Discussion
Lipid peroxidation has been implicated in the pathogenesis of numerous diseases, including SLE, rheumatoid arthritis, atherosclerosis, diabetes, and cancer, as well as in drug-associated toxicity, postischemic reoxygenation injury, and aging (Halliwell and Gutteridge, 1989; Shimozu et al., 2011). Reactive oxygen species (ROS) readily oxidize membrane phospholipids containing unsaturated fatty acids. The lipid hydroperoxides generated by this process yield several cytotoxic products including saturated aldehydes [e.g., malondialdehyde (MDA)] and unsaturated aldehydes [e.g., 4-hydroxynonenal (HNE)] (Esterbauer et al., 1991). By virtue of their increased chemical stability, these lipid peroxidation derived aldehydes (LPDA) diffuse greater distances compared with their precursor ROS and behave as secondary toxic messengers that can propagate and amplify oxidative injury. Indeed, unsaturated LPDA such as HNE react avidly with cellular nucleophiles, such as glutathione and cysteine, histidine, and lysine residues of proteins (Uchida et al., 1995, 1997; Arashiki et al., 2010). The resultant LPDA protein adducts can produce destructive functional modifications, thereby causing a variety of detrimental effects (Esterbauer et al., 1991; Uchida et al., 1995; Ruef et al., 1998; Arashiki et al., 2010). High level of HNE (10 μM–5 mM) after toxic insult in vivo often results in compromised antioxidant defense metabolisms and enhanced toxicity (Abarikwu et al., 2012). This has also been reported in vitro (Raza and John, 2006; Siddiqui et al., 2010). HNE-mediated cross-linked protein has been shown to be resistant to proteolysis and acts as a potent noncompetitive inhibitor of the multicatalytic protease/proteasome, a proteolytic complex involved in the degradation of oxidatively modified proteins (Friguet et al., 1994; Tanaka et al., 2001). Moreover, HNE can also form exocyclic DNA adducts resulting in genotoxic effects that can alter cellular function (Feng et al., 2004).

HSA is a monomeric, multi-domain protein of 66-kD, necessary for colloidal stability of blood. It is the most abundant plasma protein and acts as a transporter and disposer of many endogenous and exogenous compounds (Petitpas et al., 2001). Because of its abundance, HSA forms an attractive candidate for biomarker studies (Anderson and Anderson, 2002). The formation of aldehyde-protein adducts, in vivo, may yield biomarkers of oxidative stress and oxidant-related disease. Elevated levels of oxidized albumin have been reported in patients with various diseases (Era et al., 1995). The exposure leads to alterations in its conformation, which may result in the impairment of its biological properties (Oettl and Stauber, 2007). HNE-HSA adducts
are reported to serve as a biomarker of oxidative stress (Szapacs et al., 2006). Present work was carried out in the light of increasing evidences implicating HNE in various physiopathological conditions (Wang et al., 2007); besides projecting it as a biomarker for systemic oxidative stress (Grune et al., 1997; Toyoda et al., 2007; Wang et al., 2010; Shah et al., 2011).

In this study, HSA was modified by HNE and the structural changes were analyzed by various physicochemical techniques. Extensive damage was observed in HSA upon modification by HNE. The modified-HSA samples showed 50.87% hyperchromicity at 280 nm, compared to its native conformer. The observed hyperchromicity may be attributed to structural perturbation in HSA as a result of unfolding and protein cross-linking, due to the formation of HNE adduct with lysine, histidine and cysteine. It is reported that HNE reacts with $\varepsilon$-amino group of lysine, imidazole group of histidine and sulfhydryl group of cysteine (Toyoda et al., 2007; Wakita et al., 2009).

HNE-induced structural changes in HSA were visualized by SDS-polyacrylamide gel electrophoresis. HNE-modified HSA exhibited retardation in electrophoretic mobility as well as more extensive multimerization of bands compared to the control sample. The increase in band intensity and formation of high molecular weight species reflect cross-linking of protein; in line with earlier reports (Cohn et al., 1996; Montine et al., 1996a, 1996b). It has been reported that lipid derived aldehyde can react with amino groups of proteins and might produce intermolecular cross-links of the Schiff base type (Stewart et al., 2009). Lys-lys crosslink constitute the major entity underlying cross-linking of protein by HNE (Xu et al., 1999). The SDS-PAGE pattern of HNE-modified HSA corroborated the spectral findings.

Carbonyl content is a most commonly used marker of protein oxidation (Shacter, 2000) and accumulation of protein carbonyl has been reported in many human diseases (Beal, 2002). Carbonyl group appears as a consequence of oxidative modification of the side chain of lysine, proline, arginine and threonine (Berlett et al., 1998). These moieties are chemically stable and measured spectrophotometrically at 360 nm. The oxidation of HNE-modified HSA was evident by the significant increase in its carbonyl content in comparison to native HSA.
The aromatic amino acids tryptophan, tyrosine and phenylalanine offer intrinsic fluorescence probes of protein conformation, dynamics and intermolecular interactions. Of the three, tryptophan is the most popular probe (Chen et al., 1998). The fluorescence of the indole chromophore is highly sensitive to the environment making it an ideal choice for reporting protein conformation changes and interactions with other molecules. A single tryptophanyl residue (Trp-214) is present almost at the middle of domain II of HSA. The changes in the fluorescence spectra observed by exciting HSA at 295 nm, at which only tryptophan gets excited, can be used to evaluate changes in its microenvironment and provide a picture of local change in the protein. The observed quenching of tryptophanyl fluorescence at 295 nm might be due to modification of lys-199 and lys-195 which occur at distances of 3.7 and 7.4 Å to trp-214 in the crystal structure of HSA (Carter and Ho, 1994). Whereas, at 280 nm both tryptophan as well as tyrosine residues get excited and provide a picture of global change in the protein. Decrease in fluorescence intensity at 280 nm in modified samples as compared to the native form may be attributed to changes in global microenvironment of protein (Mir et al., 2010) resulting from HNE modification. Furthermore by exciting HSA at 280 nm and at 295 nm, the fluorescence emission maximum wavelength shifted to a shorter wavelength i.e. blue shift, which indicates that the chromophore of protein was exposed to a more hydrophobic environment (Mir et al., 2010). The wavelength strongly depends on the microenvironment especially the hydrophobicity around the protein. Also, reaction of HNE with proteins is frequently associated with their cross-linking, leading to the formation of fluorophores. A major fluorophore has been identified as a lysine derived dihyropyrrrol derivative- 2-alkyl-2hydroxy-1, 2-dihydropyrrole-3-one iminium cross-link, in the reaction of HNE with lysine, with characteristic excitation and emission wavelengths, 360 nm and 430 nm respectively (Xu et al., 1999). This is also documented from our studies with major emission band observed at 430 nm when HNE modified HSA was excited at 360 nm. Thus, modification of HSA with HNE results in the generation of fluorescent adduct, characterized by emission maxima at 430 nm.

The near-UV CD spectra for the native HSA showed two minima at 262 and 268 nm and shoulder at 283 and 293 nm, characteristic of disulphide and aromatic chromophores, which is in accordance with the previous studies (Lee and Hirose,
1992). Result shows that modification of HSA with HNE has resulted in structural perturbation in the tertiary structure; this further supports our fluorescence studies results. HNE modification also led to significant changes in the secondary structures of HSA as determined from Far-UV CD spectra. The spectrum of untreated HSA showed negative minima nearly at 208 and 222 nm, characteristic of \( \alpha \)-helical structure (Carbin et al., 1998). The untreated HSA contained about 68% \( \alpha \)-helical structure as estimated by K2d (Adrade et al., 1993), which is in agreement with the reported value (Liu et al., 2005). The \( \alpha \) helical content was found to decrease in HSA after HNE modification. This indicates HNE induced conversion of \( \alpha \)-helix to \( \beta \)-sheet structure. Previous study has also reported the formation of \( \beta \)-sheet structure in HNE-damaged protein. Alpha synuclein which is typically unfolded polypeptide chain, after modification with HNE showed substantially increased amount of \( \beta \)-sheet structure (Qin et al., 2007).

The above changes were further substantiated by FTIR spectroscopy, a powerful method for investigating the secondary structures of proteins and their dynamics. In the IR region, the frequencies of bands due to amide I, II and III vibrations are sensitive to the secondary structure of proteins. Particularly, the amide I band is useful for secondary structure studies. Amide I band is more sensitive to the change of protein secondary structure than amide II (Rahmelow and Hubner, 1996; Wi et al., 1998). The amide I peak position occurs in the 1600–1700 cm\(^{-1}\) region (mainly C=O stretch) and amide II band from 1480 cm\(^{-1}\) to 1575 cm\(^{-1}\) (C–N stretch coupled with N–H bending mode) (Liu et al., 2003; Zheng et al., 2010). FTIR spectra clearly showed a shift in amide I and amide II bands in the HNE-modified HSA when compared with its native conformer, which might be due to perturbation in the secondary structure of protein on modification with HNE. This result is consistent with the Far-UV CD data.

The thermal denaturation profile of modified HSA showed a net increase of 4°C in the Tm value as compared to the unmodified form. Thus, thermal denaturation studies demonstrated that HNE-modified HSA was thermodynamically more stable when compared with the native HSA. Formation of cross-links (Stewart et al., 2009; Xu et al., 1999) could be the main reason for the increased thermostability of HNE-modified HSA.
Increased formation and subsequent accumulation of HNE-modified protein adducts have been found in various pathologic states, including autoimmune diseases such as SLE and arthritis (Grune et al., 1997; Kurien and Scofield, 2003; Kurien and Scofield, 2008). The ε-amino group of lysine reacts with the double bond (C3) function of HNE via Michael adduct (Pillon et al., 2011). Incubation of HNE with N-acetyllysine resulted in the formation of Nα-acetyl-Nε-[1-(2-hydroxyethyl)-2-hydroxyheptyl]-L-lysine, C17H34O5N2 (MW 346). The HPLC was employed for the isolation of the standard, Nα-acetyl-Nε-[1-(2-hydroxyethyl)-2-hydroxyheptyl]-L-lysine. The retention time of the synthesized standard was found to be 22 min. The standard adduct was further characterized by nuclear magnetic resonance analysis. The HPLC of acid hydrolyzed samples of native and HNE-modified HSA showed remarkable difference in peak retention times. The extra peak at a retention time of 22.50 min in HNE-modified HSA is characteristic of Nα-acetyl-Nε-[1-(2-hydroxyethyl)-2-hydroxyheptyl]-L-lysine adduct. This is in accordance with the standard results wherein also when N-acetyllysine was exposed to HNE, a distinct peak at a retention time of 22 min was observed. However, native HSA did not show the peak at this retention time.

Generally, a majority of endogenous antigens are non-immunogenic due to immunological tolerance at T cell and/or B cell levels. However, it has been pointed out that proteins may become immunogenic if they are structurally modified post-translationally under physiologic or pathologic conditions (Ohmori et al., 2005). The modification may generate or mask antigenic epitopes and that may stimulate relevant T-cells and / or B-cells leading to breakdown or bypass of tolerance. Nonenzymatic oxidative modification of proteins, including addition with aldehydes, renders them immunogenic (Kurien et al., 2006; Mottaran et al., 2002; Wang et al., 2007; Wang et al., 2008). HNE–protein adducts are potential neoantigens and therefore could be involved in the pathogenesis of autoimmune diseases. Aldehyde-modified proteins have been demonstrated to be highly immunogenic, and autoantibodies directed against epitopes in MDA and HNE-modified low-density lipoproteins (LDL) have been shown in the plasma of rabbits and mice immunized with oxidized LDL (Esterbauer et al., 1991; Palinski and Witztum, 2000). Modification of a lupus-associated protein with HNE has been shown to increase the antigenicity and to facilitate epitope spreading (Scofield et al., 2005).
The antigenicity of native and HNE-modified HSA was ascertained by induction of antibodies in rabbits. Antibody titre was checked through direct binding ELISA. The HNE-modified HSA was found to be extremely potent antigen inducing high titre antibodies in the animals. However, immunization with native HSA resulted in moderate antigenic response. The substantially enhanced immunogenicity of HNE-modified HSA in comparison to native HSA could possibly due to the generation of potential neo-epitopes against which antibodies are raised. Thus, modification had conferred additional immunogenicity on HSA and generated neo-epitopes with increased binding affinity as compared to native HSA. Antigenic specificity of anti-HNE-HSA IgG antibodies and anti-HSA IgG antibodies was ascertained by competition ELISA. The induced antibodies were found to be immunogen specific. Visual detection of interaction of anti-HNE-HSA IgG with native and HNE-modified HSA was done by the gel retardation assay. The results reiterated specificity of induced antibodies towards the immunogen (HNE-modified HSA). Anti-HNE-HSA antibodies showed slight shift in mobility with native HSA also. This suggests that induced antibodies against HNE-modified-HSA though specific for the immunogen also show some degree of recognition for native epitopes.

Furthermore, induced antibodies showed cross-reactivity with native and HNE-modified proteins or amino-acids, a characteristic feature of polyspecific antibodies. The anti-HNE-HSA antibodies showed appreciable recognition for HNE-modified forms of BSA, N-acetyl-L-lysine, N-acetyl-histidine and cysteine. These molecules caused an inhibition of 57.3%, 73.9%, 62.1% and 64.7% respectively in antibody activity. Inhibition shown by native and HNE-modified calf-thymus DNA was moderate. However, Glycated forms of BSA, IgG, PLL and histone inhibited antibody activity to a very lesser extent, indicating glycated epitopes are not so distinctly recognized by the induced antibodies. Native counterparts of the above mentioned molecules did not cause appreciable inhibition in the activities of anti-HNE-HSA IgG. The results of cross-reactivity indicate that the induced antibodies against the HNE-modified HSA are found to be polyspecific and cross react with a variety of inhibitors sharing common antigenic determinants. This result is consistent with the previous report in which competitive fluorescence immunoassay analysis showed that anti-HNE-LDL antibodies recognized not only HNE-LDL, but also other HNE-modified proteins (e.g. HNE-HDL₃, HNE-albumin), suggesting that this
antibody is specific for HNE-derived epitopes regardless of the origin of the protein (Chen et al., 1992). Moreover, anti-HNE-HSA IgG antibodies also showed cross-reactivity with native HSA. It indicates that all epitopes of native HSA have not been converted into immunogenic neo-epitopes upon modification. In other words, HNE-modified HSA still possess some old epitopes which are scattered among neo-epitopes.

Autoimmune diseases such as SLE and RA are among the leading causes of death in young and middle-aged women (Walsh and Rau, 2000). These diseases are of unknown etiology, but are believed to be multifactorial. Several lines of evidence point to an association between oxidative modification of proteins and autoimmunity. Modification of proteins can bring about structural changes, such that the modified proteins behave like neoantigens. Such altered immunogenicity might lead to an autoimmune response by stimulating T cells (especially activation of Th1 cells) and inducing an accelerated autoantibody response (Toyoda et al., 2007). Reactive oxygen species (ROS) have been implicated in the pathogenesis of autoimmune diseases (Hadjigogos, 2003; Frostegard et al., 2005; Cuzzocrea, 2006). ROS can be produced exogenously or from a variety of intracellular processes collectively linked to the generation of superoxide anions, hydroxyl radicals, and hydrogen peroxide (Finkel and Holbrook, 2000). Such reactive oxidants can modify a variety of biological molecules, including polyunsaturated fatty acid containing lipids generating lipid peroxides, which on decomposition lead to reactive aldehydes such as HNE and MDA. These lipid peroxidation derived aldehydes (LPDA) can bind covalently with a variety of amino acids of proteins to form MDA and HNE-modified protein adducts (Grune et al., 1997; Januszewski et al., 2005; Wang et al., 2008). Increased lipid peroxidation (Koch et al., 2007) and higher levels of MDA-and HNE-modified proteins are reported in patients suffering from autoimmune diseases (Grune et al., 1997; Toyokuni et al., 2000; Kurien and Scofield, 2003; Frostegard et al., 2005; Kurien et al., 2006).

SLE is a potentially fatal systemic autoimmune disease, characterized by the increased production of autoantibodies, immune complex deposition in the microvasculature, leukocyte infiltration, and, ultimately, tissue damage in a range of organs. Of the multiple autoantibodies described in this disease, antibodies against the
native DNA are among the most characteristic (Su et al., 2007), yet the triggering antigen of the disease is still unknown. There is increasing evidence that lipid peroxidation plays a role in SLE. (i) SLE patients have an enhanced urinary excretion of isoprostanes, the well established biomarkers of lipid peroxidation (Iuliano et al., 1997), (ii) the levels of the lipid peroxidation-derived short chain aldehydes are significantly elevated in children with a high disease activity of SLE (Grune et al., 1997), and (iii) there are elevated levels of the oxidized low density lipoprotein together with elevated levels of autoantibodies related to the oxidized low density lipoprotein in female patients with SLE (Frostegard et al., 2005). Recently, evidence for the possible involvement of HNE-modified proteins as the endogenous source of the anti-DNA antibodies has been put forward (Toyoda et al., 2007).

In view of this, the possible involvement of HNE-modified HSA in SLE and RA was probed. SLE sera were screened for the presence of autoantibodies reactive to native calf thymus, native and HNE-modified HSA. Native calf thymus DNA was used as an immunochemical marker for SLE. The binding characteristic of naturally occurring SLE anti-DNA autoantibodies from forty SLE patients and twenty healthy normal subjects to native calf thymus DNA, native and HNE-modified HSA was studied by direct binding ELISA. Out of the 40 SLE sera, 67.5% showed preferentially high binding to HNE-modified HSA as compared to native DNA or native HSA. Serum samples from normal subjects did not show any appreciable binding with either of the antigens. Thus, circulating autoantibodies in the sera of SLE patients showed preferential recognition of the epitopes on HNE-modified HSA as compared to native DNA or native HSA. In competition ELISA native calf thymus DNA caused an inhibition in the range of 31.4 to 53.8% in SLE autoantibodies binding; 20.3% to 36.7% inhibition was observed with native HSA, whereas, inhibition with HNE-modified HSA ranged from 42.8% to 65.1%. These results indicate appreciable recognition of HNE-modified HSA by autoantibodies in SLE patients. The specificity of affinity purified IgG towards native calf thymus DNA, native and HNE-modified HSA was evaluated by competition ELISA. IgG from SLE patients was inhibited to the extent of 40.2% to 59.5% with native calf thymus DNA; 27% to 39.4% with native HSA, while with HNE-modified HSA the inhibition ranged from 59.9% to 87.5%. Appreciably high binding of affinity purified IgG with the HNE-modified HSA, indicates the generation of antibodies against HNE-modified
epitopes on the HSA molecules in SLE patients. Band shift assay further substantiated the preferential recognition of HNE-modified HSA over native HSA by the SLE IgG. Thus, the results confirmed that epitopes on HNE-modified HSA were preferentially recognized by SLE autoantibodies.

Albumin is the most abundant plasma protein; it could play a major role as an antioxidant in plasma (Himmelfarb and Mcmonagle, 2001). In this context, we expected that the characterization of oxidative status of serum albumin would provide, not only useful information regarding the redox state of the human body, but also alterations in the conformation and function of HSA which may result in modification of its biological properties. The oxidation of a protein typically results in an increase in carbonyl contents. Protein carbonyl groups are the biomarkers of oxidative stress (Dalle-Dome et al., 2003b). This increase is due to the oxidation of lysine, arginine, proline or other amino acid residues. In human plasma, all amino acids in the protein are susceptible to oxidative modification by oxidants (Levine et al., 1994). Present data showed that total serum protein carbonyl contents were significantly ($p<0.001$) increased in SLE patients compared with normal subjects. Further, to investigate the extent of alterations in the biological properties of HSA in SLE patients, HSA was isolated from ten SLE patients (SLE-HSA) and also from ten normal subjects (NH-HSA) and their carbonyl contents were compared. Our results are in full agreement with the previous report that redox state of HSA might change with age (Era et al., 1995; Oettl et al., 2005) and disease (Oettl et al., 2005).

Serum albumin is the most abundant protein in the circulatory system, whose redox modification modulates its physiologic function, as well as serves as biomarker of oxidative stress (Fabisiak et al., 2002). Many reported studies show the presence of elevated levels of oxidized albumin, in patients with various diseases (Era et al., 1995). Since albumin is most abundant protein of plasma, it is likely to be extensively damaged and might be responsible for the pathological conditions associated SLE. Thus HSA is continuously exposed to oxidative stress, so much so that alterations in its biological properties could result in the conformational changes of HSA. Therefore, conformational changes of purified HSA were examined. The observed hyperchromicity of SLE-HSA could be a result of structural alterations resulting due to exposure of HSA to lipid peroxidation product such as HNE. A slight decrease in
secondary structural contents was also observed in SLE-HSA. Furthermore, the observed fluorescence quenching in the case of SLE-HSA could be attributed to alterations in chromophoric microenvironments.

The experimentally induced antibodies raised against HNE-modified HSA were used as an immunochemical probe to detect the HNE mediated damage to serum albumin in SLE patients. The binding pattern of isolated HSA was quite revealing. Inhibition of anti-HNE-HSA IgG by HSA from SLE patients was recorded in the range of 63.2% to 75.3%. While, the inhibition caused by HSA from normal healthy individuals was quite low. Significantly high recognition of HSA from SLE patients by experimentally induced antibodies against HNE-modified HSA is a clear indicator of epitope sharing between the HSA modified in vitro by HNE and the HSA isolated from SLE patients. This leads to the conclusion that HNE generates neo-epitopes on the HSA molecule that are recognized as non-self by the immune system; and hence may serve as a putative antigen for autoantibody generation in SLE patients.

RA is a chronic relapsing immunoinflammatory multisystem disease with predominant synovial proliferation, bone destruction and degradation of articular cartilage. It is the most common inflammatory arthritis affecting approximately 1–2% of the general population worldwide (Harris, 1994). The exact aetiology of RA remains unknown but there is an increasing evidence to indicate that ROS play a significant role in the pathogenesis of RA (Ozturk et al., 1999). ROS are produced by neutrophils, the main cells of inflamed synovial fluid in RA (Weiss et al., 1982; Malech and Gallin, 1987). Activation of neutrophils and macrophages results in the generation of superoxide radicals, H$_2$O$_2$ and highly reactive hydroxyl radicals. Another source of ROS is hypoxic reperfusion injury from elevated synovial cavity pressure during joint movement. These ROS have been implicated as mediators of tissue damage in RA (Ozturk et al., 1999; Kamanli et al., 2004). ROS are formed during oxidative processes that normally occur at relatively low levels in all cells and tissues. If ROS are not scavenged; these species may lead to widespread lipid, protein and DNA damage (Jaswal et al., 2003; Mahajan and Tandon, 2004). Excessive ROS production disturbs redox status, damages macromolecules, including DNA and can modulate expression of a variety of immune and inflammatory molecules leading to inflammatory processes, exacerbating inflammation and affecting tissue damage.
The primary targets of ROS are double bonds in polyunsaturated fatty acids in the cell membrane, which increase lipid peroxidation and result in more oxidative damage (Perricone et al., 2009). Additionally, oxidative damage mediated by ROS resulting in generation of deleterious by-products, such as aldehydic products, lead to the formation of adducts with proteins that in turn make them highly immunogenic, thus inducing pathogenic antibodies leading to tissue damage in patients with RA (Kurien and Scofield 2008). Increased lipid peroxidation in serum/plasma and red blood cells (Sarban et al., 2005; Taysi et al., 2002; Turgay et al. 2007) and decreased antioxidant enzymes; SOD, CAT and Gpx in the patients with RA (Sarban et al., 2005) confirm the presence of oxidative stress in rheumatoid disease.

In view of this, sera from RA patients were screened for the presence of autoantibodies reactive to native and HNE-modified HSA. The binding of circulating autoantibodies from forty RA patients and twenty healthy subjects to native and HNE-modified HSA was studied by direct binding ELISA. Of the 40 RA sera, 57.5% showed preferentially high binding to HNE-modified HSA as compared to its native analogue. Serum antibodies from healthy subjects did show binding with native and HNE-modified HSA, but only to a little extent. In competition ELISA, native HSA caused 20 to 33.8% inhibition in the activity of antibodies from RA patients, whereas 41.5 to 63.2 % inhibition was observed with HNE-modified HSA. The results indicate appreciable recognition of HNE-modified HSA by the autoantibodies in RA patients. The binding specificity of isolated IgG, towards native and HNE-modified HSA, was evaluated by competition ELISA. Immunoglobulin G (IgG) from RA patients recorded an inhibition of 58 to 79.8% with the HNE-modified HSA, while with native HSA it ranged from 24.3 to 38.2%. Appreciably high binding of affinity purified IgG towards HNE-modified HSA, indicates specific recognition of HNE-modified epitopes on the HSA molecule by autoantibodies in RA patients. These results indicate that the HNE-modified HSA is an effective inhibitor of autoantibodies obtained from RA patients showing substantial difference in the recognition of modified HSA over native HSA ($p<0.001$).

Recognition of the modified epitopes was further confirmed by band shift assay on SDS-PAGE, through visual detection of the immune complexes formed
between HNE-modified HSA and IgG isolated from RA patients. An increase in the amount of high molecular weight immune complexes having retarded mobility with progressive increase of IgG content clearly establish the HNE-modified HSA as a preferred antigen for these autoantibodies as compared to the native analogue.

Carbonyl content in SLE patient’s sera was significantly higher as compared to normal subjects (\(p<0.001\)verse normal subject). Furthermore HSA was isolated from RA patients and normal subjects and their carbonyl content was estimated. The data showed that carbonyl contents were significantly increased in RA-HSA as compared to HSA from normal subjects.

Furthermore, RA-HSA showed hyperchromicity at 280 nm, revealing marked conformational alterations due to HNE-induced modifications. The secondary structure content was also found to decrease. Moreover, the decrease in fluorescence intensity observed in RA-HSA could be due to the change in the microenvironment of the chromophoric groups.

Anti-HNE-HSA antibodies were also effectively used to probe the lipid peroxidation induced damage in the HSA isolated from RA patients. HSA isolated from healthy individuals was used as the control. An inhibition in the range of 57.5 to 74.9 percent was observed in the activity of experimentally induced anti-HNE-HSA antibodies when HSA from RA patients was used as an inhibitor. The mean maximum inhibition was 66.97±5.8%. HSA samples from healthy individuals showed negligible recognition of anti-HNE-HSA antibodies.

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

1. HNE induced modification of HSA resulted in the formation of covalent adduct with nucleophilic residues of protein.
2. The modification caused gross structural changes in HSA resulting in hyperchromicity and decreased fluorescence intensity.
3. Modification of protein with HNE resulted in the generation of fluorescent adduct, 2-alkyl-2-hydroxy-1, 2-dihydropyrrole-3-one iminium, characterized by emission maxima at 430 nm.
4. HNE-modified HSA was thermodynamically more stable than its native conformer as determined by thermal denaturation studies.
5. HPLC showed the formation of the adduct, \( \text{N}^\alpha\text{-acetly-N}^\varepsilon\text{-[1-(2-hydroxyethyl)-2-hydroxyhepltyl]-L-lysine} \) in modified HSA.
6. HNE mediated modification appears to have generated highly immunogenic epitopes on native HSA, inducing high titre antibodies in experimental animals.
7. The antibodies were highly specific for the immunogen. However, they also showed cross reactivity with other inhibitors e.g. HNE modified amino-acids and proteins.
8. The preferential binding of HNE-modified HSA by autoantibodies derived from SLE and RA sera demonstrate the role HNE-modified HSA in initiation/progression of SLE and RA.
9. Lipid peroxidation leads to toxicity of HSA as confirmed by cross-reaction of isolated albumin from SLE and RA patients towards anti HNE-HSA antibodies induced in experimental animals. Thus anti-HNE-HSA IgG could be used as a potent immunological tool for probing HNE damaged HSA in SLE and RA patients.