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Relevance of Nitroxidation of Albumin in Rheumatoid Arthritis: A Biochemical and Clinical Study

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Abstract

Objective: To study the role of peroxynitrite-modified human serum albumin (nitroxidized-albumin) in rheumatoid arthritis.

Methods: Human serum albumin was exposed to peroxynitrite and changes in albumin structure were monitored by UV-visible, fluorescence and circular dichroism spectroscopy, thioflavin T, Congo red binding and attenuated total reflection-Fourier transformed infrared spectroscopy (ATR-FTIR). Antioxidant properties of nitroxidized-albumin were evaluated by free radical induced RBC hemolysis test. Markers of protein oxidation like carbonyl, thiol, dityrosine and RBC hemolysis were evaluated in RA patients' sera. Binding of autoantibodies in RA sera (n=50) with nitroxidized-albumin was studied by direct binding, inhibition ELISA and electrophoretic mobility shift assay.

Results: The nitroxidized-albumin indicated the generation of nitrotyrosine, nitrotryptophan, carbonyl, dityrosine and reduction in tyrosine and tryptophan fluorescence and α-helicity. Fluorescence emission intensities of thioflavin T and Congo red got augmented upon binding with nitroxidized-albumin. Furthermore, secondary and tertiary structures of nitroxidized-albumin were altered as evident by ATR- FTIR, far and near-UV CD. Autoantibodies in RA sera (or IgG purified from sera) showed enhanced binding with nitroxidized-albumin as determined by direct binding and inhibition ELISA. Protein carbonyls, dityrosine and RBC hemolysis were significantly high, but thiol was significantly low in RA sera compared to age- and sex- matched control.

Conclusion: Endogenously available peroxynitrite can nitrate and oxidize albumin, leading to protein nitration/oxidation and subsequent formation of crosslinks, aggregates and immunogenic nitroxidized-albumin. Therefore, nitroxidized-albumin may be a potential trigger for autoantibodies in RA patients.

Keywords: Peroxynitrite; Albumin; Rheumatoid arthritis; Nitroxidized-albumin; Nitrotyrosine; Protein carbonyl; Dityrosine

Introduction

Rheumatoid arthritis (RA) is an autoimmune, inflammatory, symmetrical polyarticular disease that affects primarily the diarthrodial joints. The disease shows prevalence in middle aged females. It has a major impact on the physical as well as mental health status of patients [1]. Its aetiology is largely unexplained. However, roles of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been suggested in the pathogenesis of RA. Elevated ROS/RNS have been reported in RA patients [2,3]. Normal cellular metabolism generates ROS and RNS and each body cell is exposed to 10000-20000 hits of free radicals per day [4]. Proinflammatory cytokines such as IL-1B and TNF-alpha can induce peroxynitrite generation by increasing nitric oxide synthetase activity [5].

The peroxynitrite can cause irreversible modification in variety of macromolecules through nitration, nitrosation or oxidation. Protein nitration mostly occurs on tyrosine residues to form 3-nitrotyrosine and is commonly used as a foot print of both *in vivo* and *in vitro* RNS generation [6]. Protein tyrosine nitration has been reported in inflammatory diseases including RA. Protein thiols are able to scavenge oxidants and thus play a significant role in protecting biomolecules from deleterious effects of oxidants. Carbonyl groups (aldehyde and ketone) are also generated on protein side chains during oxidation. Carbonyls are chemically stable and globally accepted markers of protein oxidation. Elevated protein carbonyls and reduced levels of protein thiols have been implicated in RA [7].

Trigger for RA initiation remains obscure but autoantibodies have been implicated in progression of RA as well as other autoimmune diseases that react against host tissue and promote disease pathology. The rheumatoid factor (RF), an autoantibody against Fc portion of IgG, is a specific marker of RA. Moreover, a wide variety of post translational modification (PTM) products have been reported to be involved in autoantibody generation [8-10].

During pre-clinical phase of RA there is a high uptake of albumin at the sites of inflammation because of the increased permeability of blood-joint barrier for albumin. The albumin serves as the metabolic

fuel for highly up-regulated synovial cells. We hypothesize that the peroxynitrite generated during inflammatory conditions may cause structural changes in albumin that has been trapped in synovial cells. The nitroxidized-albumin released from the cells may persist in circulation for a longer period than its native counterpart because of its poor susceptibility to proteases. The immunoregulatory cells may consider nitroxidized-albumin as foreign and start producing antibodies. Presence of autoantibodies against nitroxidized-albumin may be an indicator of the initiation or progression of RA. Therefore, this study was aimed at exploring the occurrence of autoantibodies against albumin treated with peroxynitrite, a naturally occurring nitrating-cum-oxidizing agent.

Materials and methods

Human serum albumin, silver nitrate, protein A-agarose affinity column, para-nitrophenyl phosphate, Tween-20, thioflavin T and Congo red were purchased from Sigma Chemical Company, St. Louis, MO, USA. Acrylamide, bisacrylamide, ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories, USA. Hydrogen peroxide, sodium nitrite, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithionitrobenzoic acid (DTNB) and 2,2'-azobis (2-methylamidinopropane) (AAPH) were purchased from SRL, India. ELISA modules were from NUNC, Denmark.

Modification of human serum albumin

Human serum albumin was nitroxidized by peroxynitrite prepared in the laboratory by rapid quenched flow method [11]. Albumin (15 μM) was incubated with peroxynitrite (0.75 and 1.5 mM) for 30 min at 37°C.

Characterization of native and nitroxidized-albumin

UV-visible properties of native and nitroxidized-albumin were recorded on Shimadzu spectrophotometer (UV-1700). Fluorescence characteristics of samples were evaluated on Shimadzu spectrofluorometer (RF 5301-PC). Far and near-UV CD profiles of samples were recorded on Jasco spectropolarimeter (J-815) attached to Peltier-type temperature controller (PTC-424S/15).

Congo red binding assay

Binding of Congo red to protein aggregates was analyzed from absorption profile in 300 to 700 nm range. The sample to CR molar ratio was 1:2 and the mixture was incubated at room temperature for 30 min [12].

ATR-FTIR spectroscopy

Native and nitroxidized-albumin samples were subjected to ATR-FTIR recording on Perkin Elmer machine. Infrared spectroscopy is a vital tool to understand secondary changes in protein structure. IR spectra of proteins exhibit a number of amide band vibrations which are sensitive to the secondary structure of proteins. Changes in amide I band (1600 to 1700cm^{-1}] and amide II band (1500 to 1600cm^{-1}) vibrations have more commonly been used to analyze the secondary structure of proteins [13].

Free radical induced hemolysis of red blood cells

Free radical induced hemolysis test [14] was employed to study the antioxidant properties of native and nitroxidized-albumin.

Collection of sera from human subjects

Sera of fifty adult patients having disease duration of at least five year with proven RA were selected from data bank of 200 patients maintained at the Department of Microbiology, J.N. Medical College hospital of the Aligarh Muslim University. All sera were positive for rheumatoid factor and anti-CCP and satisfied the criteria of the American Rheumatism Association [15]. They were also examined for serum CRP and ESR. Patients had a minimum ESR of 42.35 mm/hr and CRP of at least 28.65 mg/L, with proven radiographic damage. Age- and sex-matched sera of healthy subjects were used as control. All patients consented to participate in the study. The work protocol was approved by the institutional ethics committee. Prior to use all samples were heat decomplemented at 56°C for 30 min and stored.

Quantification of protein-bound carbonyls

The concentration of protein-bound carbonyls in healthy and RA sera was determined from its reaction with 2,4-dinitrophenylhydrazine (DNPH). The reaction product was read at 370 nm [16] and results were expressed in nmole/mg protein.

Quantification of thiol by Ellman's reagent

Protein thiols were determined using 5,5'-dithionitrobenzoic acid (DTNB) [17]. Briefly, wells of microtiter plate were filled with 10 μl of 1:2 and 1:4 dilutions of both RA and healthy sera. To the above preparation was added 200 μl of freshly prepared 0.5 mM DTNB (in 100 mM phosphate buffer, pH 7.4) or 200 μl of buffer alone. After incubation (in dark) for 30 min at room temperature, the 5-thiobenzoic acid that was released during the reaction was quantified from absorbance measurement at 412 nm. The thiol concentration was converted to absolute values using reduced glutathione as standard (0-0.5 mM).

Fluorescence spectroscopy of dityrosine

Dityrosine content in RA and healthy sera was determined as described earlier [18]. Samples were excited at 325 nm and dityrosine specific fluorescence (emission) was recorded at 410 nm.

Hemolysis of RBC

In vitro hemolysis of RBC of RA patients and healthy subjects were assessed as described by Buckingham [19].

Affinity purification of IgG

Immunoglobulin G was isolated from RA patients'/ healthy subjects' sera on protein A-agarose column. The fractions showing A278 / A251 ratio of 2.5 or more and a single band in denaturing polyacrylamide gel were pooled, dialyzed and used as purified IgG.

Autoantibodies detection by ELISA

Direct binding ELISA was carried out on flat bottom polystyrene maxisorp microtiter modules following the procedure of Khan et al.

[20]. The antigenic specificity of the antibodies was ascertained by inhibition ELISA [21].

Electrophoretic mobility shift assay (EMSA)

The interaction of native and nitroxidized-albumin with RA IgG was ascertained by EMSA [20] on non-reducing 7.5% SDS-polyacrylamide gel. Silver nitrate staining was carried out to visualize the bands.

Statistical analysis of data

Results were expressed as mean ± SD and data were subjected to student's t test for significance. Value at $p<0.05$ were considered significant.

Parameters	Native albumin	Nitroxidized-albumin (0.75 mM peroxynitrite)	Nitroxidized albumin (1.5 mM peroxynitrite)	Changes on modification
Absorbance at 278 nm	0.518	0.727	0.849	29 and 39% hyperchromicity
Absorbance at 428 nm	----	0.204	0.291	----
Nitrotyrosine content (nmole mg^{-1} HSA)	----	48.5	69.2	----
Nitrotryptophan content (nmole mg^{-1} HSA)	----	33.6	52.6	----
[a]Fluorescence intensity, (F.I.)				
[b]λ_{exc} = 275 nm	163	43.3	29.9	73 and 81% decrease in F.I.
[b]λ_{exc} = 295 nm	51.7	15.9	10.9	69 and 79% decrease in F.I.
Protein carbonyl content (nmole mg^{-1} HSA)	2.5	21.3[c]	30.3[c]	88 and 92% increase
Dityrosine content (nmole mg^{-1} HSA)	----	25.4	52.2	----
Dityrosine [a]F.I.				
[b]λ_{exc} = 325 nm	----	22.7	95.7	----
Far-UV CD				
[d]MRE at 208 nm	-21264.1	-18367.7	-14189.7	----
[d]MRE at 222 nm	-20057.4	-16493.7	-11733.1	----
[d]MRE at 192 nm	42517.1	38063.5	24200.6	----
α-helix (%)	68.28	60.4	40.1	----
ThT binding, [a]F.I.				
[b]λ_{exc} = 435 nm	33.9	91.7	158.5	63 and 77% increase in F.I.

[a]Fluorescence intensity (F.I.) in arbitrary units (A.U.)

[b]λ_{exc} = excitation wavelength
[c]$p<0.0001$ vs. native albumin
[d]MRE value in deg cm^2 $dmol^{-1}$

Table 1: Physicochemical characteristic of native and nitroxidized-albumin.

Results

Characterization of native and nitroxidized-albumin

UV-visible analysis of the samples suggested hyperchromicity at 278 nm in nitroxidized-albumin (Table 1). An additional peak at 428 nm was also observed. Nitrotyrosine, nitrotryptophan, carbonyl and dityrosine were found to be significantly raised in nitroxidized-albumin as compared to native albumin (Table 1). Thioflavin T binding with nitroxidized-albumin was shown to be increased. Furthermore, tyrosine and tryptophan specific fluorescence got significantly reduced in nitroxidized-albumin. The far-UV CD spectra of nitroxidized-albumin showed enhanced ellipticity at 208 and 222 nm and reduced ellipticity at 192 nm as compared to native albumin (Table 1).The findings indicate that peroxynitrite has caused decrease in α-helix conformation.

Analysis of native and nitroxidized-albumin by near-UV CD

Changes in tertiary structures of proteins are inferred from the near-UV CD spectra [22]. Near-UV CD of native albumin showed two minima at 262 nm and 268 nm respectively, accompanied by shoulders at 273 and 291 nm; characteristics of disulphide and aromatic chromophores. In addition, native albumin showed a band at around 275 nm which is specific for tyrosine residue (Figure 1). Loss of signal and disappearance of 262 nm and 268 nm minima and 275 nm peak indicate substantial loss of tertiary structure in nitroxidized-albumin.

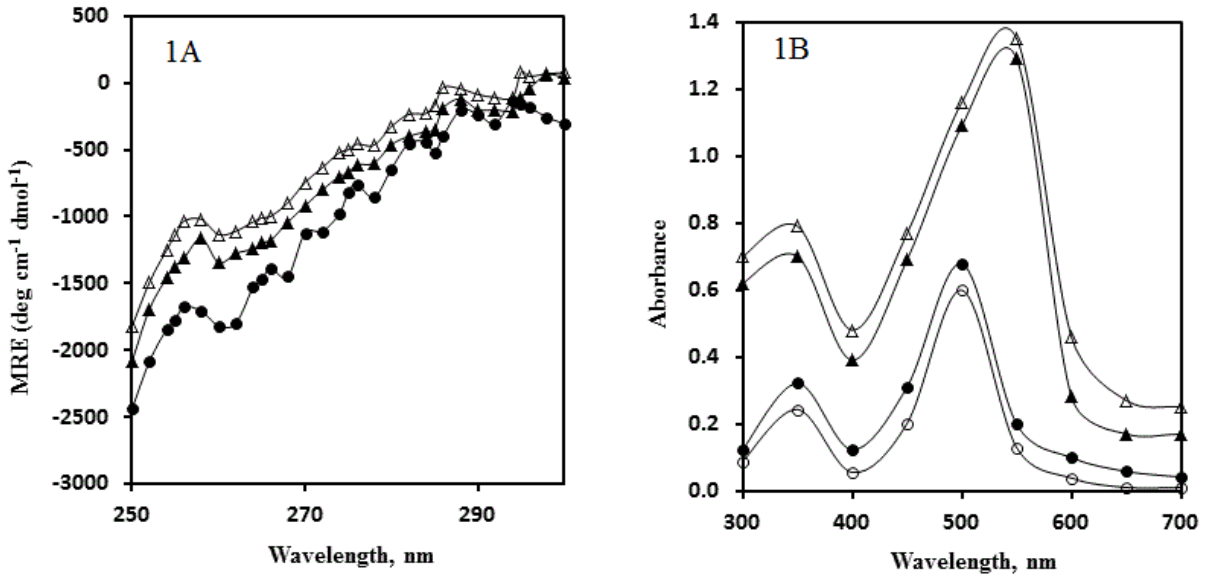

Figure 1: (a) Near UV-CD spectra of native albumin (●) treated with peroxynitrite at 0.75 mM (▲) and 1.5 mM (Δ). (b) Absorption profile of CR (○), native albumin + CR (●), albumin nitroxidized with 0.75 mM (▲) and 1.5 mM (Δ) peroxynitrite and treated with CR. Spectra represent average of three scans. Protein concentration in all samples was identical.

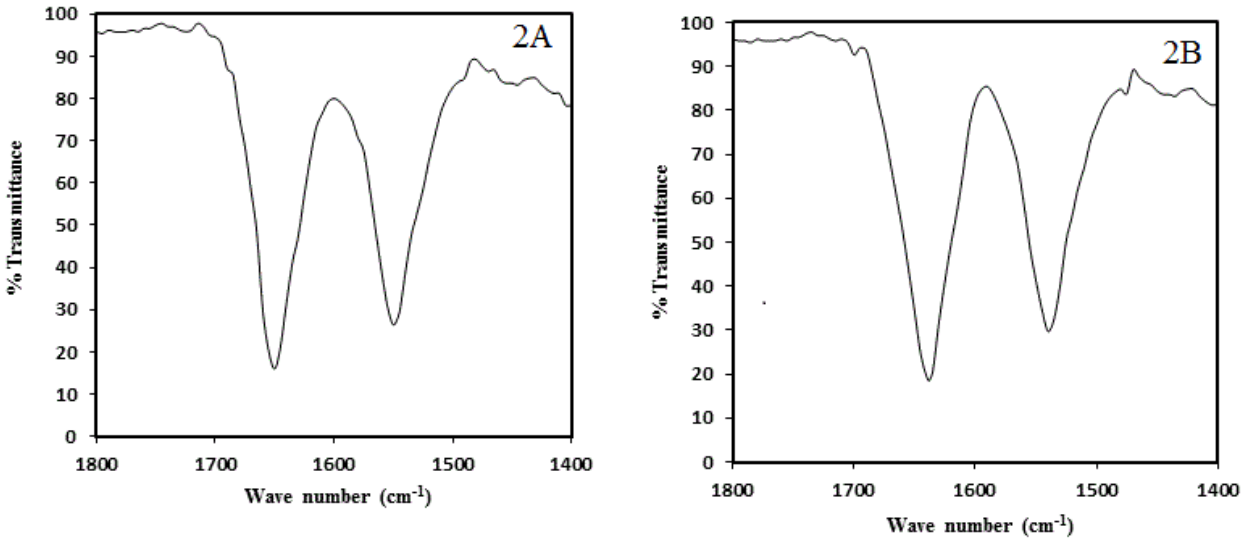

Figure 2: FTIR spectra of native albumin (A) and albumin nitroxidized by 1.5 mM peroxynitrite (B). Spectra are the average of three scans. Protein concentration in all samples was identical.

Congo red dye binding to native and nitroxidized-albumin

Congo red binding to nitroxidized-albumin caused significant increase in absorption and red shift from 490 to 540 nm [23] indicating aggregate formation (Figure 2B). Native albumin incubated with CR did not produce significant increase in absorbance and red shift in CR spectrum.

ATR- FTIR studies on nitroxidized-albumin

The effect of peroxynitrite on secondary structure of albumin was evaluated by monitoring vibrations in amide I (mainly C=O stretch) and amide II (C-N stretch coupled with N-H bending) bands. Figure 2 showed that peak position of amide I band moved from 1655 (native albumin) to 1638 cm^{-1} (nitroxidized-albumin) and amide II band moved from 1550 (native albumin) to 1540 cm^{-1} (nitroxidized-albumin). Taken together, the data indicate changes in secondary structure.

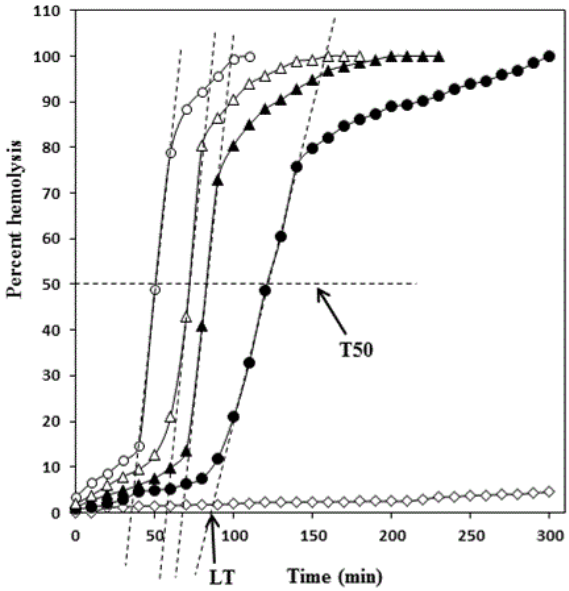

Figure 3: Representative curves of RBC hemolysis induced by AAPH in PBS, pH 7.4 at 37°C. (-o-) hemolyzed RBC, (●) effect on RBC hemolysis in presence of native albumin, (▲) effect on RBC hemolysis in presence of albumin nitroxidized by 0.75 mM peroxynitrite, (-Δ-) effect on RBC hemolysis in presence of albumin nitroxidized by 1.5 mM peroxynitrite, (◊) non hemolyzed RBC. Results represent average of three independent measurements.

Evaluation of antioxidant property of nitroxidized-albumin

The antioxidant property of native and nitroxidized-albumin was evaluated from red blood cell hemolysis test. The hemolysis was initiated with water soluble radical initiator AAPH. Figure 3 shows time dependent AAPH-induced RBC hemolysis. The sigmoid curves were used to calculate two quantitative parameters: (a) the lag time and (b) the time required to achieve 50% hemolysis (T50 min). RBCs were resistant to hemolysis for about 45 min in presence of AAPH alone due to endogenous antioxidants. Addition of native albumin to the AAPH-initiated hemolysis system significantly prolonged the lag time as well as T_{50} as compared to nitroxidized-albumin. Results suggest decrease in antioxidant potential of nitroxidized-albumin.

Protein-bound carbonyls and thiols in RA sera

Protein carbonyls are foot mark of oxidative activities and their estimation in biological fluids are helpful in understanding the disease pathology. In our study, the carbonyls in RA sera were significantly higher than healthy control (4.435 ± 0.74 nmoles/mg protein vs. 1.33 ± 0.302 nmoles/mg protein) (Figure 4A). Protein thiols are important from defence point of view because they effectively neutralize the oxidants. In RA sera, protein thiol was found to be highly reduced as compared to healthy sera (Figure 4B).

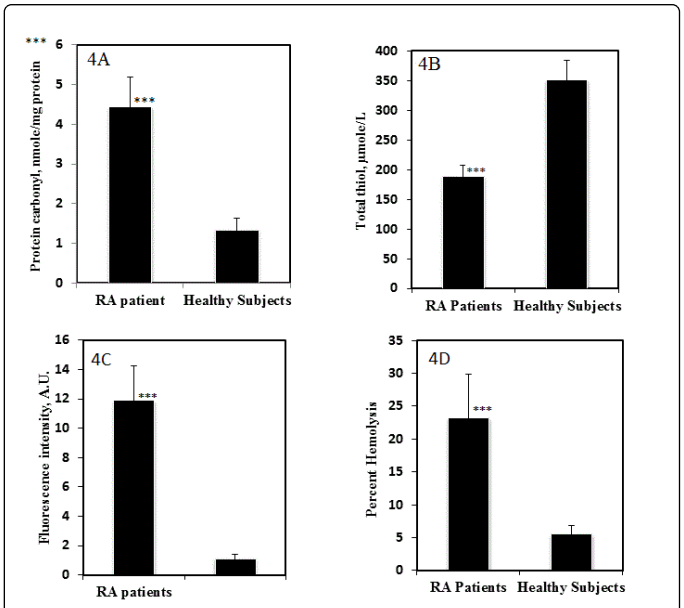

Figure 4: Protein carbonyl (A), thiol content (B), level of dityrosine(C) and rate of RBC hemolysis (D) in RA and healthy subjects. Each histogram represents the mean ± SD of 50 samples. ***p<0.0001 significantly different from healthy subjects.

Dityrosine in RA sera

Oxidants interaction with tyrosine can also generate tyrosyl radicals and the combination of two tyrosyl radicals may form dityrosine. Fluorescence spectra revealed tenfold increase in emission signal of dityrosine in diluted RA sera as compared to healthy sera of identical dilution (Figure 4C).

Rate of RBC hemolysis

Rate of hemolysis of RBC in RA patients and healthy subjects was monitored to assess the effect of oxidative stress on RBCs. The level of hemolysis was alarmingly high in RA patients as compared to control (Figure 4D). It indicates high index of oxidative stress in RA patients.

Direct binding and inhibition ELISA

Diluted sera (1:100) of RA patients and healthy subjects were subjected to direct binding ELISA on microtiter wells coated with

native and nitroxidized-albumin (albumin modified with 1.5 mM peroxynitrite) and the results are shown in Figure 5.

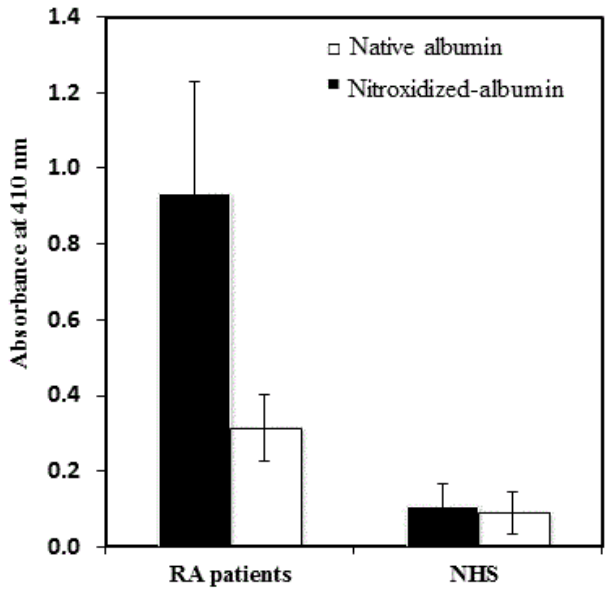

Figure 5: Direct binding ELISA of 1:100 diluted RA sera with native albumin and nitroxidized-albumin. Serum samples from normal human subjects (NHS) served as control. Microtiter wells were coated with respective antigens. The histogram represents mean ± SD of 50 samples.

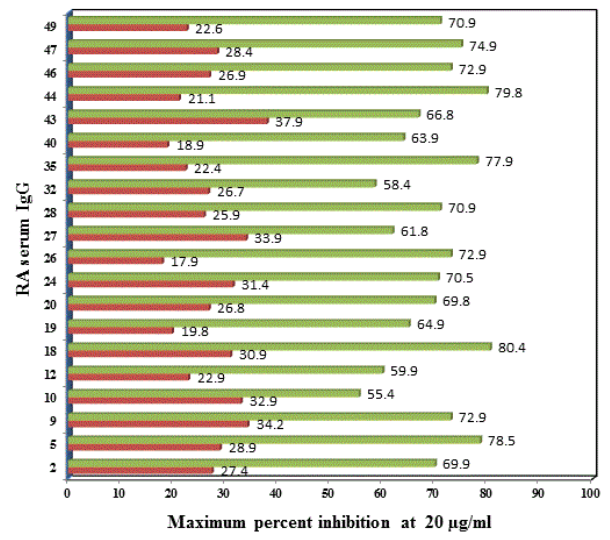

Figure 6: Inhibition ELISA of RA IgG binding with native albumin ( ) and nitroxidized-albumin ( ). Microtiter wells were coated with 100 ul of 10 ug/ml of native and nitroxidized-albumin.

RA sera showed enhanced binding with nitroxidized-albumin as compared to native albumin ($p<0.05$). Control sera showed negligible binding with either antigen. The findings indicate prevalence of anti-nitroxidized-albumin autoantibodies in RA sera. IgG purified from twenty selected RA sera were subjected to inhibition ELISA to look into the specificity of antigen–antibody interaction. The RA IgG binding with nitroxidized-albumin persisted, indicating specificity (Figure 6).

EMSA

Binding of RA IgG with nitroxidized-albumin (albumin modified with 1.5 mM peroxynitrite) was visualized. RA IgG (0-40 µg) was incubated with 10 µg of either native or nitroxidized-albumin. The complex was electrophoresed on SDS-PAGE. The retarded mobility of nitroxidized-albumin and RA IgG with simultaneous decrease in the intensity of unbound nitroxidized-albumin was taken as an indicator of specific interaction (Figure 7B). However, under identical conditions binding of native albumin with RA IgG did not show prominent immune complex formation (Figure 7A). In this case no significant decrease in intensity of unbound antigen was observed.

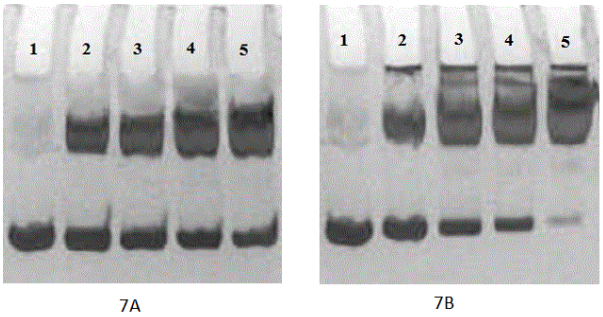

Figure 7: Electrophoretic mobility shift assay of RA IgG with native and nitroxidized-albumin on SDS-polyacrylamide gel. Electrophoresis was carried out on 7.5% SDS-PAGE for 3.5 h at 70 V. (A) native albumin (10 µg, lane 1) and (B) nitroxidized-albumin (1.5 mM peroxynitrite) (10 µg, lane 1) were incubated with 10, 20, 30 and 40 µg RA IgG (lane 2-5) at 37°C for 2 h and overnight at 4°C.

Discussion

Role of reactive oxygen and nitrogen species in inflammatory diseases have been well documented [24]. Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of joints. Elevated levels of interleukins may induce nitroxidative stress; a condition characterized by an imbalance between reactive oxygen and nitrogen species and antioxidants [25]. Furthermore, high concentration of nitric oxide [26] superoxide [27] and peroxynitrite [28] have also been reported in RA. Peroxynitrite is a strong oxidant and a powerful nitrating agent and capable of causing oxidation and nitration of biomolecules. Elevated levels of nitrotyrosine in plasma/tissue of RA patients have been reported [29]. It has been argued that high tissue nitrotyrosine in various diseases may be responsible for chronic inflammatory conditions [30]. In such a situation nitrotyrosine may act as a potential autoantigen and trigger autoimmunity [31].

In this study, peroxynitrite reaction with albumin formed a stable yellow protein-bound adduct, identified as nitrotyrosine from its maximum absorbance at 428 nm under alkaline conditions. The secondary and tertiary structure of nitroxidized-albumin was severely affected as shown by the results of ATR-FTIR, far-UV and near-UV

CD respectively. Increased carbonyl content and dityrosine in nitroxidized-albumin substantiates the oxidizing nature of peroxynitrite. Aggregate formation in nitroxidized-albumin was supported by Congo red and thioflavin T binding. The antioxidant properties of albumin were greatly reduced upon modification with peroxynitrite as shown by radical initiated RBC hemolysis assay. Here, it may be pointed out that the antioxidant properties of albumin have been mainly attributed to Cys34 residue. The oxidation of the very residue by peroxynitrite may have grossly affected the antioxidant property of albumin. Furthermore, radical generation under oxidative stress may lead to protein modifications since they are essential targets of free radical attack, both intra- and extra-cellularly [32]. Sera of RA patients included in this study showed high carbonyl, dityrosine, low thiols and increased hemolysis of RBC. It goes in favour of high index of oxidative stress [33].

Role of oxidized and/or nitrosylated proteins are being actively researched to understand their contribution in human pathologies, especially autoimmune diseases. The oxidation and/or nitrosylation may alter proteins' secondary and tertiary structure, biological functions and may be critical in generating immunogenic or tolerogenic self proteins [34]. Several post translational modifications may contribute in the initiation and/or maintenance of autoimmune diseases by breaking immunological tolerance. In autoimmune diseases, post translationally modified self antigens initiate an adaptive response through activation of autoreactive T cells that, in turn, promote the activation of B cells via cytokines to generate autoantibodies. Under proinflammatory conditions simultaneous generation of superoxide and NO is known to increase the formation of peroxynitrite several fold [35]. During proinflammatory and/or inflammatory phase the synovial cells recruit more albumin to meet the growing energy needs of cells and its encounter with already present peroxynitrite can alter its biochemical and immunological properties in a way similar to what we observed during our *in vitro* studies on modification of albumin with peroxynitrite. Furthermore, binding of RA sera autoantibodies with well characterized nitroxidized-albumin (albumin modified with 1.5 mM peroxynitrite) supports the likely origin of potentially immunogenic nitroxidized-albumin from within the synovial tissue. Earlier *in vitro* studies have demonstrated the highly immunogenic nature of peroxynitrite-modified protein antigens [36,37]. Therefore, peroxynitrite induced changes on albumin (nitration/oxidation, aggregation) in totality may favour autoantibodies' induction. Monitoring of the progression of anti-nitroxidized-albumin autoantibodies in RA may be helpful in understanding the disease process and effect of treatment regimen.

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Conflict of interest

There is no conflict of interest.

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