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Biochemical and microscopy evidence on adverse effects of nitroxidized human serum albumin

Akhlas Tarannum[1], Zarina Arif[1]*, Moinuddin[1], Khursheed Alam[1] & Tajalli Ilm Chandel[2]

[1]Department of Biochemistry, Faculty of Medicine, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh-202 001, Uttar Pradesh, India

[2]Interdisciplinary Biotechnology Unit, Faculty of Life Sciences, Aligarh Muslim University, Aligarh-202 001, Uttar Pradesh, India

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Earlier researches have pointed about the accumulation of peroxynitrite modified proteins and their aggregates in the etiopathogenesis of many age-related neurodegenerative and several autoimmune diseases. Human serum albumin (HSA) is present in abundance in plasma and is susceptible to modification by peroxynitrite. In this study, HSA modified with peroxynitrite (nitroxidized-HSA) formed aggregate besides other gross structural changes. Aggregation or assembly of aberrant proteins is responsible for increase in production of reactive species and is often correlated with toxicity in neurodegenerative diseases. However, lack of literature on the cytotoxicity of aggregated nitroxidized-HSA led us to explore its toxicity using human peripheral blood lymphocytes. Elevated protein carbonyl coupled with decreased protein thiol, and release of antioxidant enzymes and lactate dehydrogenase (LDH) were observed upon incubation of lymphocytes with nitroxidized-HSA. Trypan blue exclusion and MTT assays indicated nitroxidized-HSA induced injury/death of lymphocytes. This may be attributed to the observed reactive oxygen species generation during the interaction of nitroxidized-HSA with lymphocytes. Moreover, the analysis of the cellular morphology by dual staining, fluorescence and confocal microscopy further confirms the cytotoxicity of nitroxidized-HSA. Since various age-related degenerative diseases are characterized by deposition of protein aggregates, the demonstrated toxicity of nitroxidized-HSA may be an important driver in the pathophysiology of neurodegenerative diseases.

Keywords: Aggregation, Nitration, Oxidation, Peroxynitrite, Toxic protein aggregates

The role of free radicals in the pathogenesis of various inflammatory, autoimmune and neurodegenerative diseases is well established[1,2]. These radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS). Peroxynitrite (RNS) is physiologically produced in the body *via* the interaction of nitric oxide and superoxide radicals where it plays the role of strong antimicrobial agent[3]. However, increased levels of peroxynitrite have deleterious effects[3]. Peroxynitrite can potentially cause irreversible modification to almost all macromolecules through nitration and/or oxidation[3-5].

Peroxynitrite has been implicated in a variety of diseases, including neurodegenerative disorders[6], atherogenesis/cardiovascular diseases[7], diabetes mellitus[8], immune and inflammatory disorders[9]. Protein modification by peroxynitrite causes conformational changes that affect either the protein function and/or the tendency of a protein to misfold and aggregate[10,11]. These protein aggregates, regardless of biochemical composition, have the common property of causing cellular damage because of loss of its biological function and/or gain of toxic activity[12]. The aggregates cause cellular damage via interaction with cell and their components, generation of reactive species and/or disruption of cell membranes[13]. Although no single factor can determine the onset of autoimmune/neurodegenerative diseases, increased oxidative/nitrosative damage to protein may play a prominent role[14].

The toxicity of many modified proteins and their aggregates has been evaluated earlier in variety of cells such as erythrocytes, lymphocytes, and various cell lines[15-17]. *In vitro* cell culture methods provide excellent model systems for studying the physiology and biochemistry of cells or the effects of pharmaceutical and toxic compounds on the cells or evaluating microbial infections quantitatively/qualitatively[18]. The cells can be taken directly from the tissue and processed to establish them under optimized culture conditions.

*Correspondence:
E-mail: zarif66@hotmail.com

Because they are derived from tissue and not modified, they mimic *in vivo* state. However, some issues are associated with culture technique, incubation, and media which might lead to abnormal growth patterns, uneven cell attachments, or unexplainable changes in growth rate. Human peripheral blood lymphocytes are comparatively easier to manage because they can be freshly isolated and used in controlled culture environments without affecting the quality of the results[16].

Human serum albumin (HSA) is a multi-functional predominant plasma protein and a prime antioxidant in plasma[19]. Because of the large pool of HSA and its long circulating half-life (21 days), HSA is a target for various oxidants/nitrosants[19]. Reactive species have devastating effects on the structure and function of proteins that eventually leads to the aggregation of proteins. Considering the fact that aggregated proteins are pathogenic, we hypothesized that aberrant HSA aggregates may be a cause of initiation and/or progression of autoimmune/neurodegenerative diseases. Therefore, the present study was carried out to determine the toxic effect of nitroxidized-HSA using human peripheral blood lymphocytes.

Materials and Methods

Materials

Human serum albumin, thioflavin T, 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), dithiothreitol (DTT), RPMI-1640 medium, histopaque 1670, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide], trypan blue, guanidine hydrochloride, sodium pyruvate, nicotinamide adenine dinucleotide (reduced), acridine orange, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), propidium iodide, pyrogallol were purchased from Sigma Chemical Company, St. Louis, MO, USA. Hydrogen peroxide, sodium nitrite, 2,4-dinitrophenylhydrazine (DNPH), and 5,5'-dithionitrobenzoic acid (DTNB) were purchased from SRL, India. ELISA modules were purchased from NUNC, Denmark.

Methods

Nitroxidation of HSA

Peroxynitrite was prepared in our research laboratory by rapid quenched flow method from sodium nitrite and acidified hydrogen peroxide[17]. Concentration of the peroxynitrite was determined from the absorbance at 302 nm using the molar extinction coefficient of 1670 M^{-1} cm^{-1}. Human serum albumin (15 µM) was incubated with peroxynitrite (1.5 mM) for 30 min at 37°C[24]. At the end of incubation, products were extensively dialyzed in sodium phosphate buffer, pH 7.4, and were characterized by various biochemical and biophysical techniques.

Isolation of lymphocytes and treatment with native/nitroxidized-HSA

Lymphocytes were isolated[20] from the whole blood of healthy human donor employed at J.N. Medical College, A.M.U., Aligarh, India. Freshly isolated lymphocytes (1×10^6 cells/mL) were dissolved in RPMI-1640 media and incubated with HSA/nitroxidized-HSA (15 µM) at 37°C for 2 h. Following incubation, the mixture was centrifuged at 4000 rpm for 10 min. The supernatant and lymphocyte pellet (suspended in PBS) were used for various analyses.

Estimation of total protein carbonyl, thiol, and glutathione of lymphocytes treated with native/nitroxidized-HSA

Protein carbonyl in native and nitroxidized-HSA treated lymphocyte suspension was determined according to the method described elsewhere[21]. Briefly, 250 µL of lymphocyte suspension was mixed with 250 µL DNPH (2.5 M stock) and incubated in dark. After 15 min, 125 µl of trichloroacetic acid (50%; *w/v*) was added. The samples were then kept at −20°C for 15 min followed by centrifugation. The pellet was collected and washed three times with ice-cold ethanol/ethyl acetate mixture (1:1; *v/v*). Residual ethanol/ethyl acetate mixture was removed and the pellet was redissolved in 3 mL guanidine-hydrochloride (6 M). Absorbance was recorded at 370 nm and protein carbonyl content was calculated using the molar extinction coefficient of 22000 M^{-1} cm^{-1}. The results are expressed in terms of nmoles per mg of protein.

Lymphocyte suspension treated with native/nitroxidized-HSA was used to determine total protein thiol by Ellman's method[22]. Absorbance of the reaction product was read at 412 nm and the P-SH was expressed as nmoles per mg of protein.

Glutathione (GSH) was determined with DTNB reagent[23]. Treated lymphocyte cell suspension was mixed with sulphosalicylic acid (0.6%) and incubated at 4°C for 1 h. The precipitate was removed by centrifugation at 1200 g for 15 min and supernatant was separated. Supernatant (50 µL) was mixed with 1 mL of DTNB solution and incubated in dark for 30 min. The absorbance was measured at 412 nm and glutathione was expressed as nmoles per mg of protein.

Estimation of released superoxide dismutase and catalase

Superoxide dismutase (SOD) was assayed in the supernatant by measuring the inhibition of autoxidation of pyrogallol[24]. Briefly, 40 µL of supernatant was mixed with 820 µL of Tris-HCl buffer (0.05 M, pH 7.4) and 40 µL of 1 mM EDTA. The reaction was initiated with 120 µL of Pyrogallol (25 mM). The absorbance was read at 325 nm for 5 min at an interval of 30 sec and the result was expressed as units per mg of protein.

Similarly, catalase was measured in the supernatant by observing the rate of degradation of H_2O_2[25]. Briefly, to 40 µL of the supernatant, 610 µL of potassium phosphate buffer (50 mM, pH 7.4) was added and gently mixed. The reaction was initiated by adding 350 µL of H_2O_2 (54 mM) and the absorbance was read at 240 nm. The result is expressed as units per mg of protein.

Detection of cell death by Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) was determined in the supernatant of native and nitroxidized-HSA treated lymphocytes according to the method of Mitchell, Santone & Acosta[26]. Briefly, 2.3 mM sodium pyruvate (in 0.1 M phosphate buffer pH 7.5) was added to the respective samples and the reaction was initiated by adding NADH (0.13 mM). The absorbance was monitored at 340 nm for 5 min at an interval of 30 sec. Lymphocytes without any treatment served as negative control.

Live-cell counting by trypan blue exclusion assay

The viability of lymphocytes treated with native and nitroxidized-HSA was tested by the trypan blue[27]. In this method, viable cells do not take up the trypan blue dye because their membrane is intact, whereas dead ones do[28]. The number of cells stained with trypan blue was counted under the microscope using a hemocytometer and the cell viability was estimated[29].

The number of viable cells/mL was calculated from the following equation:

$$\text{Percent cell viability} = \left(\frac{\text{Average number of live cells per large square (treated)}}{\text{Average number of live cells per large square (untreated)}}\right) \text{x}100$$

Cell viability testing by MTT assay

The MTT assay was carried out as described earlier[30]. The tetrazolium dye, MTT is reduced to its insoluble formazan (purple color) by the enzymes NAD(P)H dependent cellular oxidoreductase[31]. These enzymes reflect the number of viable cells present and hence the quantity of formazan formed is proportional to the number of viable cells in the population. The resulting formazan is exported from the cell by exocytosis and is deposited on the cell surface forming crystals[32]. The formazan crystals were dissolved in dimethyl sulfoxide and the absorbance was measured at 570 nm on microplate reader. The viability of cells devoid of native or nitroxidized-HSA was considered to be 100 percent.

Acridine orange and ethidium bromide staining of lymphocytes

To further explore the effect of nitroxidized-HSA on lymphocytes viability, a combination of acridine orange (AO) and ethidium bromide (EtBr) was used[33]. The AO can penetrate the viable cells and stain them green, while EtBr stain only the dead cells (red). The dual staining method allows the identification of viable and non-viable cells. Briefly, a stock solution was prepared by mixing 100 µg/mL each of AO and EtBr. On a separate glass slide, 20 µL of lymphocytes treated with native or nitroxidized-HSA was carefully placed and 1.0 µL of stock staining solution was poured, material was air-dried, and then covered with a coverslip[34]. The slides were placed under a fluorescent microscope and the excitation/emission wavelengths were set. The excitation/emission wavelengths for AO and EtBr are 502/526 nm and 510/595 nm, respectively[33]. The emitted fluorescence and the morphology of cells were captured.

Measurement of ROS

The intracellular ROS generation was detected by dichloro-dihydro-fluorescein-diacetate (DCFH-DA) dye, a sensitive fluorescent probe[35]. First, the lymphocytes suspension (1×10^6 cells/mL) was pre-incubated with 10 µM DCFH-DA at 37 °C for 1 h and centrifuged. The excess dye was removed by washing with phosphate buffer, pH 7.4. The pelleted cells were resuspended in PBS and incubated with native or nitroxidized-HSA for 2 h. The reaction mixture was diluted in PBS (50 times) and emission was recorded at 530 nm by exciting the samples at 485 nm on a spectrofluorimeter. For the microscopy study, samples were air-dried on glass slides and visualized under fluorescence microscope at 40X magnification.

Confocal laser scanning microscopy

The lymphocytes (1×10^6 cells/mL) previously treated with native/nitroxidized-HSA were stained with a mixture of propidium iodide and acridine orange (1 mg/mL each), for 15 min in dark. The residual dye was removed by centrifugation and samples were washed with PBS, pH 7.4. The image

was recorded with the help of confocal laser scanning microscope (Zeiss, Germany).

Statistical analysis

The statistical analysis of the results was carried out using GraphPad Prism 9 data analysis software. One-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey's test) was done to evaluate statistical differences in this study. All data were expressed as mean ± standard deviation (SD) of at least three experiments. The difference was considered to be significant at $P < 0.05$.

Results

Characterization of nitroxidized-HSA

In an earlier study, Arif *et al*. [36] have thoroughly characterized the nitroxidized-HSA and similar changes have been observed in this study. The results obtained are summarized in (Table 1). Hyperchromicity at 278 nm with an additional peak at 428 nm was observed in nitroxidized-HSA. The formation of nitro-tyrosine and nitro-tryptophan, quenching in tyrosine and tryptophan fluorescence, decreased surface hydrophobicity, reduction in the α-helical content, shift in hydrodynamic radii, increased carbonyl content, reduction in free sulfhydryl groups, and low affinity towards ligand indicates secondary structural/conformational changes in nitroxidized-HSA. Moreover, ThT assay and scanning electron microscopy were performed that showed highly altered surface morphology and aggregation in nitroxidized-HSA (data not shown).

Table 1 — Characterization of HSA and nitroxidized-HSA by various biophysical and biochemical techniques

Parameters	Native HSA	Nitroxidized-HSA
Absorbance at 278 nM	0.490	0.817
Absorbance at 428 nM	---	0.196
Nitrotyrosine content (nmol mg^{-1} HSA)	---	49.5
Nitrotryptophan content (nmol mg^{-1} HSA)	---	34.03
FI_{tyr} (λ_{exc}=275nM, λ_{ems}=280-500nM)	286.9	11.3
FI_{trp} (λ_{exc}=295nM, λ_{ems}=300-500nM)	342.3	37.016
FI_{ANS} (λ_{exc} = 380 nM, λ_{ems}=400-600 nM)	135.001	28.1
α-helix (%)	67.89	39.07
Hydrodynamic radius (nM)	3.0	3.8
Protein carbonyl content (nmol mg^{-1} HSA)	3.14±0.13	14.2±0.58**
Protein thiol content (nmol mg^{-1} HSA)	164.33±2.08	101.6±1.1**
Cobalt binding affinity (μM)	122.6±2.6	103.01±4.4**
FI ThT assay	72.016	100.598

FI= Fluorescence intensity of in arbitrary units (A.U.)
λ_{exc}= excitation wavelength
λ_{ems} = emission wavelength range
** indicates highly significant difference from native HSA at $P \leq 0.01$

Estimation of carbonyl, thiol, glutathione, catalase, SOD, and LDH in lymphocytes treated with native/nitroxidized-HSA

Besides having nitrating properties, peroxynitrite is also a strong oxidant. An important marker for oxidation is protein carbonylation. Lymphocytes treated with native/nitroxidized-HSA were assayed for total protein carbonyl. Protein carbonyl was higher ($P < 0.0001$) in lymphocytes treated with nitroxidized-HSA (142.49±0.85 nmoles/mg protein) as compared to lymphocytes treated with native-HSA (16.17±1.46). Also, untreated lymphocytes (13.37±1.71 nmoles/mg protein) showed negligible amount of protein carbonyls (Table 2).

Thiols are highly nucleophilic and hence are vulnerable targets of reactive species and related oxidants. Lymphocytes treated with nitroxidized-HSA showed a decrease in total protein thiol (1417.57±31.12 nmoles/mg protein) ($P < 0.0001$) as compared to untreated lymphocytes (4057.35±168.9 nmoles/mg protein) or lymphocytes treated with native-HSA (3802.74±167.64 nmoles/mg protein) (Table 2). Similar results were obtained when reduced glutathione content was checked in lymphocytes treated with nitroxidized-HSA. Lymphocytes treated with nitroxidized-HSA showed a reduction ($P < 0.01$) in total reduced glutathione content (413.76±9.68 nmoles/mg protein) as compared to untreated lymphocytes (859.5±37.12 nmoles/mg protein) or lymphocytes treated with native-HSA (815.0933±13.64 nmoles/mg protein) (Table 2).

Apart from non-enzymatic antioxidants, several antioxidant enzymes are also present in cells that prevent or repair the damage caused by various stressors. These antioxidant enzymes play an important role in redox-sensitive signaling pathways as well. Enzymes such as SOD are essential for life in all oxygen metabolizing cells. The enzymes convert toxic superoxide anion to less toxic hydrogen peroxide and molecular oxygen. Another important antioxidant enzyme, *i.e.*, catalase catalyzes the decomposition of hydrogen peroxide into water and

Table 2 — Effect of nitroxidized-HSA treatment on some biochemicals of lymphocytes

Parameters	Lymphocytes		
	Untreated	Treated with native HSA	Treated with nitroxidized-HSA
Carbonyl (nmoles/mg protein)	13.37±1.71	16.17±1.46	142.49±0.85****
Thiol (nmoles/mg protein)	4057.35±168.9	3802.74±167.64	1417.57±31.12****
Glutathione (nmoles/mg protein)	859.5±37.12	815.09±13.64	413.76±9.68**
Catalase (units/mg protein)	3.4±0.15	3.83±0.16	19.5±0.38****
Superoxide dismutase (units/mg protein)	2.03±0.07	2.66±0.09	10.39±0.43****
Lactate dehydrogenase (%)	1.87±0.179	2.27±0.34	49.93±0.72****

All data has been expressed as mean ± SD of three independent experiments. Data were analysed by ANOVA followed by Tukey's multiple comparison test. Siginificantly different compared to untreated and native-HSA treated lymphocytes at **$P \leq 0.01$ and **** $P \leq 0.0001$

oxygen. Exposure of lymphocytes to nitroxidized-HSA might have caused damage to the cell membrane and hence the release of enzymes. SOD and catalase activity was found to be increased ($P < 0.0001$) when lymphocytes were treated with nitroxidized-HSA (10.39±0.43 units/mg protein and 19.5±0.38 units/mg protein, respectively) (Table 2). However, SOD and catalase activity did not significantly change in untreated lymphocytes (2.03±0.07 units/mg protein and 3.4±0.15 units/mg protein, respectively) and lymphocytes treated with native HSA (2.77±0.09 units/mg protein and 3.83±0.16 units/mg protein, respectively).

Similarly, LDH release was estimated to be 49.93%±0.72 ($P < 0.0001$) in nitroxidized-HSA. This enzyme is released into the surroundings through damaged plasma membrane or lysed cells[37]. The lymphocytes incubated with native HSA did not show any significant release of LDH (Table 2).

Estimation of live/dead cells by cell viability assays

The viability of lymphocytes under the influence of nitroxidized-HSA was assessed by trypan blue exclusion method and compared with the native HSA treated and untreated cells. Results of viable cell count are presented in (Fig. 1) which indicates significantly low percentage of live cells ($P < 0.0001$) in lymphocytes treated with nitroxidized-HSA (44.93%±1.7) compared to untreated cells (100%). However, the percentage of viable cells in lymphocytes treated with native HSA (94.33%±1.07) was not significantly different from that of untreated cells (99.19%±0.19).

The metabolic activity and hence the viability of the lymphocytes was also evaluated by MTT assay. Our results show that lymphocytes treated with nitroxidized-HSA had decreased ($P < 0.0001$) viability

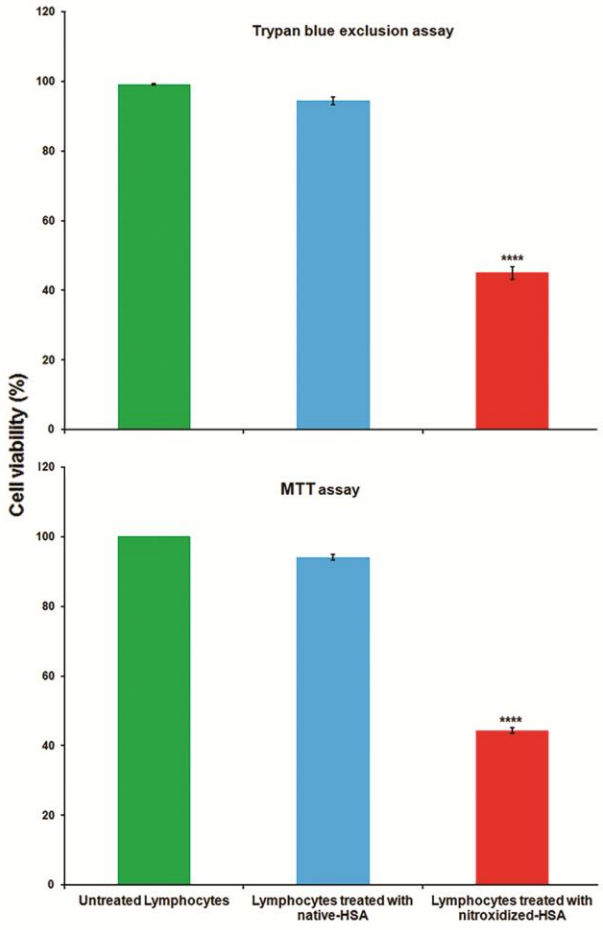

Fig. 1 — Percentage viability levels of lymphocytes treated with native/nitroxidized-HSA as measured by Trypan blue exclusion and MTT proliferation assay. All data has been expressed as mean ± SD of three independent experiments. Data were analysed by ANOVA followed by Tukey's multiple comparison test. Siginificantly different compared to untreated and native-HSA treated lymphocytes at **** $P \leq 0.0001$

(44.29%±0.86), whereas, no significant difference in cell viability was observed in lymphocytes treated with native HSA (94.06%±0.86) with respect to the untreated cells (100%).

Dual staining and fluorescence microscopy of untreated and treated lymphocytes

The results obtained from fluorescence microscopy studies suggest the detrimental nature of nitroxidized-HSA (Fig. 2). AO penetrates viable cells and stains them green while EtBr, which is excluded by viable cells, stains the nonviable cells red. The differential uptake of these two dyes allows the identification of viable and non-viable cells. Untreated lymphocytes and lymphocytes treated with native HSA show little to none EtBr stained cells, whereas, lymphocytes treated with nitroxidized-HSA showed large number of EtBr stained cells (Fig. 2).

Estimation of intracellular ROS generation in untreated and treated lymphocytes

DCFH-DA is itself non-fluorescent which diffuses inside the cell membrane and is converted to 2,7

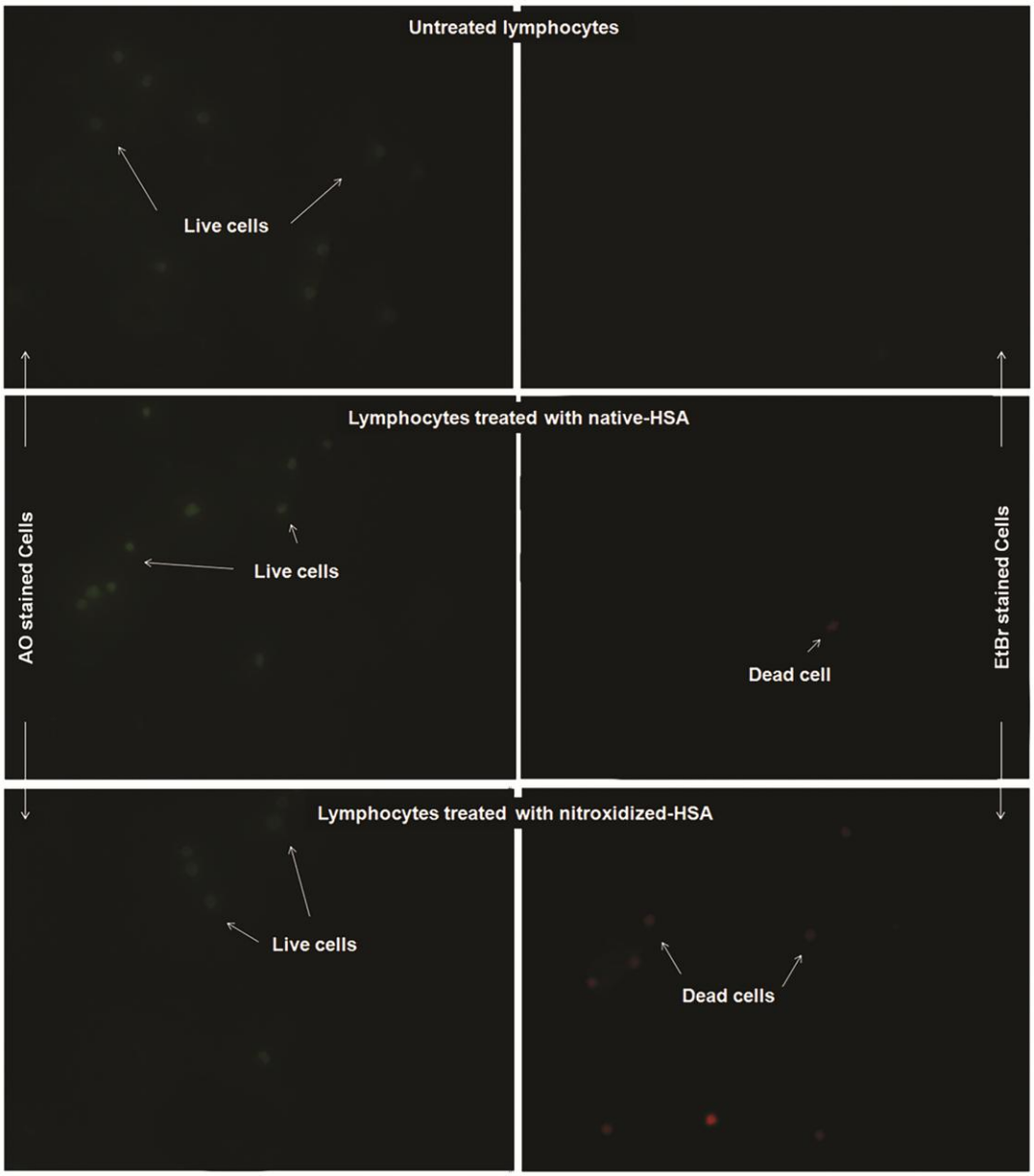

Fig. 2 — Visualisation of live (green) and dead cells (red) in lymphocytes treated with native/nitroxidized-HSA by AO/EtBr dual staining and fluorescence microscopy. Untreated lymphocytes and lymphocytes treated with native-HSA shows mainly green fluorescence (viable cells) while lymphocytes treated with nitroxidized-HSA shows both green (viable cells) and red fluorescence (dead cells)

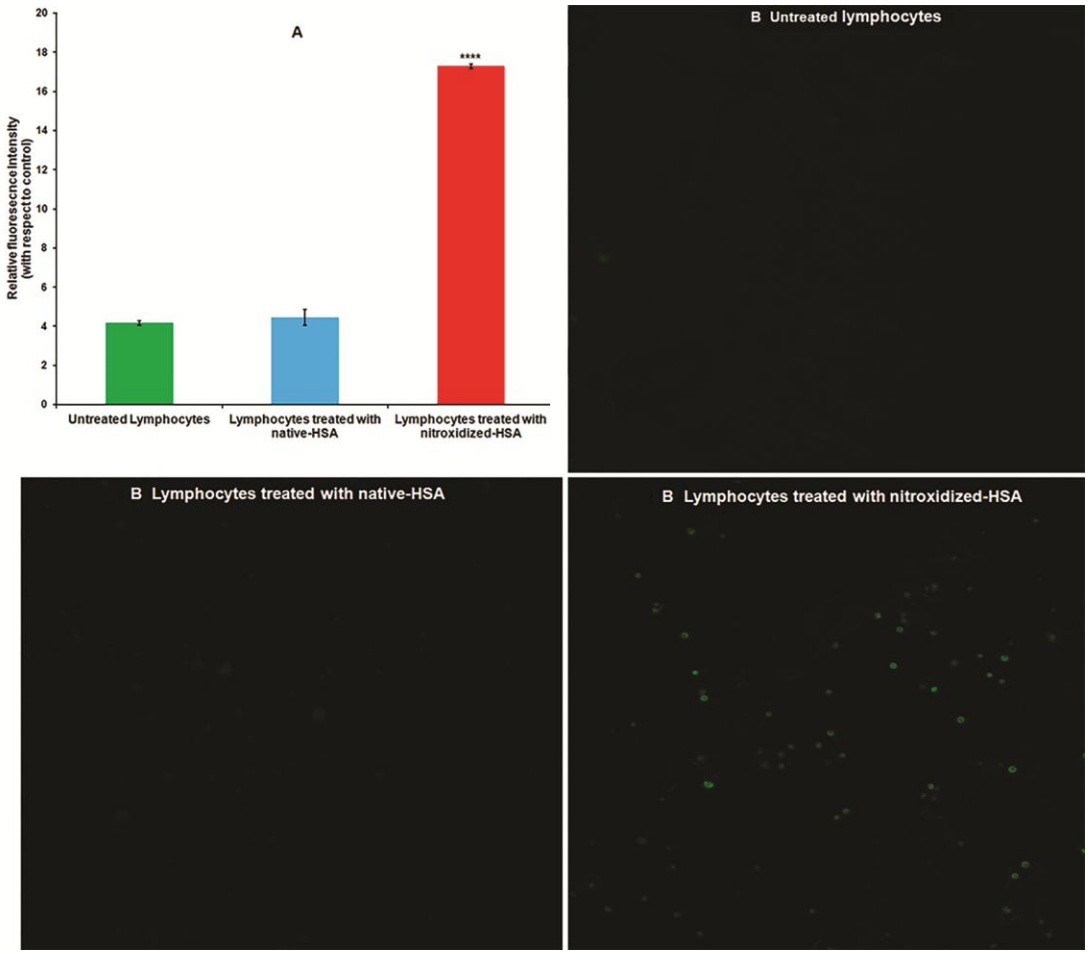

Fig. 3 — Detection of intracellular ROS generation in lymphocytes treated with HSA/nitroxidized-HSA. (A) Relative fluorescence intensities of lymphocytes treated with native/nitroxidized-HSA. All data has been expressed as mean ± SD. Data were analysed by ANOVA followed by Tukey's multiple comparison test. Siginificantly different compared to untreated and native-HSA treated lymphocytes at **** $P \leq 0.0001$; and (B) Microscopic visualisation of intracellular ROS in untreated lymphocytes; and lymphocytes treated with native/nitroxidized-HSA

dichlorofluorescein (DCF) in presence of ROS that makes the cell fluorescent. The fluorescent emission signals given by lymphocytes treated with nitroxidized-HSA were 3.5-folds higher when compared to native-HSA treated lymphocytes (Fig. 3). Fluorescence micrographs of lymphocytes treated with native/nitroxidized-HSA confirm our spectrofluorometric results. In presence of nitroxidized-HSA, green fluorescence (*i.e.* ROS generation) was observed (Fig. 3) but not in presence of native-HSA (Fig. 3).

Detecting mechanism of cell death by confocal laser scanning microscopy

AO stains live cells and emit green fluorescence while PI stains dead cells and emit red fluorescence. Untreated lymphocytes and lymphocytes exposed to native-HSA are viable with intact membranes and uniform green color in their nuclei. However, most of the cells treated with nitroxidized-HSA showed membrane blebbing with an unapparent outline. Condensed chromatin in some cells suggests preparation for apoptosis. Moreover, many cells already died of necrosis (Fig. 4).

Discussion

Peroxynitrite is both a strong oxidant and a nitrating agent and thus is proficient in causing nitroxidation of biomolecules[38]. The extent of protein nitroxidation is dependent on the age and level of oxidants/nitrosants present in the body. High levels of peroxynitrite can be seen in many autoimmune, inflammatory, and neurodegenerative diseases[6,39]. Peroxynitrite modifies biomolecules (for instance protein) that lead to crosslinks/aggregate formation. HSA is the major

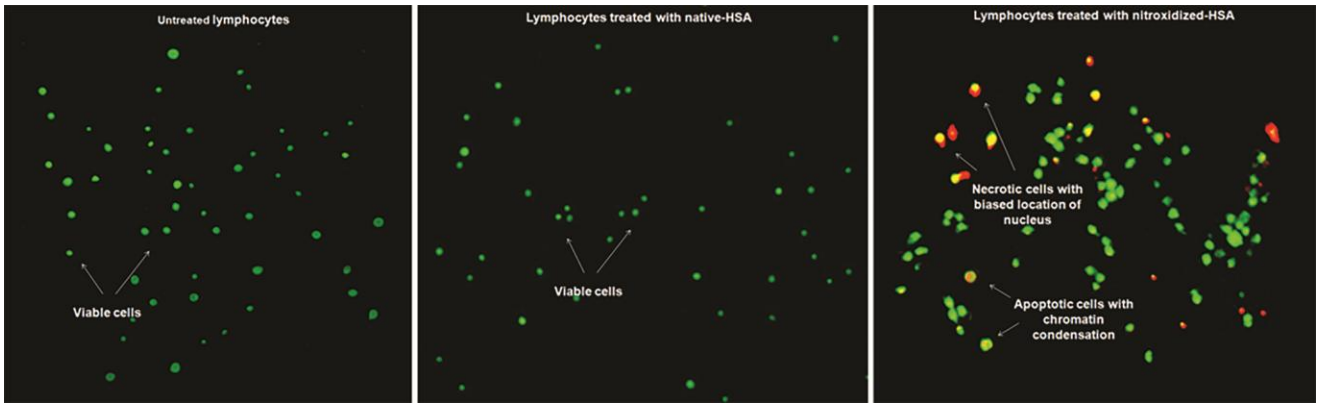

Fig. 4 — illustrates confocal images of differential stained lymphocyte cells with AO and PI dye. Untreated lymphocytes and lymphocytes treated with native-HSA showing similar distribution of cells with circular nucleus, lymphocytes treated with nitroxidized-HSA showing increased cells' volume, uneven orange-red fluorescence with biased location of nucleus indicating many cells died with necrosis

target of oxidative/nitrosative stress and is manifested by an increase in protein oxidation/nitroxidation[19]. The alteration in physico-chemical properties of HSA and formation of protein aggregates after nitroxidation has been already reported in earlier studies[36,40] but, the noxious nature of nitroxidized-HSA on cells/tissues was not explored. Therefore, the toxic potential of physico-chemically altered nitroxidized-HSA is evaluated in the present study.

The increased carbonyl and decreased thiol/reduced glutathione content in lymphocytes treated with nitroxidized-HSA are suggestive of reactive species generation and hence oxidative stress. Reactive species are capable of causing damage to biomolecules; however; the major target is intracellular thiols[41]. Thiols play a crucial role in maintaining cellular redox homeostasis. Apart from protein thiols, non-protein thiols are also targets of oxidative stress. In almost every aerobic cell, the major non-protein thiol is glutathione (0.1 to 10 mM)[42], which is present in abundance in mammalian cells. This tripeptide (γ-glutamyl-cysteinyl-glycine) protects cells from reactive species-mediated oxidative damage[42]. In healthy cells and tissues, ~ 90% of the total glutathione pool is in the reduced (GSH) form[41]. It has been studied that depletion of thiols (specifically glutathione) enhances oxidative stress and thereby initiates cell death in distinct neuronal population[43]. The enhanced release of intracellular enzymes (SOD, catalase, and LDH) suggests oxidative stress and consequent damage to lymphocytes treated with nitroxidized-HSA. In the physiological state, cell membrane is impermeable to enzymes[44], however, a small amount of enzyme may get released when membrane blebs detach with resealing of the membrane. But, the substantial release indicates a severe disruption of the membrane integrity[44], and the same was observed in our study.

MTT is a sensitive chromogenic indicator and has long been used for the assessment of cell viability both *in vitro* and *ex vivo* studies[32]. Also, cell counting using viability dyes such as trypan blue provides the percentage of viable cells[27]. Our results show that lymphocytes treated with nitroxidized-HSA had decreased viability compared to those treated with native-HSA. The nucleic acid binding dyes *i.e.* AO and EtBr can accurately determine cell viability. The AO/EtBr dual staining and fluorescence imaging of the untreated and treated cells conforms the results of the viability tests. Our results clearly show that lymphocytes exposed to nitroxidized-HSA had high levels of dead cells. The protein aggregates exert their cytotoxic effects via the generation of ROS and the interaction of such aggregate with cells causes oxidative damage[45]. Naeem *et al.*[46] have reported that treatment of lymphocytes with prefibrillar aggregates of HSA produces free radicals. Free radicals, especially ROS, generated during protein aggregation is a common, fundamental molecular mechanism involved in the pathogenesis of oxidative damage and cell death in several diseases[45]. Therefore, the levels of ROS generated intracellularly in lymphocytes were assessed using a fluorescent probe *i.e.* DCFH-DA. From the results it has been inferred that ROS is produced in lymphocytes treated with nitroxidized-HSA but not in untreated or lymphocytes exposed to native-HSA.

We observed that the interaction of aggregated nitroxidized-HSA with lymphocytes is responsible for

causing oxidative damage and cell death; however; the assays utilized (trypan blue and MTT assays) cannot differentiate between the mechanisms of cell death. Therefore, to identify the mechanism(s) of cell death, dual staining by AO/PI and confocal laser scanning microscopy was employed. Here, we observed that all viable nucleated cells fluoresce green and all necrotic/dead nucleated cells fluorescence red[47]. However, apoptotic cells have yellowish areas of condensed chromatin in the nucleus[48]. The method clearly distinguishes viable, apoptotic, and necrotic cells. Therefore, we reached to the conclusion that nitroxidized-HSA is cytotoxic and its accumulation in cells and tissues may be injurious. Moreover, the leakage of cytosolic components into the extracellular space may arouse an inflammatory response[49]. These liberated cytosolic components, after disintegrations, may be modified and further add to the disease pathophysiology. A wide number of autoimmune and degenerative diseases are characterized by the accumulation of aggregated proteins. Aggregation-induced cellular toxicity has been implicated in various diseases such as alzheimer[6], Parkinson's[6], type II diabetes[8] and in certain forms of heart diseases[7]. It can, therefore, be speculated that the accumulation of nitroxidized-HSA may directly/indirectly contribute to the pathogenesis of various autoimmune and neurodegenerative diseases.

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