

Antibodies against 4-hydroxy-2-nonenal modified epitopes recognized chromatin and its oxidized forms: Role of chromatin, oxidized forms of chromatin and 4-hydroxy-2-nonenal modified epitopes in the etiopathogenesis of SLE

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Abstract. *Objectives:* This study was undertaken to investigate the role of lipid oxidative-by-product 4-hydroxy-2-nonenal (HNE)-modified human serum albumin (HSA), chromatin, reactive oxygen species (ROS)-modified chromatin and nitric oxide (NO)-modified chromatin in systemic lupus erythematosus (SLE).

Methods: HSA was modified by HNE. Immunogenicity of modified HSA was probed by inducing polyclonal antibodies in rabbits. Chromatin was isolated from goat liver and modified by ROS or NO. Immunocross-reactions of Protein-A purified anti-HNE-HSA-IgG with chromatin, ROS-chromatin and NO-chromatin were determined. Autoantibodies from 74 SLE patients were screened. HSA was isolated from SLE patients (SLE-HSA) and immunocross-reactions of isolated SLE-HSA with HNE-specific antibodies were investigated.

Results: HNE-HSA was found to be highly immunogenic in rabbits. The notable feature of anti-HNE-HSA-IgG showed cross-reactions with chromatin, ROS-chromatin and NO-chromatin ($p < 0.01$). High degree of specific binding to HNE-HSA, chromatin, ROS-chromatin or NO-chromatin was observed with antibodies from 55% of SLE patients. SLE anti-native/oxidized chromatin antibodies showed specificity towards HNE-HSA. Furthermore, SLE-HSA showed binding with HNE-specific antibodies.

Conclusions: This is the first study to demonstrate that chromatin and its oxidized forms have been recognized by antibodies against HNE modified epitopes. Our results provide an important insight into the immunological basis of the reported HNE-modified epitopes in SLE.

Keywords: Systemic lupus erythematosus, autoimmunity, 4-hydroxy-2-nonenal, chromatin, free radicals

1. Introduction

4-Hydroxy-2-nonenal (HNE) is one of the most prominent lipid peroxidation-specific aldehydes, is be-

lieved to be largely responsible for the pathological effects observed during oxidative stress [1]. HNE exerts these effects because of its facile reactivity with biological materials, including proteins [2]. It is reported that the sequence of an anti-HNE monoclonal antibodies (mAb) has highly homologous to the anti-DNA autoantibodies [3]. Excess generation of reactive oxygen species (ROS) have the ability, either directly or indirectly, to damage proteins, DNA, lipids

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and other cell biomolecules [4,5]. Protein oxidation, which results in functional disruption, is not random but appears to be associated with increased oxidation in specific proteins [5]. Human serum albumin (HSA) is the most abundant serum protein whose redox modification modulates its physiologic function, as well as serves as biomarker of oxidative stress [6,7]. Therefore, HSA is continuously exposed to oxidative stress, so that alterations of the conformation and function of HSA could occur, resulting in the modification of its biological properties.

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disease with several clinical manifestations [8,9]. The initial immunizing immunogen (s) that drive the development of SLE are unknown, but it was accepted that intermolecular epitope spreading was one of the mechanisms for the amplification and diversification of autoantibody responses in SLE [10]. Anti-double stranded DNA (anti-dsDNA) autoantibodies are a prime feature of SLE [9]. However, evidence has accumulated in recent years that anti-chromatin autoantibodies are correlated even better with SLE than anti-dsDNA autoantibodies [11,12] and anti-chromatin antibodies are known to occur in SLE before the appearance of anti-dsDNA antibodies [10]. Studies have shown that 65% of anti-DNA negative SLE patients are anti-chromatin antibody positive [12].

In the present study, commercially available HSA was modified by lipid oxidation product HNE. Immunization of rabbits with either HNE-modified HSA (HNE-HSA) or native HSA (nHSA) and an antibody response was established faster and more vigorously in the animals that were immunized with the HNE-HSA. Chromatin was isolated from goat liver and modified by ROS or nitric oxide (NO). Cross-reactions of affinity purified anti-ROS-HSA IgG or anti-nHSA IgG with chromatin, ROS-modified chromatin, NO-modified chromatin were determined and found that anti-ROS-HSA IgG showed immunospecificity with chromatin and its oxidized forms. This is our novel finding and has not been reported previously. In addition, the antigen(s) binding characteristics of naturally occurring SLE autoantibodies to HNE-HSA, nHSA, ROS-chromatin, NO-chromatin and unmodified chromatin were evaluated. Antibodies against chromatin, ROS-chromatin and NO-chromatin from SLE patients were also tested for the recognition of *in-vitro* generated HNE-HSA. Furthermore, immunocross-reactions of induced anti-HNE-HSA IgG and anti-nHSA IgG with HSA from SLE patients (SLE-HSA) and HSA from normal human controls (NH-HSA) were also attempted.

2. Materials and methods

2.1. Preparation of protein-HNE antigen

4-Hydroxy-2-nonenol (HNE; Cayman Scientific, Ann Arbor, MI, USA) modified protein (protein-HNE) antigen was prepared in Tris-HCl buffer as previously described [13]. Briefly HSA (Sigma-Aldrich, Co, USA) (1 mg) was incubated with Tris-HCl (5 mM, pH 8.3), and HNE (5 mM) for 7 h at 37°C. The unbound HNE was removed by centrifuging the sample with a 10,000-molecular-weight cut-off spin microfuge from Millipore (MA, USA). Generation of HNE in the reaction mixture was confirmed by L-carnosine (catalog # C9625, Sigma-Aldrich, CO, USA), a well known HNE inhibitor [14]. Concentration of L-carnosine in the reaction mixture was 20 mM.

2.2. Immunization schedule

The immunization of random bred, New Zealand white female rabbits (2–2.5 kg) was performed as described previously with some modifications [15]. Rabbits ($n = 4$; two each for native and HNE-modified HSA antigens) were immunized intramuscularly at multiple sites with 50 μ g of antigen, emulsified with an equal volume of Freund's complete adjuvant. The animals were boosted in Freund's incomplete adjuvant at weekly intervals for 6 weeks with the same amount of antigen. Test bleeds were performed 7 days post boost, which gave an appropriate titre of the antibody. The animals were bled and the sera were separated and heated at 56°C for 30 min to inactivate complement proteins and stored at –80°C. Pre-immune serum samples were collected prior to immunization.

2.3. Isolation and modification of chromatin

Chromatin was isolated from fresh goat liver as described by Bonner et al. [16]. Briefly, 10 g of goat liver was homogenized with 200 ml of saline-EDTA (75 mM NaCl, and 24 mM EDTA, pH 8.0). The homogenate was strained through 6–8 layer of cheese cloth. The filtrate was centrifuged at 1500 g for 15 min. the pellet was homogenized in 40 ml of Tris buffer (50 mM, pH8.0) and sedimented at 10,000 g for 15 min. This step was repeated once. The final pellet was suspended in 30 ml of Tris buffer (50 mM, pH 8.0). Five milliliter aliquots of the above suspension were layered on 25 ml portions of 1.7 M sucrose (in 10 mM Tris buffer, pH 8.0) contained in centrifuge tubes. The upper two thirds

of each tube were gently mixed and the tubes were then centrifuged at 21,000 rpm for 3 h at 4°C. The pellets were suspended in 10 mM Tris buffer, pH 8.0, and dialyzed overnight against the same buffer. The dialyzed suspension was sheared in Viritis homogenizer for 90 s, stirred for 30 min and then centrifuged at 10,000 g for 30 min. The supernatant of the above was referred to as sheared liver chromatin.

Isolated chromatin was modified by hydroxyl radicals (ROS), generated by UV irradiation (254 nm) of hydrogen peroxide (15.1 μ M) for 1 h at room temperature in PBS as described previously [17]. Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4. Chromatin was nitrated according to Ohshima et al. [18]. The pH of the solution was adjusted to 3.5 with acetic acid. Sodium nitrite (200 mM) was added to the final concentration of 1 mM and the solution was incubated at 37°C for 24 h. Excess sodium nitrite was removed by extensive dialysis against PBS, pH 9.0. Generation of hydroxyl radicals or nitric oxide was further checked by the addition of specific inhibitor of hydroxyl radicals (D-Mannitol, catalog # M9546, Sigma-Aldrich, CO, USA) [19] or nitric oxide (Carboxy-PTIO, catalog # C221, Sigma-Aldrich, CO, USA) [20] in their reaction mixtures. The concentration of D-Mannitol and Carboxy-PTIO used in their specific reactions were 286 mM and 30 mM, respectively.

2.4. Assay of carbonyl formation

Carbonyls contents of native and HNE-modified HSA, chromatin and its oxidized forms of chromatin were analyzed as previously described [6]. Briefly, the reaction mixture containing 15 μ M of native and modified samples, 0.5 ml of 10 mM 2, 4-dinitrophenylhydrazine (DNPH)/2.5 M HCl was added and thoroughly mixed. After addition of 250 μ M TCA (20%) and centrifugation, the pellet was collected and washed three times with 1 ml ethanol: ethylacetate (1:1) mixture. The pellet was then dissolved in 1 ml of 6 M guanidine solution and incubated at 30°C for 15 min. After centrifugation, the supernatant was collected and the carbonyl contents were estimated from the absorbance at 370 nm using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$. Samples were spectrophotometrically analyzed against a blank of 1 ml of guanidine solution (6M). Protein concentration was determined in the samples and carbonyl contents were expressed as nmol/mg protein.

2.5. Patients and serum preparation

Present study has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for humans and EC Directive 86/609/EEC for animals. The study group included 74 patients (69 female and 5 male) with SLE, as defined by the American College of Rheumatology 1997 revised criteria [21], and the age range was 29–67 years (mean \pm SD 49.4 \pm 12.2 years). All SLE samples showing high titre anti-DNA antibodies ($>$ 1:12,800). No patient had an active infection, known malignancy, tuberculosis, pregnancy, or cirrhosis. The patients were informed beforehand written consents were collected. The control group comprised 35 healthy subjects (33 female and 2 male, age range 26–68 years, mean \pm SD age 42.3 \pm 17.8 years). The mean ages were not significantly different between the groups. The racial/ethnic and sex compositions of the SLE groups were comparable with those of the control group. Venous blood samples from the control subjects and SLE patients were collected, and the serum from individual subjects was stored in small aliquots at $-80^{\circ}C$ until analyzed further.

2.6. ELISA

An enzyme-linked immunosorbent assay (ELISA) was performed on flat bottom 96-well, polystyrene immunoplates (NUNC, Denmark) as described previously [7]. Briefly, polystyrene polysorp immunoplates were coated with 100 μ l of native or HNE-modified HSA (10 μ g/ml) in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plates were coated for 2 h at 37°C and overnight at 4°C. Each sample was coated in duplicate and half of the plates served as control devoid of only antigen coating. Unbound antigen was washed thrice with TBS-T (20 mM Tris, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20) and unoccupied sites were blocked with 2% fat free milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4–6 h at 37°C. After incubation, the plates were washed four times with TBS-T. The test serum (1:100 or serially diluted) in TBS-T or affinity purified IgG in TBS (100 μ l/well) was adsorbed for 2 h at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-rabbit/human alkaline phosphatase conjugate (Sigma-Aldrich, Co, USA) using p-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader (Anthos Zenyth 3100 Multimode Detectors, Salzburg, Austria). Each sam-

ple was run in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of $A_{test}-A_{control}$.

2.7. *In-vitro* modification of solid phase protein by HNE

In-vitro modification of solid phase HSA by HNE was performed as described previously with slight modifications [22]. Briefly, HSA was coated at room temperature for 2 h on ELISA plates in carbonate coating buffer, the plates were washed twice with PBS, pH 7.4 and were tapped dry and HNE (5 mM) in PBS was added per test well. Each sample was coated in duplicate and half of the plates served as control which contained PBS, pH 7.4 alone. The plates were incubated at room temperature in a humid chamber for 4 h and were washed with TBS-T. The remaining steps were the same as in direct binding ELISA.

2.8. Competitive inhibition ELISA

Antibody specificity was determined by competitive binding assays as previously described [23,24]. Varying concentrations of inhibitors (0–20 $\mu\text{g}/\text{ml}$) were allowed to interact with a constant amount of antiserum or affinity purified IgG for 2 h at 37°C and overnight at 4°C. The immune complex thus formed was coated in the wells instead of serum/IgG. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculated using the formula:

$$\text{Percent inhibition} = 1 - [(A_{inhibited}/A_{uninhibited})] \times 100$$

2.9. Purification of immunoglobulin G

IgG from rabbit/human sera was isolated by affinity chromatography using Protein A-Sepharose CL-4B column (Sigma-Aldrich, Co, USA) as described previously [25]. Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to the column (12 mm \times 45 mm) equilibrated with the same buffer. The flow through was reloaded onto the column 2–3 times. Unbound proteins were removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride and neutralized with 1.0 ml of 1.0 mol/l Tris-HCl, pH 8.5, 1 ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering $1.38 \text{ OD}_{278} = 1.0 \text{ mg IgG}/\text{ml}$. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at -20°C . The homogeneity of isolated IgG was checked by polyacrylamide gel electrophoresis.

2.10. Immunoprecipitation

Affinity purified SLE IgG (200 μg) was incubated with 200 μg of chromatin, ROS-chromatin, or NO-chromatin in different reaction tubes with gentle rocking overnight at 4°C. Protein A/G agarose beads (Pierce, Rockford, IL, USA) were added (40 μl of 50% bead slurry in 500 μl of total reaction volume) and incubated with gentle rocking for 3 hours at 4°C. Microcentrifuged the reaction tubes for 30 seconds at 4°C and washed the pellets five times with 500 μl of PBS, pH 7.4. After the final wash, supernatant was removed. Desired SLE antibodies were eluted from the solid support using low-pH and analyzed with direct binding ELISA by *in-vitro* modification of solid phase HSA by HNE as described previously [22].

2.11. Purification of HSA from SLE patients and normal healthy controls

Albumin was isolated by ammonium sulphate fractionation followed by gel exclusion chromatography on Sephacryl S-200 HR column as previously described [26]. Protein in each fraction was monitored by the method of Lowry et al. [27]. The purity of the isolated HSA was checked by SDS-PAGE.

2.12. Western immunoblot analysis

Immunoblot of antibodies from SLE and normal human (NH) serum samples binding to HNE-modified HSA or unmodified HSA as described previously [28]. HNE-HSA or nHSA (40 μg) was resolved on 4%–20% gradient SDS-PAGE (Bio-Rad) and PVDF immobilon-P transfer membranes (Millipore Corporation, Bedford, MA, USA). The membrane was then excised into strips and each membrane strip was blocked with non-fat dry milk powder in Tris buffered saline and 0.1% Tween-20 (TBS-T). Each strip was probed with a different serum samples diluted 1:100. Immunoreactive blot strips were visualized by using 1:1000 diluted HRP-linked secondary antibodies and enhanced chemiluminescence (GE Healthcare, Milwaukee, WI, USA). Images were captured using AFP-Imaging System (Minimedical Series, Elms Ford, NY, USA).

2.13. Statistical analysis

All measurements were performed in duplicates and repeated at least 3 times using age- and sex-matched

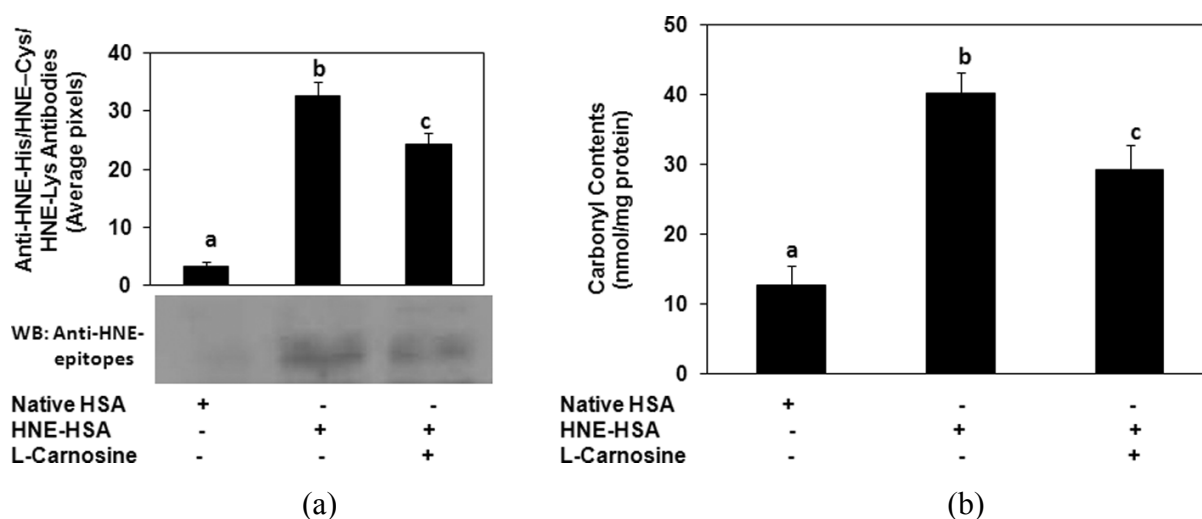


Fig. 1. Characterization of 4-hydroxy-2-nonenal (HNE) modification of human serum albumin (HSA). (a) Immunoblot analysis of HNE-modification of HSA. Native HSA, HNE-modified HSA and HNE-HSA in the presence of L-Carnosine were immunoblotted with anti-HNE-monoclonal antibodies. These monoclonal antibodies were specific for 4HNE-lysine, 4HNE-histidine and 4HNE-cysteine adduct. Band images were digitally captured and the band intensities (pixels/band) were obtained using the Un-Scan-It software. Average pixel values presented as Mean \pm SD; data without a common letter differ, $p < 0.05$. (b) Carbonyl contents in native HSA, HNE-HSA and HNE-HSA in the presence of L-Carnosine. The protein concentration in all samples was 15 μ M. Each histogram represents the mean \pm SD of five independent assays. Data without a common letter differ, $p < 0.01$.

SLE or control samples. Comparisons were performed using Origin 6.1 software package (Northampton, MA, USA) and Graph Pad Prism-5 (San Diego, CA, USA) (one paired two tailed t -test with ANOVA analysis and Tukey's post-hoc analysis). P values less than 0.05 were considered significant, and P values less than 0.001 were considered highly significant. Values shown are mean \pm SEM unless stated otherwise.

3. Results

3.1. Characterization of HNE-modified HSA

Earlier reports [29–31] showed alterations in HSA following exposure to the HNE. HPLC analysis showed that HNE was rapidly quenched by HSA because of the covalent adduction to the different accessible nucleophilic residue of the protein, as demonstrated by electrospray ionization mass spectrometry (ESI-MS) [29]. A liquid chromatography-tandem mass spectrometry (LC-MS) analysis identified 11 different HNE-adducts, 8 Michael adducts and 3 Schiff bases and it was found that HNE forms stable protein adducts with histidine, lysine and cysteine side chain [29,30]. Lipid peroxidation generated epitopes on HSA was further characterized by Western immunoblot analysis using commercially available HNE-specific monoclonal antibodies

(JalCA, Shizuoka, Japan). These antibodies were specific for 4HNE-lysine, 4HNE-histidine or 4HNE-cysteine adduct. Our results clearly showed that *in-vitro* generated HNE-HSA was well recognized by anti-HNE-His/lys/cys antibodies. The generation of HNE was further confirmed by using L-Carnosine, well known quenchers of HNE-products. The data showed a significant loss ($p < 0.05$) in the band intensity, when the modification was done in the presence of L-Carnosine, whereas band intensity was high in the absence of this quencher (Fig. 1a). Carbonyl contents estimation in native HSA, HNE-HSA and HNE-HSA with L-Carnosine further reiterate the Western immunoblotting results (Fig. 1). The data showed a significant increase ($p < 0.001$) in carbonyl formation in HNE-HSA as compared with unmodified HSA. Addition of L-Carnosine to the reaction mixture significantly decrease ($p < 0.05$) the carbonyl contents (Fig. 1b).

3.2. Antigenicity of HNE-modified epitopes

Immunizations of HNE-modified HSA adduct or unmodified HSA in rabbits induced high titre antibodies. HNE-modified HSA was found to be a potent immunogen in rabbits inducing high titre antibodies (titre = 51200), whereas with native HSA the titre was low (titre = 25600) under identical experimental conditions (Fig. 2a). Preimmune serum serve as negative con-

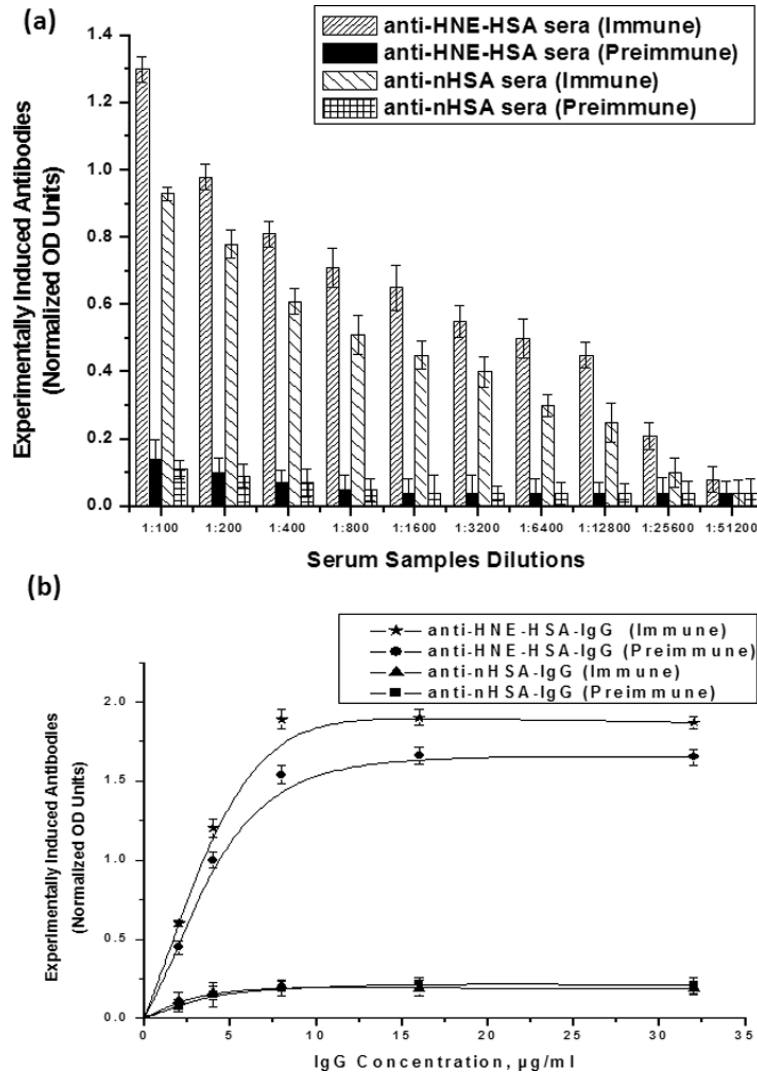


Fig. 2. Level of experimentally induced antibodies against 4-hydroxy-2-nonenal-modified human serum albumin (HNE-HSA) and native human serum albumin (nHSA). (a) Binding of HNE-HSA or nHSA with their respective immune and preimmune sera. (b) Binding of HNE-HSA or nHSA with their respective immune and preimmune affinity purified IgG. New Zealand white rabbits ($n = 4$; two each for HNE-HSA or nHSA antigens) were immunized intramuscularly as described in methods. Microtitre plates were coated with their respective antigens ($10 \mu\text{g/ml}$). Each point represents a mean \pm SEM of four independent assays.

control, showed negligible binding with modified or unmodified proteins. Direct binding ELISA of Protein A-Sepharose isolated anti-HNE-HSA IgG and anti-nHSA IgG showed strong binding with their respective immunogens. Preimmune affinity purified IgG showed negligible binding under identical conditions (Fig. 2b).

3.3. Characterization of ROS-modified and NO-modified chromatin

Modifications in chromatin by ROS or NO have been studied by carbonyl contents estimation. The average

carbonyl contents (\pm SD) of five independent assays in unmodified chromatin were 18.4 ± 3.3 nmol/mg protein. Whereas, the carbonyl contents (\pm SD) of five independent assays in ROS and NO-modified chromatin were 53.6 ± 3.3 and 49.5 ± 3.8 nmol/mg protein, respectively (Fig. 3), a p-value of < 0.001 indicates significant difference in the carbonyl contents of native and modified chromatin. The generation of hydroxyl radicals in ROS modification of chromatin was confirmed by using D-mannitol, specific inhibitor of hydroxyl radicals. The data showed significant decreased ($p < 0.05$) in carbonyl formation, when the modifica-

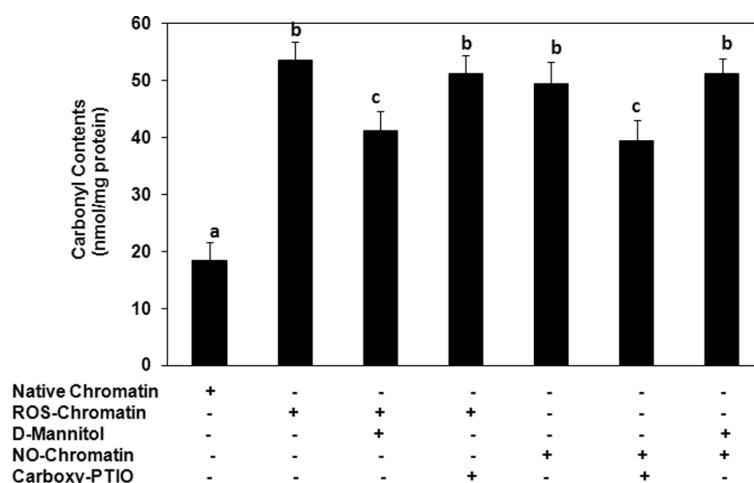


Fig. 3. Modification of chromatin by reactive oxygen species and nitric oxide. Carbonyl contents in native chromatin, reactive oxygen species-modified chromatin (ROS-chromatin), ROS-chromatin in the presence of D-mannitol (catalog # M9546, Sigma-Aldrich) or carboxyl-PTIO (catalog # C221, Sigma-Aldrich), nitric oxide-modified chromatin (NO-chromatin) and NO-chromatin in the presence of carboxyl-PTIO or D-mannitol. The protein concentration in all samples was 15 μ M. Each histogram represents the mean \pm SEM of five independent assays. Data without a common letter differ, $p < 0.01$.

tion was done in the presence of this inhibitor. Whereas, high carbonyl formation occurred in its absence (Fig. 3). Similarly, generation of nitric oxide was also confirmed by using NO inhibitor, Carboxy-PTIO. Carbonyl formation was significantly decreased ($p < 0.05$), when NO modification was done in the presence of this inhibitor (Fig. 3). We also cross checked the effect of NO inhibitor on the generation of hydroxyl radicals or hydroxyl radicals inhibitor on NO generation. The data showed D-mannitol had no effect on the generation of NO. Likewise, Carboxyl-PTIO was also failed to inhibit the generation of hydroxyl radicals (Fig. 3).

3.4. Recognition of chromatin, ROS-modified chromatin and NO-modified chromatin by anti-HNE-protein antibodies

Induced IgG against HNE-modified HSA or unmodified HSA was purified by affinity chromatography on Protein A-Sepharose CL-4B column. The purified IgG was found to elute in a single symmetrical peak and migrated as a single homogenous band on SDS-PAGE (data not shown). Affinity purified anti-HNE-HSA IgG and anti-nHSA IgG were tested for binding to native or oxidized chromatins by direct binding ELISAs. Our data showed that chromatin, ROS-chromatin and NO-chromatin were well recognized by anti-HNE-HSA IgG, but the recognition was more with ROS-chromatin or NO-chromatin (Fig. 4a; $p < 0.05$). Whereas, anti-

nHSA IgG showed no binding with chromatin and its oxidized forms (Fig. 4b; $p > 0.05$). Native and HNE-modified HSA showed strong binding with their respective induced antibodies and were used as positive controls in their respective binding assays (Fig. 4a&b).

The antigenic specificity of induced anti-HNE-HSA antibodies was further characterized by competitive inhibition assays (Fig. 4c). A maximum of 97.8% inhibition of the anti-HNE-HSA IgG with the immunogen as inhibitor was observed at 20 μ g/ml of inhibitor concentration. The anti-HNE-HSA antibodies exhibited a variable recognition of chromatin, ROS-modified chromatin and NO-modified chromatin in competition ELISA. Inhibition of affinity purified anti-HNE-HSA IgG by native chromatin, ROS-chromatin and NO-chromatin was 44.2%, 67.8%, and 66.8%, respectively at 20 μ g/ml of inhibitor concentration (Fig. 4c). 8.8 μ g/ml of ROS-chromatin was required for 50% inhibition, whereas 8.2 μ g/ml of NO-chromatin was required for 50% inhibition of anti-HNE-HSA IgG. Competition ELISA with anti-HSA IgG showed a maximum inhibition of 89.3% with the immunogen as inhibitor and 12.9 μ g/ml of immunogen was required for 50% inhibition in antibody binding. The anti-HSA IgG didn't recognized chromatin and its oxidized forms (Fig. 4d).

3.5. Detection of SLE autoantibodies directed to HNE-modified epitope(s)

To probe the possible role of the lipid oxidative-by product HNE and its associated autoimmunity in the

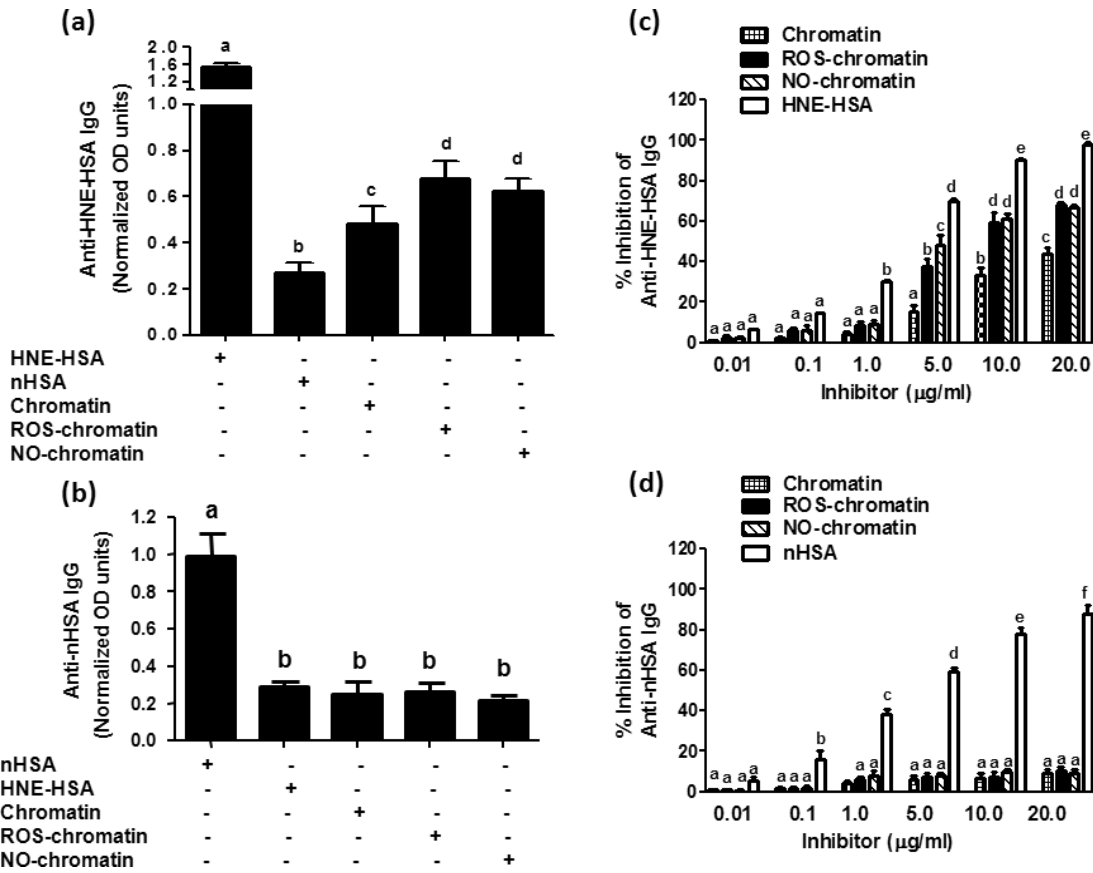


Fig. 4. Recognition of chromatin and its oxidized forms by anti-HNE-HSA IgG. Binding characteristics of affinity purified anti-HNE-HSA IgG (a,c) and anti-nHSA IgG (b,d) to native chromatin (chromatin), reactive oxygen species-modified chromatin (ROS-chromatin), nitric oxide-modified chromatin (NO-chromatin) determined direct binding (a,b) and competitive inhibition (c,d) ELISAs. HNE-HSA and nHSA were used as positive controls with their respective immunogens. Each histogram represents a mean \pm SEM of three independent assays. Data without a common letter differ, $p < 0.05$.

pathogenesis of SLE, 74 sera of anti-DNA autoantibodies high titre were selected for the binding to native and HNE-modified protein. Direct binding ELISA showed majority of SLE sera (41/74) showed strong binding to HNE-HSA over native HSA (nHSA) at 1:100 serum dilution ($p < 0.05$) (Fig. 5a). The average absorbance at 410 nm (\pm SD) of 41 SLE sera binding to HNE-HSA and native HSA was 0.66 ± 0.23 and 0.31 ± 0.01 , respectively. Whereas, the absorbance at 410 nm (\pm SD) of 35 normal human (NH) sera binding to HNE-HSA and nHSA was 0.18 ± 0.21 and 0.19 ± 0.12 . SLE autoantibodies directed to HNE-HSA was further evaluated by competitive inhibition ELISA. The average percent inhibition (\pm SD) in the binding of 41 SLE sera to HNE-HSA and nHSA was 47.1 ± 9.5 and 14.8 ± 7.9 , respectively. The data reveals striking differences in the recognition of HNE-HSA and nHSA by SLE autoantibodies ($p < 0.001$). We have also con-

ducted similar experiments with NH sera under identical experimental conditions, NH sera showed negligible binding with either of the antigen (Table 1). Native DNA was used as an immunochemical marker for SLE. The average absorbance at 410 nm (\pm SD) of 41 SLE sera binding to native DNA was 0.71 ± 0.25 . Whereas, the absorbance at 410 nm (\pm SD) of 35 normal human (NH) sera binding to DNA was 0.16 ± 0.15 . Competition assays with DNA reiterate the direct binding ELISA results that SLE autoantibodies showed high degree of specificity towards native DNA (data summarized in Table 1). IgG was purified from 15 most active SLE sera by affinity chromatography using Protein A-Sepharose CL-4B column. The interaction of HNE-modified HSA with affinity purified SLE IgG was ascertained by direct binding ELISA. Figure 5b shows the binding of affinity purified SLE IgG to HNE-HSA. The average absorbance at 410 nm (\pm SD) in

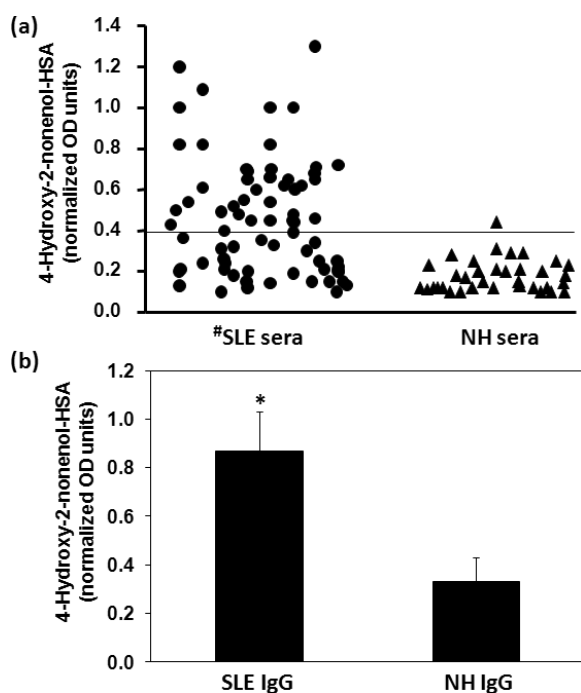


Fig. 5. Binding of SLE autoantibodies to HNE-modified HSA. (a) Direct binding ELISA of circulating antibodies of SLE patients (●) and normal human subjects (NHS) binding to HNE-modified epitopes. Seventy four SLE patients and thirty five NH sera (1:100 diluted) were analyzed for the binding to HNE-HSA. Values above 0.4 were considered positive (above the line). (b) Direct binding ELISA of affinity purified SLE IgG and normal human (NH) IgG binding to HNE-modified HSA. IgGs were purified from fifteen most active SLE patients and thirteen normal health subjects using protein-A Sepharose CL-4B affinity column. ELISA plates were coated with HNE-HSA (10 μ g/ml). # $p < 0.01$ Vs NH sera; * $p < 0.001$ Vs NH IgG.

the binding of 15 SLE IgG to HNE-HSA was 0.87 ± 4.5 . Similar experiments were done with affinity purified normal human (NH) IgG used as a control. The average absorbance at 410 nm (\pm SD) in the binding of 13 NH IgG to HNE-HSA was 0.43 ± 2.3 (Fig. 5b). Native HSA under identical experimental conditions did not showed negligible binding with either SLE IgG or NH IgG and was used as a negative control (data not shown). The data reveal striking differences in the recognition of native and HNE-modified HSA by SLE antibodies ($p < 0.01$).

The interaction of HNE-modified epitope (s) with SLE autoantibodies was also evaluated by Western immunoblot analysis. Figure 6 shows the binding of most active SLE or NH antibodies to HNE-HSA and nHSA. The data showed striking difference in the recognition of HNE-modified HSA by SLE serum autoantibodies as compared with NH antibodies present in the

serum samples (Fig. 6a). Whereas neither SLE nor NH serum antibodies showed reactivity with nHSA (Fig. 6b). Experimentally induced antibodies against HNE-HSA or nHSA used as positive controls. Interaction of HNE-HSA with purified SLE IgG was also determined by Western immunoblot analysis under identical experimental conditions as with SLE sera. Results obtained were same as we obtained with serum samples (Figs 6c&d). The data showed that 4-hydroxyl-2-nonenol modified epitope(s) were well recognized by SLE autoantibodies (Fig. 6).

3.6. Recognition of chromatin, ROS-chromatin and NO-chromatin by SLE autoantibodies

The binding specificity of Protein-A sepharose purified SLE IgGs from 22 selected sera of SLE patients was evaluated by competitive inhibition ELISA using native chromatin, ROS-chromatin and NO-chromatin as inhibitors (Fig. 7a). Whereas, HNE-HSA was used as positive control and nHSA was used a negative controls. Results showed higher reactivity of autoantibodies in patient's sera towards HNE-HSA, n-chromatin, ROS-chromatin and NO-chromatin, whereas nHSA didn't recognized by SLE IgG. The average percent inhibition (\pm SEM) in the binding of 22 SLE sera to HNE-HSA by HNE-HSA, chromatin, ROS-chromatin, NO-chromatin and nHSA was 61.1 ± 6.5 , 39.2 ± 4.7 , 50.5 ± 4.7 , 54.5 ± 2.8 and 13.3 ± 4.7 , respectively. Native DNA (nDNA) was used as immunochemical marker for SLE and was strongly recognized by affinity purified SLE IgGs. Furthermore, we have also tested affinity purified NH antibodies for the recognition of the same chromatin and its oxidative forms under identical experimental conditions as with SLE antibodies. Results revealed that NH IgGs showed insignificant binding to all the tested inhibitors ($p > 0.05$; Fig. 7b). Data pointed out a significant difference in the recognition of HNE-HSA, chromatin and its oxidative forms by SLE antibodies, when compared with the antibodies from normal human controls ($p < 0.0001$) (Fig. 7).

3.7. Recognition of HNE-modified epitope(s) by anti-chromatin, anti-ROS-chromatin or anti-NO-chromatin SLE antibodies

Direct binding ELISA of immunoprecipitation purified anti-chromatin, anti-ROS-chromatin and anti-NO-chromatin SLE IgGs showed strong binding with HNE-modified HSA as compared with nHSA ($p < 0.001$) (Fig. 8). The average absorbance at 410 nm (\pm SEM) of

Table 1
Immunological details of study subjects

Parameters		SLE-sera	NH-sera
Age		45.2 ± 17.6 (n = 41)	42.1 ± 18.2 (n = 35)
Sex		39F/2M	32F/3M
Detection of anti-HNE-HSA antibodies	A ₄₁₀ (HNE-HSA)	0.66 ± 0.23 ^a	0.18 ± 0.0 ^b
	MPI (HNE-HSA)	47.1 ± 9.5 ^d	13.3 ± 4.7 ^e
Detection of anti-nHSA antibodies	A ₄₁₀ (nHSA)	0.31 ± 0.10 ^c	0.19 ± 0.12 ^b
	MPI (nHSA)	14.8 ± 7.9 ^e	12.9 ± 8.2 ^e
Detection of anti-dsDNA antibodies	A ₄₁₀ (dsDNA)	0.71 ± 0.25 ^a	0.16 ± 0.15 ^b
	MPI (dsDNA)	56.7 ± 10.3 ^f	12.3 ± 5.7 ^e

ELISA plates were individually coated with native HSA, HNE-HSA or native DNA. Unbound protein or DNA were washed with tris buffer saline containing tween-20 (TBS-T) and unoccupied sites were blocked with 2% fat free milk solution. Test serum in direct binding assays or antigen-serum mixture in inhibition immunoassays were then coated and bound antibodies were assayed using anti-human alkaline phosphatase conjugate (catlog # A3187; Sigma-Aldrich) and p-nitrophenyl phosphate (catalog # 71768; Sigma-Aldrich) as described in methods section. Percent inhibition in competitive inhibition assays was calculated using the formula: Percent inhibition = $1 - [(A_{inhibited} / A_{uninhibited})] \times 100$. Abbreviations: SLE-sera: sera from systemic lupus erythematosus patients; NH-sera: sera from normal human subjects; n: number of samples tested; F: females; M: males; nHSA: native HSA; A₄₁₀: absorbance at 410 nm calculated by direct binding ELISA; MPI: maximum percent inhibition at 20 µg/ml of inhibitor concentration calculated by competitive inhibition ELISA; dsDNA: native calf thymus double stranded DNA used as a positive control for SLE patients. Data represents as mean ± SD. Data without a common letter differ, $p < 0.05$.

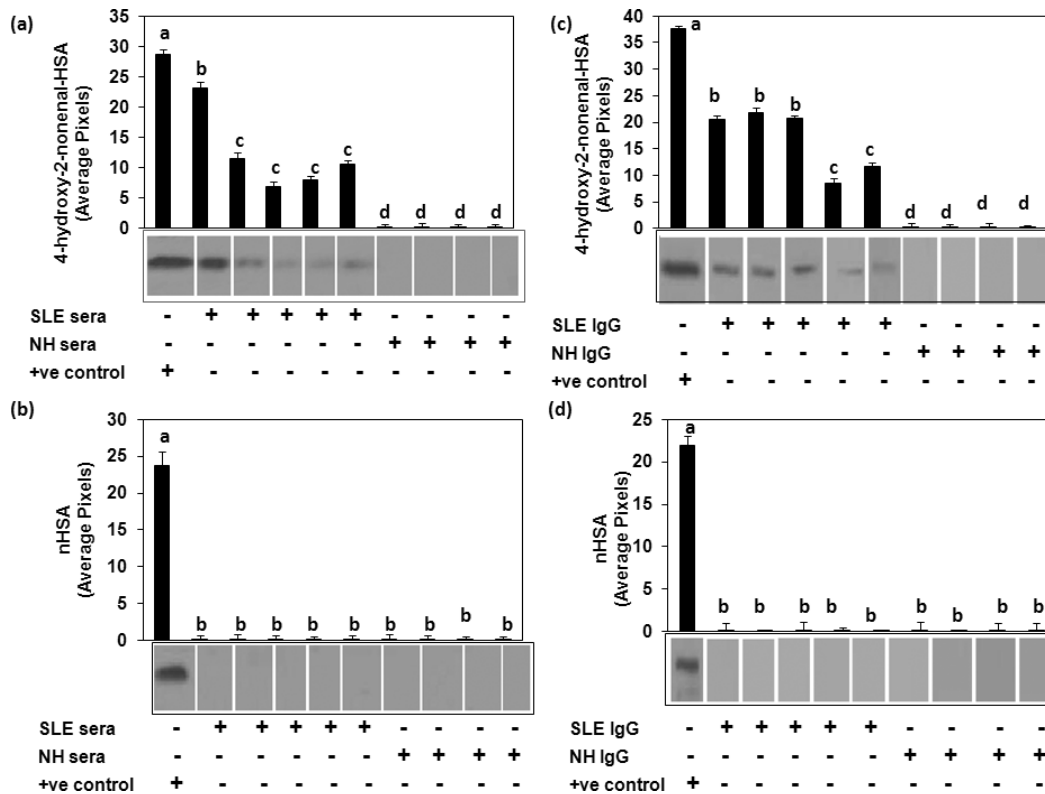


Fig. 6. Immunoblot analysis of SLE autoantibodies binding to HNE-modified HSA. Western immunoblot of antibodies from SLE and normal human serum samples binding to HNE-modified protein (a) and unmodified protein (b). Western immunoblot of affinity purified IgG from SLE and normal human subjects binding to HNE-HSA (c) and HSA (d). HNE-HSA or nHSA (40 µg) was resolved on 4%–20% gradient SDS-PAGE and transfer to PVDF immobilon-P membranes. The membrane was then excised into strips and each membrane strip was blocked with non-fat dry milk powder in Tris buffered saline and 0.1% Tween-20 (TBS-T). Each strip was probed with serum samples (1:100 diluted) or affinity purified IgG samples (1:500 diluted). Experimentally induced anti-HNE-HSA antibodies or anti-nHSA antibodies were used as positive controls. Band images were digitally captured and the band intensities were obtained using UN-San-It software (Silk Scientific Corporation, Orem, UT, USA) and are expressed in average pixels/band. Average pixel values presented as Mean ± SD; data without a common letter differ, $P < 0.05$.

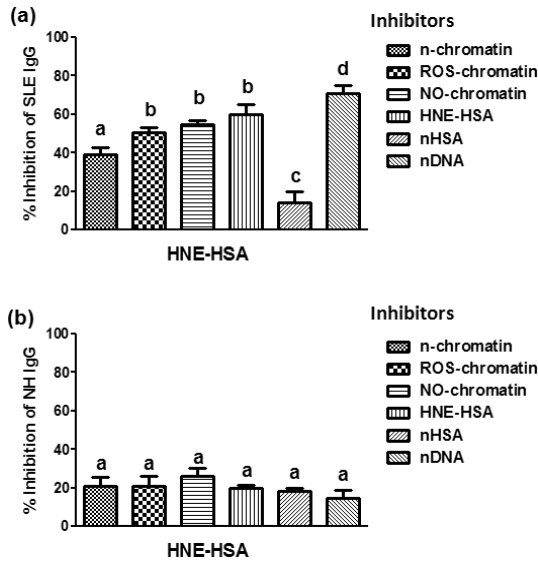


Fig. 7. Competitive inhibition of SLE IgG. Inhibition of affinity purified SLE IgG (a) and normal human IgG (b) binding to HNE-modified protein. Inhibitors used were native chromatin (n-chromatin), ROS-modified chromatin (ROS-chromatin), nitric oxide-modified chromatin (NO-chromatin), HNE-modified protein (HNE-HSA) and native protein (nHSA). Native calf thymus dsDNA used as a positive control for SLE patients. Inhibitors concentration used in the competitive binding assays was 20 $\mu\text{g/ml}$. Microtitre plates were coated with HNE-HSA (10 $\mu\text{g/ml}$). The number of SLE samples used was 22. Each histogram represents a mean \pm SEM of four independent assays. Data without a common letter differ, $p < 0.05$.

three independent assays with anti-chromatin SLE IgG binding to HNE-HSA and native HSA was 0.35 ± 0.06 and 0.09 ± 0.04 , respectively. Whereas, the average absorbance at 410 nm (\pm SEM) of three independent assays with anti-ROS-chromatin SLE IgG binding to HNE-HSA and native HSA was 0.48 ± 0.03 and 0.09 ± 0.03 , respectively and with anti-NO-chromatin SLE IgG the average absorbance was 0.54 ± 0.05 and 0.10 ± 0.05 , respectively (Fig. 8).

3.8. Identification of lipid peroxidative damaged in albumin purified from SLE patients

To investigate the HNE modifications in HSA of SLE patients, HSA was isolated from 15 most active SLE patients (SLE-HSA) and also from 13 normal healthy subjects (NH-HSA). The purified HSA was found to elute in a single symmetrical peak on Sephacryl S-200 HR column (Fig. 9). SDS-PAGE of purified HSA showed a single homogenous band (Fig. 9 inset). HNE generated epitope(s) on SLE-HSA were identified by Western immunoblot analysis using commercially HNE-specific monoclonal antibodies. Our results showed that SLE-

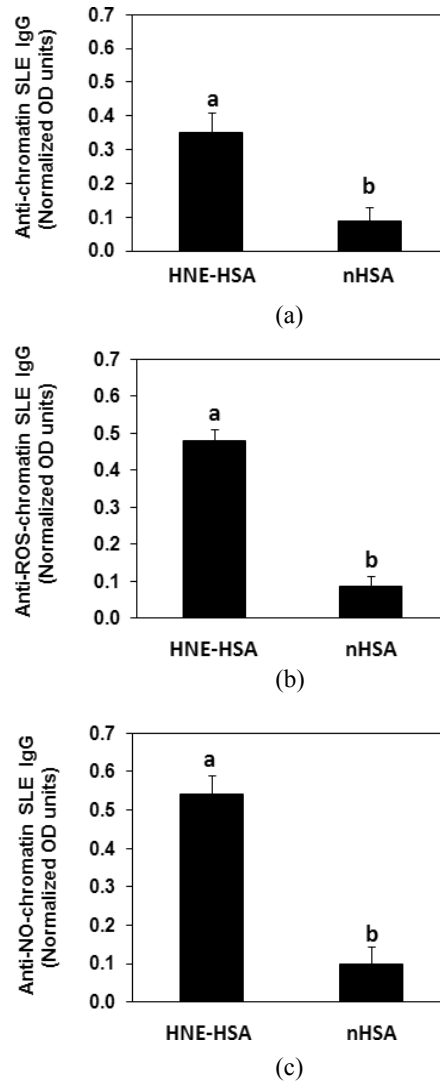


Fig. 8. Immunoprecipitation of SLE autoantibodies with chromatin or its oxidized forms, followed by direct binding ELISA with native and HNE-modified HSA. Immunoprecipitated SLE anti-chromatin IgG, anti-ROS-chromatin IgG and anti-NO-chromatin IgG were eluted from Protein-G beads by changing the pH and were used in ELISA with native and HNE-HSA as antigens. Data shown are cumulative of three experiments and values are presented as Mean \pm SEM; data without a common letter differ, $p < 0.05$.

HSA was well recognized by anti-HNE-specific antibodies, whereas these antibodies were failed to recognize NH-HSA (Fig. 10a). We have also demonstrated, HNE-induced modifications in SLE-HSA by carbonyl estimation. The average carbonyl contents (\pm SD) of five independent assays of SLE-HSA and NH-HSA were 2.3 ± 0.15 and 1.69 ± 0.11 nmol/mg protein, respectively (Fig. 10b), a p value of 0.01 indicates significant difference in the carbonyl contents of SLE-HSA

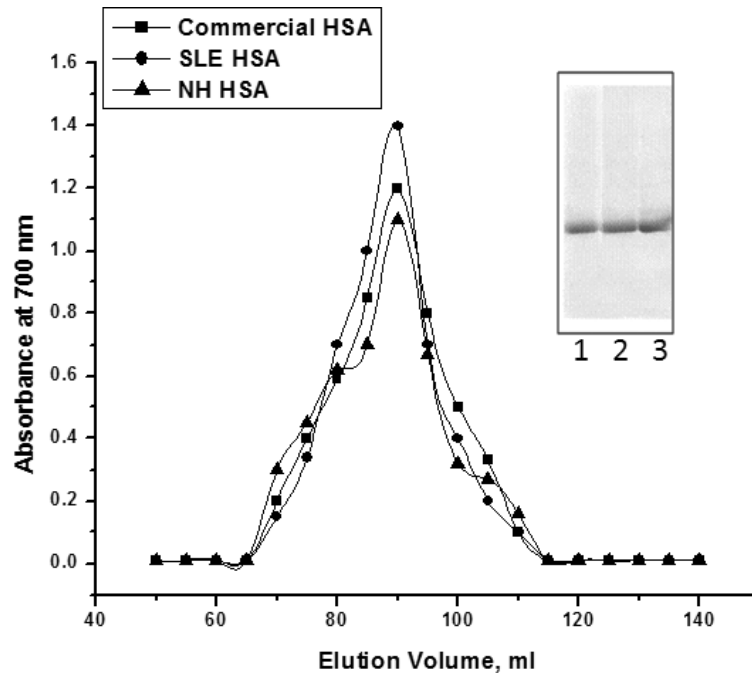


Fig. 9. Purification of HSA from SLE patients. Elution profiles of purified HSA from SLE patients (▲) and normal humans subjects (■) on Sephacryl S-200 HR column. Commercial HSA (●) was also eluted from the same column and was used as a marker. Protein in each fraction was monitored by the method of Lowry *et al.* (1951). Inset: Native PAGE of purified HSAs, and commercial HSA on 7.5 % gel. Lane 1 contains eluted commercial HSA, lane 2 and 3 contain purified HSA from normal human and SLE patient's, respectively.

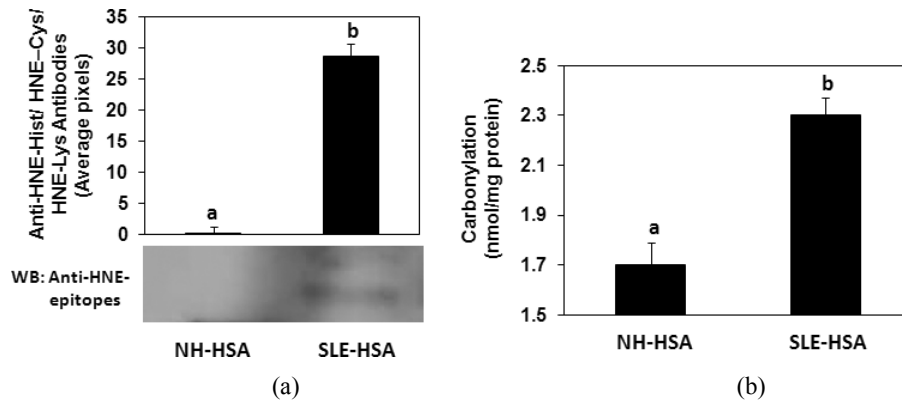


Fig. 10. Characterization of HSA from SLE patients. (a) Immunoblot analysis of HSA from SLE patients (SLE-HSA) and normal human subjects (NH-HSA). SLE-HSA and NH-HSA were probed with commercial anti-HNE-monoclonal antibodies. Band images were digitally captured and the band intensities (pixels/band) were obtained using the Un-Scan-It software. Average pixel values presented as Mean \pm SD; data without a common letter differ, $p < 0.05$. (b) Carbonyl contents in SLE-HSA and NH-HSA. The protein concentration in both HSA samples was 15 μ M. Each histogram represents the mean \pm SEM of five independent assays. Data without a common letter differ, $p < 0.001$.

and NH-HSA. In addition, HNE generated epitopes on SLE-HSA were further screened by experimentally induced rabbit anti-HNE-HSA IgG. The average absorbance at 410 nm (\pm SEM) of three independent assays of anti-HNE-HSA IgG binding to SLE-HSA and NH-HSA was 0.45 ± 0.06 and 0.097 ± 0.05 , respec-

tively (Fig. 11a). Whereas, the absorbance at 410 nm (\pm SEM) of three assays with induced anti-nHSA IgG binding to SLE-HSA and NH-HSA was 0.18 ± 0.03 and 0.16 ± 0.02 , respectively (Fig. 11b). Data clearly pointed out that SLE-HSA was well recognized by anti-HNE-HSA IgG. No appreciable binding was ob-

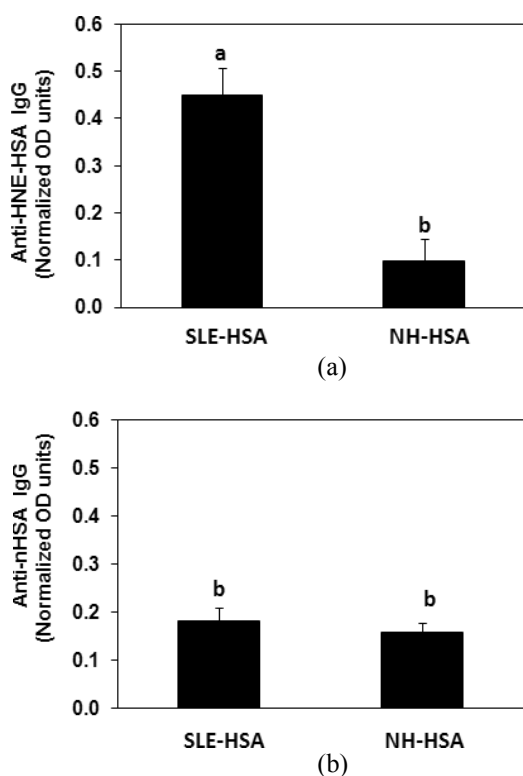


Fig. 11. Binding characteristics of anti-HNE-HSA IgG binding to SLE-HSA. Direct binding ELISA of experimentally induced rabbit anti-HNE-HSA IgG and anti-nHSA IgG to purified HSA samples from SLE patients (SLE-HSA) and normal human subjects (NH-HSA). ELISA plates were coated with SLE-HSA or NH-HSA (10 μ g/ml). Each histogram represents the mean \pm SEM of four independent assays. Data without a common letter differ, $p < 0.001$.

served between anti-nHSA IgG and SLE-HSA. Whereas, NH-HSA under identical experimental conditions didn't recognized by neither anti-HNE-HSA IgG or nor by anti-nHSA IgG (Fig. 11).

4. Discussion

In the present study, commercially available HSA was modified with HNE *in-vitro*, HNE generated neo-epitope(s) were characterized by commercially available HNE-specific antibodies (Shizuoka, Japan) and by carbonyl formation. Modified HSA was found to be a potent antigenic stimulus inducing high titre antibodies in rabbits, whereas with nHSA, low titre antibodies were obtained. The antigenic specificity of affinity purified anti-HNE-HSA IgG and anti-nHSA IgG reiterated that induced antibodies were immunogen specific. The substantially enhanced immunogenicity of

HNE-HSA in comparison to nHSA could possibly be due to the HNE-generated neo-epitopes. Anti-dsDNA antibodies are often considered to be a hallmark of SLE [32]. However, evidence has accumulated in recent years that anti-chromatin autoantibodies are correlated even better than anti-dsDNA in SLE [33,34]. Chromatin is an antigen for T and B cells from patients with SLE [35]. Additionally, anti-chromatin antibodies are a ubiquitous feature of murine SLE [35] and are necessary but not sufficient for the development of glomerulo nephritis in one strain of mouse [36]. It was found that anti-chromatin always preceded the appearance of anti-dsDNA antibodies in two strains of mice, and the suggestion was made that anti-dsDNA antibodies were a subset of anti-chromatin antibodies [37]. For these reasons, we have tested the specificity of experimentally induced antibodies against HNE-modified epitope(s) towards chromatin and its oxidized forms. Our results showed that affinity purified anti-HNE-HSA antibodies showed cross reactions with chromatin, ROS-chromatin and NO-chromatin. Whereas, induced antibodies against nHSA showed negligible binding to these nucleic acid antigens. Therefore, it was thought worthwhile to investigate the binding characteristics of natural occurring SLE autoantibodies to HNE-damaged HSA, so that the possible involvement of HNE-modified epitopes could be ascertained. Sera of 74 SLE patients having high anti-DNA antibodies titre and 35 normal human (NH) subjects were collected for the present study. Of these, 55.4% SLE sera showed preferentially high binding to HNE-modified HSA as compared to its native analogue as determined by direct binding ELISA. No appreciable binding was observed with the normal human subjects. These results were further confirmed with Protein-A purified IgGs from the same SLE patients. Competitive inhibition ELISA reiterated the direct binding results, that the HNE-modified HSA was an effective inhibitor, showing substantial difference in the recognition of SLE IgG over normal human IgG. Previously it was reported that the differential immobilization of HNE-modified proteins and unmodified proteins to ELISA plates (since binding to plastic is charge/conformation dependent) [22]. With the modification of histidine and lysines by HNE, the protein tends to lose positive charges due to these amino acids, and thereby become more negatively charged. This enables the modified proteins to bind to ELISA plates better than the unmodified proteins, thereby skewing the results obtained and it was also reported that the conformation of the protein also changes following modification, which may also affect the binding of the protein

to the solid surface [22]. To rule out this possibility of our ELISA results with HNE-modified HSA, we also performed some of ELISA experiments by modifying a solid phase protein as described previously [22]. Results obtained with this methods were little bit difference in the binding of antibodies with pre- and post-immobilization of the HNE-antigens but the overall pattern for the recognition of antibodies to HNE-modified antigen was same. Furthermore, this possibility of differential immobilization of HNE-modified antigen was further ruled out by Western immunoblot analysis. Immunoblot data further substantiates the enhanced binding of HNE-modified epitopes towards SLE autoantibodies as compared with antibodies present in normal human subjects. In addition, we have also tested the same SLE sera or SLE IgG for the detection of SLE autoantibodies specificity towards chromatin and its oxidized forms. All the sera or IgGs tested showed preferentially high binding to ROS-modified or NO-modified chromatin as compared to unmodified chromatin. Thus by employing these immunological techniques, the data clearly demonstrates a substantial increase in the recognition of HNE-modified HSA, ROS-modified chromatin and NO-modified chromatin by circulating SLE autoantibodies.

Furthermore, we have also investigated the HNE-induced alterations of HSA in SLE patients, HSA was isolated from 15 most active SLE patients (SLE-HSA) and also from 13 normal human subjects (NH-HSA) and their carbonyl contents or immunoreactivity towards HNE-specific antibodies were compared. Our data showed that SLE-HSA contained significantly high carbonyl contents as compared with NH-HSA ($p < 0.01$). In this context, it is important to point out that the oxidation of a protein typically results in an increase in carbonyl contents. This increase is due to the oxidation of many amino acid residues including Lys, His, Cys [38,39]. Now, it is also well reported that HSA adducted with HNE in human serum is considered to be a potential biomarker of carbonylation damage in human [29,40] and many investigators are now trying to make their strategies that will inhibits the formation of advanced lipoxidation end-products based on carbonyl-sequestering agents and it seems to be the most promising pharmacological tool [41,42]. In view of these, we have estimated HNE-induced modifications in term of carbonylation. HNE generated eipitope(s) were further analyzed by detection of HNE-adducted His, Cys, or Lys by Western immunoblot analysis using commercially available anti-HNE-specific monoclonal antibodies. These HNE-specific antibodies showed strong

binding with SLE-HSA, but not with NH-HSA. This clearly indicated that SLE-HSA was HNE modified. In addition, we have also confirmed HNE-induced modifications in SLE-HSA by experimentally induced rabbit anti-HNE-HSA IgG. Anti-HNE-HSA IgG showed binding with SLE-HSA, but not with NH-HSA. Whereas, experimentally induced IgG against nHSA did not showed binding either with SLE-HSA or with NH-HSA. All these data confirmed that SLE-HSA was HNE modified. Furthermore, we have also demonstrated that immunoprecipitated anti-chromatin IgG, anti-ROS-chromatin IgG, or anti-NO-chromatin IgG from SLE patients showed immunocross-reactions with *in-vitro* generated HNE-HSA. Therefore, all these results demonstrated that HSA and chromatin in SLE patients were oxidatively modified, which might play an active part in the progression of disease. The present study further propose that, in addition to HSA in serum concentration and chromatin in nucleus of affected cells, the quality of HSA or chromatin molecules may be not only a crucial factor affecting their normal functions, but also a risk factor as a pro-oxidant in lupus patients. In conclusion, our data demonstrated that exposure of HNE caused oxidative damaged to proteins and presents unique epitopes for the generation of autoantibodies specific to chromatin and its oxidized forms. HSA modified with HNE and chromatin modified with reactive oxygen/nitrogen species may be important factors for the induction of circulating SLE autoantibodies. These findings contribute to a paradigm of lipid peroxidation and ROS/RNS products causing at least some autoimmune diseases.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

HAA, AAR, AAA carried out the experimental work, collection, interpretation and manuscript drafting. ZR conceived of the study, its design, experimentation, coordination, data interpretation and manuscript drafting. All authors have read and approved the final manuscript.

Acknowledgements

This work was supported in part by Research Dean-ship Grant # 952 and funds from College of Medicine,

Qassim University. ZR also thanks Professor A. Alghasham (Dean, College of Medicine) for providing research facilities. Thanks are also due to Dr. R. Mehala, School of Medicine, University of South Carolina, USA. for performing some of our Western Immunoblotting experiments.

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