complex is less than the respective band intensities after modification. Our gel retardation assay results also point to the generation of neoepitopes on glycoxidised IgG inducing autoimmune response. Cross reactivity studies performed threw a light on sharing of epitopes with other antigens encompassing a set of proteins and amino acids. Maximum binding of anti-OH'-MG-IgG for OH'-MG modified immunogen demonstrates high specificity of the antibody. Native immunogen also showed appreciable binding to OH'-MG-IgG suggesting old epitopes are still left over on the protein after modification. Infact modified immunogen possess a mixture of old and new epitopes spread all over the molecule. An appreciable binding with OH'-MG modified arginine and lysine residues is indicative of maximum involvement of these residues in modification. OH'-MG modified BSA and HSA also showed a good inhibition of 40.8 and 42.9 % respectively. It is probably the OH'-MG derived adducts that form neoepitopes. These neoepitopes are common in all OH'-MG modified proteins. OH'-MG modified forms of phenylalanine, tyrosine and tryptophan showed less binding than OH'-MG modified arginine and lysine indicating comparatively less involvement in protein modification / less abundance or lesser availability for ligand to react. Same set of antigen were tested for cross reactivity against N-IgG and a comparatively lesser binding was reported in all cases. However, native antigens showed more binding than modified counterparts. Cross reactivity studies have been performed earlier also and the results obtained were in agreement with that of ours (Khatoon et al., 2012; Ahmad et al., 2013). Along with immunogenicity, inflammatory response was also studied extensively in the present study.
Discussion

Inflammation occurs when AGE interacts with its receptor called RAGE. AGE – RAGE interaction via downstream signaling pathways converge into NF-κB activation which in turn initiates inflammatory pathways (Guedes-Martins et al., 2013). RAGE is a pattern recognition receptor found on monocytes, macrophages, neurons, astrocytes, microglia, neurons, endothelial cells and smooth muscle cell. The signaling pathway activated on AGE-RAGE interaction may cause many age related diseases (Younessi and Younessi., 2011). The increased count of hematological parameters (WBC, RBC, Neutrophils, Basophils, eosinophils, platelets) in post immune blood of rabbits injected with glycoxidised IgG compared to native counterpart gives a clear idea of inflammatory response activated due to AGE-RAGE interaction. It is this inflammation that caused a 2 °F hike in rectal temperature in OH’-MG-IgG treated rabbits in comparison to native counterpart. Thus, glyoxidation of IgG results in AGEs formation, and these AGEs bind to their receptors. It is due to the interaction mediated inflammation that lead to increase count of haematological parameters and a rise in rectal temperature observed in our study.

Type 2 diabetes mellitus (T2DM) is a complex multifactorial metabolic disease whose prevalence is increasing continually at a global level. The disease arises from defects in secretion or action of insulin. These defects raise blood glucose which in turn leads to hyperglycemia (ADA., 2009). Early diagnosis of the disease is very important as prolonged hyperglycemic conditions have been found to affect nervous system, eyes, kidneys and cardiovascular system (Nathan., 1993). Glucose has several fates : 1) It can either react with biological macromolecules forming adducts which ultimately transform into advanced glycoxidation products (Sadowska-Bartosz et al., 2014) 2) It can undergo auto-oxidation releasing ROS (Bonnefont-Rousselot., 2002) 3) It can be degraded into reactive dicarbonyl species like methylglyoxal, glyoxal, 3-Deoxyglucosone. Thus, patients with T2DM have elevated level of these reactive dicarbonyl compounds (Tauer et al., 2005). Oxidative stress has long been associated with complications of diabetes. It accelerates AGEs forming process, can cause β cell dysfunction and promote insulin resistance (Tangvarasittichai., 2015). As per estimates, 4.9 million people die of the disease every year (IDF., 2014). Due to its rapid rise, associated complications and several other factors its early diagnosis is very important. However, contrary to it, most people recognize it only at later stage making it difficult to handle (Nowotny et al.,
The disease also imposes a financial burden on health care system. Thus to combat the disease effectively, it is very important to gain more advanced knowledge about the risk factors, biochemistry of disease progression and molecular mechanism for its pathogenesis. It is well established that the disease revolves around hyperglycemia and glycoxidised serum proteins. So, this work aimed to investigate the relevance of glycoxidised IgG as a marker for diabetes. Related studies have been performed earlier also. Till now, many modified proteins have served as markers for disease prediction (Lyssenko and Laakso., 2013; Zhang et al., 2015). Autoantibodies against glutamic acid decarboxylase have been found as important factor in disease development (Khan et al., 2009; Towns and Paeitrapaolo., 2011). Borg et al., reported islet cell antibody in 85% of children with IDDM (Borg et al., 1997). The presence of CML IgG against CML-HSA adduct indicated CML to be a potent marker for diabetes (Vay et al., 2000). In the light of above description and keeping in mind the immunogenicity of glycoxidised IgG in experimental animal, we studied binding of N-IgG, OH⁻-IgG, MG-IgG and OH⁻-MG-IgG with circulating autoantibodies in T2DM patients. Direct binding ELISA studies were performed on 80 diabetic patients and 20 healthy subjects aged ≥ 20 years with exclusion criteria of micro and macrovascular complications, T1DM, GDM. Around 66.25% of the total tested diabetes sera showed higher binding to OH⁻-MG-IgG. However, sera from healthy human subjects did not show any appreciable binding with N-IgG or its modified counterparts. Thus our results clearly suggest that circulating autoantibodies from sera of T2D patients are better recognized by OH⁻-MG-IgG followed by MG-IgG and OH⁻-IgG. Native forms showed least binding. The plausible explanation for this is that the glycoxidative modification of IgG results in the generation of neo-epitopes on the protein and these may be responsible for induction of a population of auto-antibodies in diabetes patients. Furthermore, sera showing very high binding were selected for competitive inhibition ELISA. The inhibition range and mean percent inhibition was maximum for OH⁻-MG-IgG followed by MG-IgG, OH⁻-IgG in comparison to N-IgG. These results are in full agreement with direct binding ELISA results and indicate appreciable recognition of glycoxidatively modified IgG followed by glycated and hydroxylated IgG in comparison to N-IgG. 10 of 18 diabetic sera showing very high percent inhibition were chosen for further studies. IgG was affinity purified to exclude any interference from serum proteins in the antibody binding studies. Appreciably high binding was observed in competitive inhibition assay with purified IgG. These
results reiterate glycoxidative damage to IgG in diabetes patients, resulting in generation of neoepitopes on the protein which are now recognized as foreign by the immune system leading to elevated titres of circulating antibodies against these epitopes. Furthermore, to gain insight about structural damage, the selected samples were examined physicochemically by UV and fluorescence spectroscopy. The observed hyperchromicity in UV and quenching in fluorescence spectroscopic analysis in IgG isolated from selected T2D serum samples compared to healthy controls is a clear indication of exposure of chromophoric aromatic amino acid residues and alteration in tyrosine and tryptophan microenvironment in the IgG of T2D patients. The selected IgG was also examined for changes in secondary structure (Far-UV CD and FTIR spectroscopy), tertiary structure (Near-UV CD) and aggregation propensity (TEM). From the FTIR and far-UV CD spectroscopic analysis, it can be said that IgG of diabetes patients has slight alteration in secondary structure. Besides, the tertiary structure also showed certain alteration, as was evident by near-UV CD studies. Comparatively less aggregates in transmission electron microscopy in the IgG sample from diabetes patient, as compared to the in vitro glycoxidatively modified IgG, may be explained due to the presence of a protein quality control mechanism in the body that eliminate protein aggregates either by refolding it back by the chaperone system or degrading it by ubiquitin-proteasome system (Gregersen et al., 2005). Similar structural perturbations have been reported in HSA isolated from diabetes patients with or without CKD (Neelofar et al., 2016). Glyoxidation induced damage in IgG isolated from T2D patients was also effectively probed using experimentally induced anti-\(\text{OH}^•\)-MG-IgG antibodies. IgG isolated from healthy subjects served as control. Compared to IgG from healthy subjects, IgG isolated from T2D patients exhibited higher recognition of experimentally induced anti-\(\text{OH}^•\)-MG antibodies. These results further confirm that the structural perturbations observed in T2D-IgG are due to its glycoxidation in the body. These altered epitopes are recognized as alien by the immune system leading to the induction of auto-antibodies in diabetes. It can be said that the structural perturbations in IgG of diabetes patients due to glyoxidative damage make it a potential antigenic candidate for eliciting autoimmune response in type 2 diabetes patients.

RA is a chronic autoimmune disease that mainly targets diarthrodial joints causing destruction of bones and cartilages. In spite of its growing incidence, the
etiology and pathogenesis is not yet known (Komatsu and Takayanagi., 2012; Mc Innes and Schett., 2007). Similar to diabetes, the disease is associated with long term complications like skeletal disorder, psychological disturbances, cardiovascular disease etc. Females are more prone to the disease (Mc Innes and Schett., 2011). RA has both genetic and environmental predispositions which in association causes inflammation and damages synovium (Edwards and Cooper., 2006). Role of ROS in disease progression is well established. As per reports mitochondrial and monocytic ROS production increases by 5 fold in the blood of RA patients (Miesel et al., 1996) indicating free radicals as pathological hallmark of RA. Free radicals can cause several disturbances like targetting and degrading proteoglycan in joint cartilage (Quinonez-Flores et al., 2016). Increased oxidative stress can also result in accumulation of glyoxidation products such as pentosidine and CML. CML has been reported to be produced by HOCl mediated conversion of serine to glycoaldehyde (Anderson et al., 1999). ROS, such as O$_2^\cdot$-, HOCl, OH$^-$ can also generate reactive carbohydrate intermediates called dicarbonyls (methylglyoxal, glyoxal, 3-deoxyglucosone). These reactive compounds accelerate AGE production (Yang et al., 2014; Nowonty et al., 2015). Also an increase in the content of total protein is observed in synovial fluid and severely inflamed joints in RA. So interaction occurs between free radicals and proteins stimulate maillard reaction which is again a source of AGEs (Rodriguez-Garcia et al., 1998).

In view of this the involvement of glyoxidated IgG in disease progression of RA has been studied. Direct binding ELISA was performed on 80 RA serum samples and 20 healthy subjects. Compared to healthy controls, appreciably high binding to glyoxidated IgG was observed in RA serum samples. Serum autoantibodies also showed appreciable binding to MG and OH$^-$ modified IgG; however, it was less than glyoxidatively modified IgG. Antibodies showed least binding with N-IgG as immunogen. Thus, the circulating autoantibodies in the sera of RA patients were best recognized by glyoxidatively modified IgG. High binding RA sera were selected for competitive inhibition ELISA and the range of inhibition so obtained is maximum for OH$^-$-MG-IgG followed by OH$^-$-IgG and MG-IgG. Percent inhibition varied in a narrow range in case of N-IgG. The results indicate appreciable recognition of OH$^-$-MG-IgG by the autoantibodies in patients suffering from RA. 7 of the 12 RA sera showing very high percent inhibition against glyoxidated IgG were selected for
further experiments. The IgG isolated from these sera recorded an inhibition range of 26.8-37.8 %, 47.2-60.1 %, 50.3-59.4 % and 60.6-79.9 % when N-IgG, OH\(^{-}\)-IgG, MG-IgG and OH\(^{-}\)-MG-IgG respectively were used as inhibitors. Appreciably high binding of RA sera to glycoxidated immunogen indicate generation of antibodies against these neo-epitopes on the IgG molecule in RA patients. To confirm the above results, band shift assay was done. The results of assay further confirmed the preferential binding of glycoxidatively modified IgG to antibodies in RA sera. The strong binding of RA antibodies to OH\(^{-}\)-MG-IgG point towards the involvement of advanced glycoxidation products in pathogenesis and progression of the disease. These neo-epitopes are recognized as non-self by the immune system of the body resulting in autoantibody production. It can be said that the production of neo-epitopes is a consequence of structural changes in the protein, hence we further studied the nature and extent of structural change in IgG of RA patients. The IgG from serum sample 5, 14, 25, 34, 47, 67 and 71 showing high specificity in competitive inhibition ELISA studies were examined by UV and fluorescence spectroscopic analysis, with IgG from normal human sera (NHS-IgG) serving as parallel control. The hyperchromicity and quenching as observed in UV absorbance analysis and fluorescence spectroscopy points to exposure of chromophoric aromatic amino acid residues and alteration in tyrosine and tryptophan micro environment of RA-IgG. Furthermore, in comparison to NHS-IgG; RA-IgG presented an altered secondary structure (loss of β pleated sheet) as evident by far-UV CD and FTIR spectroscopic analysis. The slight variation in near-UV CD signals of RA-IgG in comparison to NHS-IgG is indicative of a change in overall tertiary structure of IgG from RA patients. Furthermore, transmission electron micrograph of RA-IgG showed slight variations from NHS-IgG indicating aggregation. Compared to OH\(^{-}\)-MG modified IgG, isolated RA-IgG showed few aggregates. As protein quality control mechanism in the body keeps a check of misfolded and aggregated proteins, efficiently removing them from the body, no visible aggregates were found. Furthermore, high recognition of experimentally induced anti-OH\(^{-}\)-MG-IgG antibodies by RA-IgG compared to NHS-IgG confirm that the observed structural damage in RA-IgG is due to its glycoxidation. From the physicochemical and immunological analysis of RA-IgG it can be said that oxidative stress increases accumulation of AGEs in RA patients having damaging effect on IgG. This abundant serum protein undergoes changes in its structure at both secondary and
tertiary level. These changes causes misfolding and aggregation of IgG leading to generation of neo-epitopes, rendering it immunogenic.

**Conclusion**

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

1. The damaging potential of glycoxidation is quite high as compared to glycation or oxidation alone.

2. Modifications by MG, OH⁻ and their cumulative effects result in gross structural changes in IgG. Maximum modification was, however, observed when glycation and oxidation reactions were performed simultaneously.

3. Glycation and oxidation, as well as the combined effect of both, generate highly immunogenic epitopes on N-IgG inducing high titre antibodies in experimental rabbits. The immunogenicity of glycoxidatively modified IgG, however, was quite high compared to that of glycated IgG and oxidatively modified IgG.

4. The induced antibodies against glycoxidatively modified IgG showed high specificity towards the immunogen as well as cross reactivity with other glycoxidated proteins and amino acids.

5. The preferential binding of glycoxidatively modified IgG by autoantibodies present in diabetes and RA sera point towards the possible role of OH⁻-MG modified IgG in the initiation and/or progression of diabetes and/or RA.

6. The altered structure of IgG from diabetes and RA patients as observed from physico-chemical analysis, in comparison to IgG isolated from healthy human sera further suggests the role of glycoxidatively modified IgG in disease etiopathogenesis.

7. The glycoxidatively modified IgG may serve as a possible biomarker for disease diagnosis and evaluation of therapeutic efficiency.