ABSTRACT
Non-enzymatic glycation and oxidation play an important role in the pathogenesis of several diseases like diabetes and rheumatoid arthritis (Newkirk et al., 2003; Jakus, 2003; Schmitt et al., 2005). They also induce the accelerated accumulation of AGE products in tissues of diabetic patient, particularly with secondary complications like retinopathy, nephropathy and artherosclerosis (Cohen et al., 2005; Defraigne, 2005). In diabetes mellitus and rheumatoid arthritis protein glycation and the formation of AGEs are accompanied by increased free radical activity that contributes toward the biomolecular damage (Ahmed, 2005; Drinda et al., 2005; Sunahori et al., 2006). AGE formation is an inevitable process in vivo and can be accelerated under pathological conditions such as oxidative stress. Oxidative stress and oxidative damage to tissues are common end points of chronic diseases such as diabetes and rheumatoid arthritis (Baynes and Thorpe, 1999; Ahmed et al., 2005).

In the present study, human serum albumin (HSA) was glycated non-enzymatically by incubating with glucose for 10 weeks. Glycated HSA was further modified by ROS generated by the irradiation of hydrogen peroxide with UV light at 254 nm. Under these experimental conditions, the major species would be hydroxyl radical (OH), the most reactive of ROS. The modified HSA samples showed remarkable biophysical changes analyzed by gel electrophoresis, spectral analysis, circular dichroism spectropolarimetry and thermal denaturation studies. Estimation of ketoamine, carbonyl and free amino groups revealed that glycation and oxidation attributed to the conformational and structural changes in HSA.

The electrophoretic pattern of both modified HSA showed formation of high and low molecular weight aggregates. However, ROS-glycated HSA showed almost similar changes but with considerable decrease in their intensity which can be attributed to fragmentation of glycated HSA on ROS modification. UV spectra of glycated HSA showed hypochromicity, whereas, ROS-glycated HSA exhibited hyperchromicity as compared to unmodified HSA. These changes are indicative of conformational changes in HSA on modifications. Glycation causes shielding of aromatic amino acids contributing to hypochromicity. However, fragmentation by OH causes exposure of aromatic amino acids in glycated HSA towards solvent
system resulting in hyperchromicity. The fluorescence spectral studies of HSA also showed the same pattern as observed in UV spectra as the same aromatic amino acids are involved in both spectral analyses.

Tryptophan specific fluorescence was also carried out. Glycated HSA showed lower fluorescence intensity and quantum yield, as compared to ROS glycated HSA showing higher tryptophan specific fluorescence and quantum yield. The significant observation in this result was the blue shift on modifications. The blue shift was more in case of glycated HSA than ROS-glycated HSA. The results reiterate the earlier observation of conformational changes in glycated HSA whereas fragmentation appears to be one of prominent phenomena on ROS exposure.

The melting temperature profile of glycated and ROS-glycated HSA showed a net increase of 18.1°C and 8.8°C, respectively, as compared to unmodified protein. Once again it can be attributed to conformational stability of both modified HSA over their native form. However, ROS may causes disruption of weak bonds or fragmentation of glycated HSA and might be one of the reasons for increase in Tm value.

Furthermore, colorimetric estimations were carried out to support the biophysical analysis. Ketoamine level was found to be significantly higher in case of both modified samples (with slight difference) as compared to native HSA, which showed negligible ketoamine content. Levels of carbonyl groups were also elevated in both cases, an important marker of both glycation and oxidative stress. However, the increase was more in ROS modified glycated HSA. Number of free amino groups in modified HSA samples was found to be half as compared to native HSA. ROS modification of glycated HSA resulted in appreciable increase in amino groups reiterating once again the fragmentation and structural changes in 'OH-modified glycated polymer.

Studies with various antioxidants, scavengers and metal chelators showed inhibition of different parameters in both the modified samples. Moreover, maximum inhibition with aminoguanidine and combination of two enzymatic antioxidant
(catalase and SOD) was observed showing a definite role of ROS in the modification of glycated HSA and AGE formation.

Native and modified HSA samples were used to induce antibodies in rabbits and were found to be immunogenic, producing high titer antibodies. The antigenic specificity of the induced antibodies was studied by direct binding ELISA, immunodiffusion and competition ELISA. The immunogen showed a high degree of specificity for the induced antibodies, reiterated by gel retardation assay. Anti-glycated and ROS-glycated HSA antibodies showed preferential recognition of glycated and ROS-glycated HSA in a competition assay. The induced antibodies were polyspecific in nature.

Sera of diabetic patients were tested for the presence of antibodies reactive with native and both the modified samples of HSA. Direct binding ELISA showed greater recognition for modified HSA samples as compared to the native form ascertained by competition ELISA. Moreover, significantly higher recognition of modified HSA was observed in the sera of diabetic patients having secondary complications like retinopathy, nephropathy and artherosclerosis. The higher binding to both modified HSA over native HSA of antibodies in the sera of diabetic patients suggests the involvement of modified HSA in the production of autoantibodies in these patients. The binding specificity of glycated and ROS-glycated HSA with diabetic patient’s IgG was reiterated by gel retardation assay.

Glycation and ROS damage to human blood proteins was detected immunochemically using anti-glycated and anti-ROS-glycated HSA antibodies as probes. Albumin and IgG from different diabetic patients inhibited antibodies binding to their respective immunogen demonstrating the presence of glycated and ROS-glycated epitopes on albumin and IgG molecules. Data obtained from our studies correlates to the earlier studies that glycation and glycoxidation causes in vivo protein modifications.

The binding of circulating autoantibodies in rheumatoid arthritis with native, glycated and ROS-glycated HSA was also analyzed. Direct binding ELISA results showed preferential binding of rheumatoid arthritic autoantibodies to both modified
HSA in comparison to native HSA. Inhibition ELISA reiterated the direct binding results. Gel retardation assay further substantiated the binding of both modified HSA with rheumatoid arthritic autoantibodies.

In conclusion, glycation and oxidation causes damage to HSA and render it highly immunogenic. Polyclonal antibodies generated against modified antigens showed preferential recognition of the immunogens. The induced antibodies as immunochemical probes detected glycation and oxidative damage to the blood proteins. Recognition of modified HSA samples by antibodies in sera of patients with diabetes and rheumatoid arthritis suggests glycation and oxidation induced blood proteins damage in these patients. It is, therefore, postulated that glycation and ROS modification of blood proteins appears to play a major role in the production of autoantibodies in disease state(s).