

Full Length Research Paper

Autoantibodies against reactive oxygen species modified immunoglobulin G in patients with type 1 diabetes mellitus

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The role of hydroxyl radicals ($\cdot\text{OH}$) damaged immunoglobulin G (IgG) in insulin dependent diabetes mellitus has been investigated. IgG was isolated from normal human serum and modified by hydroxyl radicals, generated by UV irradiation of hydrogen peroxide. $\cdot\text{OH}$ -induced modification on IgG has been studied by ultra-violet (UV) absorption spectroscopy, tryptophan/tyrosine fluorescence, circular dichroism, SDS-PAGE and carbonyl groups estimations. $\cdot\text{OH}$ caused extensive damage to IgG. The binding characteristics of circulating antibodies in type 1 diabetes patients against native and modified IgG were assessed. Type 1 diabetes patients ($n = 36$) were examined by direct binding ELISA and the results were compared with healthy age-matched controls ($n = 22$). High degree of specific binding of diabetes sera towards $\cdot\text{OH}$ modified IgG, in comparison to its native analogue ($p < 0.05$). Sera from normal human subjects showed negligible binding with either antigen. Our results conclude that $\cdot\text{OH}$ -modification of IgG causes structural perturbations, resulting in the generation of neo-epitopes and making it a potential immunogen. IgG modified with $\cdot\text{OH}$ may be one of the factors for the induction of circulating type 1 diabetes autoantibodies.

Key words: Autoantibodies, hydroxyl radicals, immunoglobulin G, $\cdot\text{OH}$ -IgG, type 1 diabetes mellitus.

INTRODUCTION

Type 1 diabetes is an autoimmune disease resulting from the selective destruction of pancreatic β cells. Its incidence in different ethnic groups is extremely variable, suggesting the involvement of genetic as well as environmental elements (Park, 2007). In type 1 diabetes, during the long prodromal period, multiple autoantibodies to pancreatic β cell antigens may arise and persist even after the disease becomes clinically over. In humans, glutamic acid decarboxylase (GAD), IA-2 and insulin would be the most important target molecule against which autoantibodies, as well as autoreactive T cells are directed (Park, 2007). The precise mechanism of β -cells

destruction leading to diabetes remains unclear (Kawasaki et al., 2004). In fact, the oxidative stress plays a central role in the onset of diabetes mellitus (DM) as well as in diabetes associated complications (Phillips et al., 2004). Various studies have shown that DM is associated with increased formation of free radicals and decrease in antioxidant potential. This leads to oxidative damage of cell components such as proteins, lipids and nucleic acid (Nazirogilu and Butterworth, 2005). Increased oxidative stress in diabetic patients leads to protein oxidation (Telci et al., 2000). The conversion of protein to protein carbonyl (PCO) derivatives occurs through direct oxidation by ROS, with the eventual formation of oxidized amino acids (Carrard et al., 2002). Immunoglobulin G (IgG) is a major protein of serum whose function lies in the specific interactions with and clearance of antigens. In rheumatoid arthritis (RA)

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Table 1. Biochemical details of study subjects.

Variables	Control	Type 1 diabetes
N	22	36
Age (yr)	26 - 36	24 – 32
Sex (M:F)	14:8	26:10
Blood glucose (mg/dl)	110.2 ± 7.5*	352.0 ± 24.3*
HbA _{1c} (%)	4.5 ± 0.4*	8.3 ± 1.2*
Carbonyl contents in IgG (nmol/mg protein)	0.49 ± 0.08*	1.2 ± 0.17*

M: male, F: female, n: number of samples tested, *Mean ± S.D.

patients, IgG dysfunction has been reported; it behaves not only as an antibody, but also as a putative antigen for rheumatoid factor (Chou, 2002). It has been well documented that IgG is quite vulnerable to ROS (Uesugi et al., 2000; Kleinveld et al., 1991). Many studies show the presence of elevated levels of oxidized IgG in patients with RA (Rasheed, 2008). Therefore, IgG is continuously exposed to oxidative stress, so that alterations of the conformation and function of IgG could occur, resulting in modification of its biological properties. It is well documented that oxidative damage of protein and DNA presents unique neo-epitopes, which is one of the factors in antigen-driven autoimmune response in autoimmune diseases (Scofield et al., 2005; Kurien and Scofield, 2008). Recent studies from our laboratory have demonstrated that after modification with ROS, proteins became highly immunogenic and oxidative by-products with proteins could lead to neoantigens which would initiate autoimmunity in diseases such as RA (Rasheed, 2008; Rasheed et al., 2006, 2008; Rasheed and Ali, 2006; Rasheed et al., 2007a, 2009a). In view of these, present study was based upon a hypothesis that oxidative by-products, hydroxyl radical damage IgG, help to initiate autoimmunity in patients with type 1 diabetes mellitus. To test this hypothesis, IgG was purified from normal human serum by affinity chromatography. Affinity purified IgG was modified with reactive oxygen species. ROS-induced modification of IgG has been characterized by physico-chemical techniques. We studied circulating autoantibodies in type 1 diabetes sera directed against native and ROS-modified IgG.

RESEARCH DESIGN AND METHODS

Reagents

SDS-molecular weight markers, anti-human IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Folin-ciocalteu reagent, Coomassie Brilliant Blue G-250, Tween-20 and Protein A-Sepharose CL-4B were purchased from Sigma Aldrich (St. Louis, MO, USA). Polysorp microtitre flate bottom ELISA plates having 96 wells were from NUNC (Denmark). Acrylamide, ammonium persulphate, bisacrylamide, N,N,N,N-tetramethyl-ethylene diamine (TEMED) were purchased from Bio-Rad Laboratory U.S.A. All other reagents/chemicals were of the highest analytical grade.

Human subjects

Blood was collected from voluntary donors with history of type1 DM attending outdoor and indoor in Jolly Grand Hospital, Dehradun, UK, India. The control samples were collected from healthy subjects. Complete details of subjects have been summarized in Table 1. Informed consent was obtained from each patient or from patient's family members. The study protocol had the approval of the ethics review committee of J.N. Medical College. All sera were decplemented by heating at 56°C for 30 min and stored in aliquots at -20°C.

Purification of immunoglobulin G

IgG from normal human sera was isolated by affinity chromatography on protein A-Sepharose CL-4B column as previously described (Rasheed, 2008). Briefly, serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to the column (12 × 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, 3 ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.38 OD₂₇₈ = 1.0 mg IgG/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.

Modification of IgG

IgG was modified in PBS (10 mM sodium phosphate buffer containing 150 Mm NaCl, pH 7.4) by our published procedure (Rasheed, 2008). An aqueous solution of IgG (1.2 µM) was modified by hydroxyl radical, generated by the irradiation (30 min) of hydrogen peroxide (15.1 mM) at 254 nm. Excess hydrogen peroxide was removed by extensive dialysis against PBS. The ultra-violet (UV) absorption characteristics of native and modified wererecorded on a Shimadzu UV-240 spectrophotometer.

Fluorescence measurements

Fluorescence measurements were performed on Hitachi F-200 spectrofluorimeter (Japan). The fluorescence spectra were measured at 25 ± 0.1°C with a cell of 1 cm path length. The excitation and emission slits were set at 5 and 10 nm, respectively. Intrinsic fluorescence was measured by exciting the protein solution at 280 or 295 nm and emission spectra were recorded in the range of 300 - 400 nm. Increase of fluorescence intensity (F.I.) was calculated using the following equation:

% Increase of F.I. = $[(F.I. \text{ Modified sample} - F.I. \text{ Unmodified sample}) / F.I. \text{ Modified sample}] \times 100$

Circular dichroism measurements

Far-UV CD measurements were carried out with Jasco spectropolarimeter, model J- 720 equipped with a microcomputer. The instrument was calibrated with D-10- camphorsulfonic acid. The CD measurements were made at 25°C with a thermostatically controlled cell holder attached to Neslab's RTE 110 water bath with a temperature accuracy of $\pm 0.1^\circ\text{C}$. Spectra were taken with a scan speed of 20 nm/min at a response time of 1 s. Each spectrum was the average of four scans. CD spectra were taken at protein concentration of 3.0 μM with a cell of 1 mm path length. The MRE (mean residual ellipticity) was expressed in $\text{deg.cm}^2.\text{mol}^{-1}$.

Assay of carbonyl formation

Carbonyl contents of native and ROS-modified IgG were analyzed according to Levine et al. (1994) with slight modifications. The reaction mixture containing 6.7 μM native IgG or ROS-IgG, 0.5 ml of 10 mM 2, 4- dinitrophenylhydrazine (DNPH) / 2.5 M HCl was added and thoroughly mixed. After addition of 250 μM 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 carbonyl contents were estimated from the absorbance at 370 nm using a molar absorption coefficient of 22,000 $\text{M}^{-1}\text{cm}^{-1}$. Samples were spectrophotometrically analyzed against a blank of 1 ml of guanidine solution (6 M). Protein concentration was determined in the samples by the method of Lowry et al. (1951). Carbonyl contents were expressed as nmol/ mg protein.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The modification of IgG was further analyzed by SDS-PAGE on 7.5% polyacrylamide gel according to the method of Laemmli (1970). The protein was electrophoresed at 50 V for 3 h at room temperature and the bands were visualized by silver staining (Oakley et al., 1980)

Detection of antibodies against ROS-IgG in diabetes patients

Presence of antibodies against native and ROS modified IgG in sera of diabetes patients was evaluated by solid phase enzyme immunoassays. Enzyme Linked Immunosorbent Assay (ELISA) was carried out on polystyrene polysorp plates as described previously (Rasheed et al., 2006). Polystyrene polysorp microtitre plates were coated with 100 μL of native or modified IgG (10 $\mu\text{g ml}^{-1}$) 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 h at 37°C. After incubation the plates were washed four times with TBS-T. The test serum serially diluted in TBS-T (100 μL well-1) was adsorbed for 2 h at 37°C and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. Absorbance (A) was monitored at 410 nm on an automatic microplate reader and mean of duplicate readings for each sample was recorded. Results have been

expressed as a mean of Atest–Acontrol. The antigen binding specificity of the antibodies was evaluated by competition ELISA (Rasheed et al., 2007). Varying amount of inhibitor (0 - 20 mg ml⁻¹) was mixed with a constant amount of antiserum. The mixture was incubated at room temperature for 2 h and overnight at 4°C. The immune complex thus formed was coated in the wells instead of the serum. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculated using the formula:

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

Statistical analysis

Data are presented as mean \pm SD. Significance of differences from control values were determined with the Student's t-test (Statgraphics, Origin 6.1). A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

IgG was purified from normal human sera on protein-A Sepharose CL-4B affinity column. The purified IgG was found to elute as a single symmetrical peak and gave a single band on SDS-PAGE as shown previously (Rasheed, 2008). Affinity purified IgG was then modified by $\cdot\text{OH}$ and the induced changes in IgG were analyzed by UV absorption spectroscopy. The UV absorption spectra of ROS-modified IgG revealed a marked hypochromicity (45.4%) at 280 nm (Figure 1a). ROS-modified IgG on SDS-PAGE under non-reducing conditions, showed significant change in the mobility as compare to its native analogue (Figure 1b). Intensity of ROS-IgG band decreased considerably as compared to native IgG. The oxidation of tryptophan residues on damaged IgG was evident by the loss of 61.2% fluorescence intensity (FI) at 340 nm using an excitation wavelength of 295 nm (Figure 1c). The damage to tryptophan residues was confirmed by the loss of 64.2% FI at 340 nm using an excitation wavelength of 280 nm (Figure 1c).

The far-UV CD spectra of ROS-modified IgG showed increased in the ellipticity value in the range 210 to 230 nm as compared to CD spectra of unmodified IgG, indicating a significant loss of secondary structure of protein on damage by $\cdot\text{OH}$ radical (Figure 1d). Oxidation of protein typically results in an increase in protein carbonyl contents, a known biomarker of oxidative stress. Oxidation induced carbonyl formation in IgG has been shown in Figure 2a. The amount of carbonyl/protein was increased with increase of H_2O_2 concentration. Generation of $\cdot\text{OH}$ radical was confirmed using mannitol and dimethyl sulfoxide (DMSO) as they are specific quenchers of hydroxyl radical. IgG (6.7 μM) was modified in absence and in presence of 286 mM mannitol and DMSO and their carbonyl content was determined. The data showed decrease in carbonyl formation in presence of mannitol and DMSO (Figure 2b), whereas high carbonyl formation occurred in the absence of these quenchers.

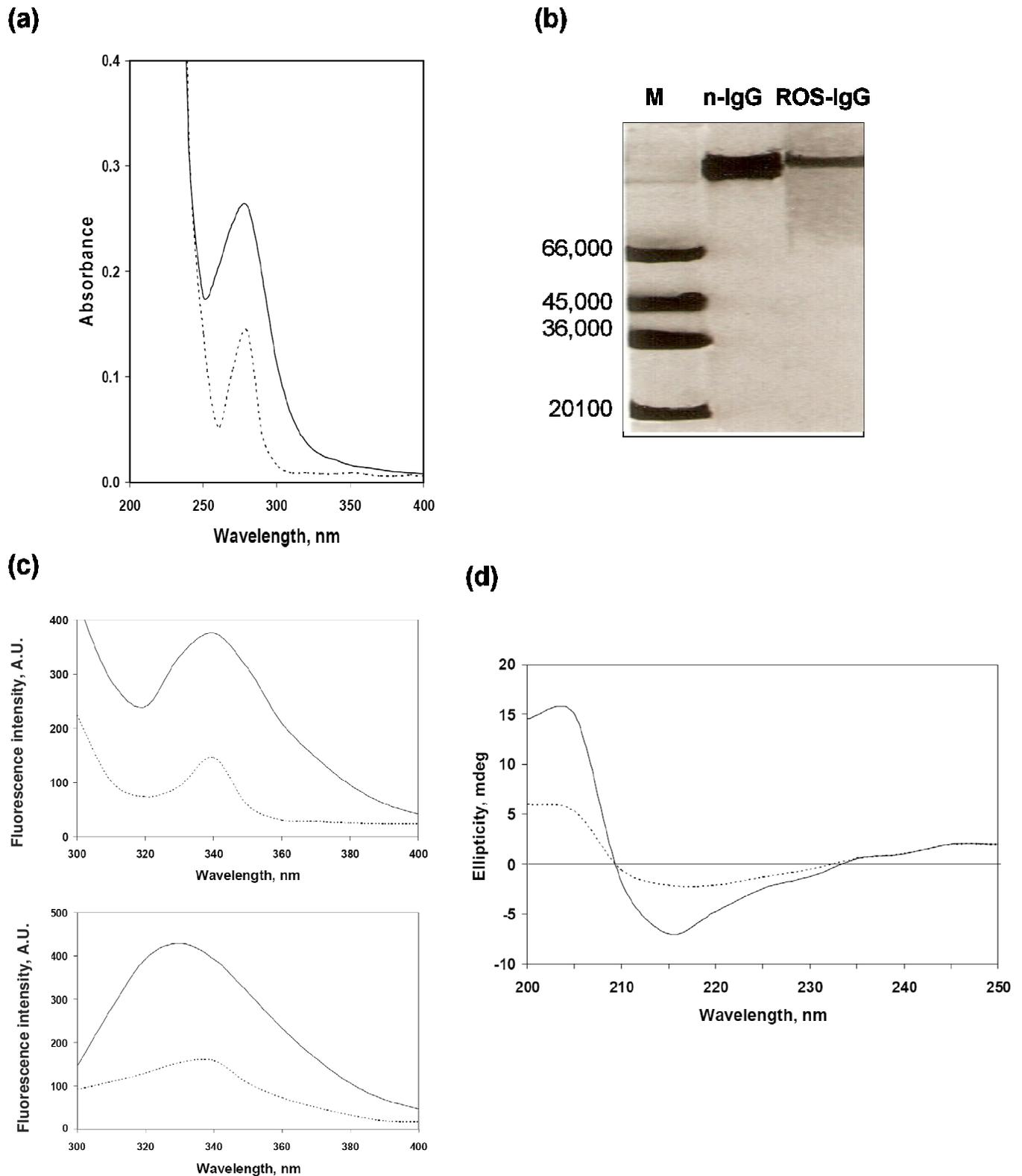


Figure 1. Characterization of ROS-modified IgG. (a) UV-absorption spectra of native (-) and ROS-modified (---) IgG. The samples were in PBS, pH 7.4. (b) SDS-polyacrylamide gel electrophoresis of native and ROS-modified IgG under non-reducing conditions. M represents standard molecular weight markers. (c) Fluorescence emission spectra of native (-) and ROS-modified (---) IgG. Protein was in PBS, pH 7.4 at a concentration of 1.5 μM . The excitation wavelength was 295 nm (upper panel) and 280 nm (lower panel). (d) Far UV circular dichroic spectra of native (-) and ROS-modified (---) IgG. The samples were in PBS, pH 7.4 at a concentration of 3 μM . (Figures 1 a, c and d were adopted from Rasheed (2008) Clin Biochem 41:663-9).

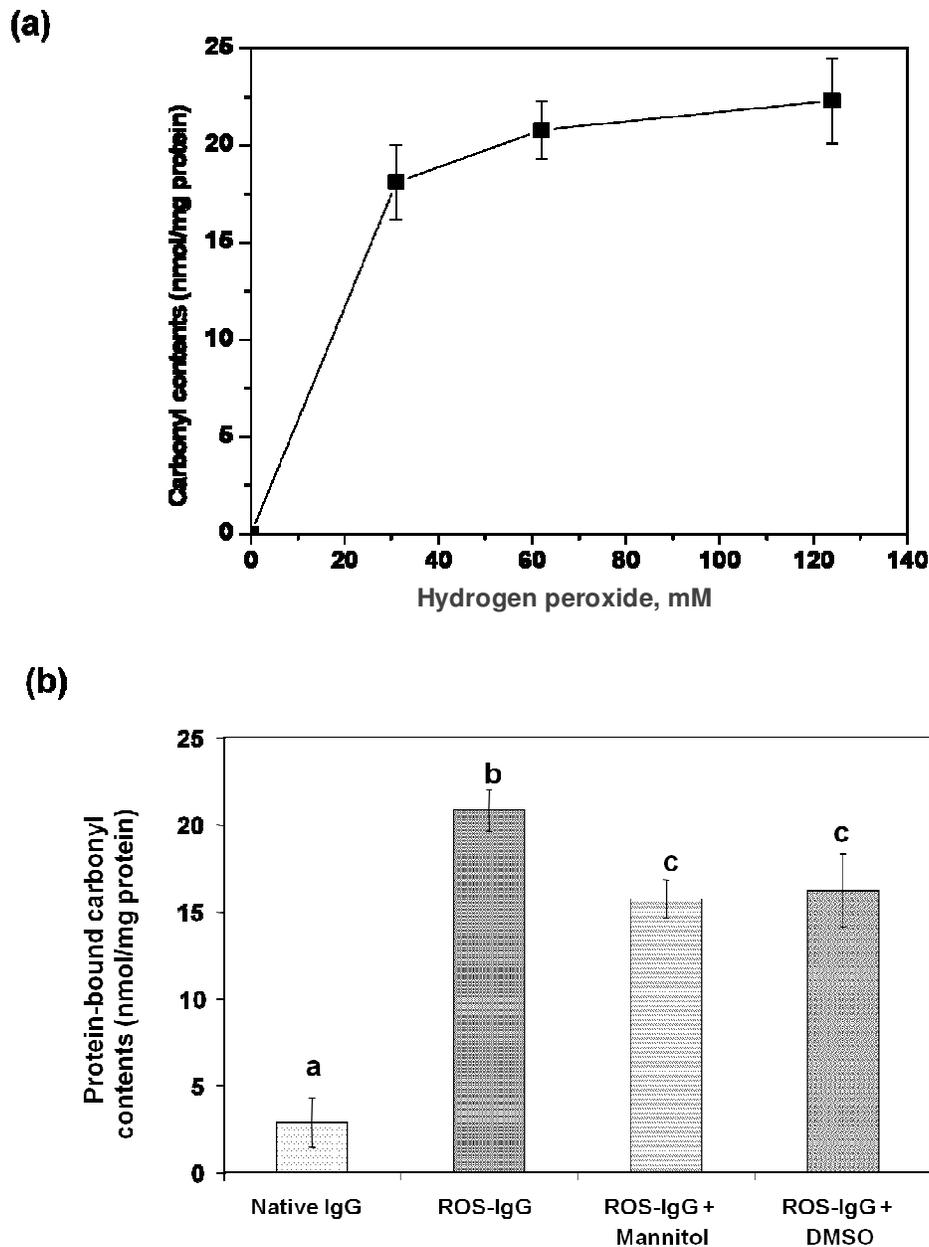


Figure 2. Protein-bound carbonyl contents in ROS-modified IgG (a) Estimation of protein-bound carbonyl groups in ROS-IgG by 30 min UV exposure of native IgG with the indicated H_2O_2 concentrations. (b) Protein-bound carbonyl groups present in native and ROS-modified IgG samples and effect of OH^\cdot quenchers on ROS modification of IgG. The protein concentration was 15 μ M. Results are representative (mean \pm SEM) of five independent experiments and differ without a common letter $P < 0.05$.

Our study comprised of 36 serum samples from patients suffering from type 1 diabetes. Each sample was obtained after careful clinical examination. Control serum samples were obtained from 22 healthy individuals. All sera were tested for binding to native and ROS-modified IgG by direct binding and competitive inhibition ELISA. A majority of diabetes sera (24/36) showed strong binding to ROS-modified IgG over native IgG at 1:100 serum

dilution ($p < 0.05$) (Figure 3). No appreciable binding was observed with the sera from normal subjects. The average absorbance at 410 nm (\pm SD) of 24 DM sera binding to native and ROS-damaged IgG was 0.56 ± 0.05 and 0.81 ± 0.06 , respectively. Whereas, the average absorbance at 410 nm (\pm SD) of 22 normal human sera binding to both protein antigens was 0.23 ± 0.05 and 0.26 ± 0.04 , respectively. The binding specificity of antibodies

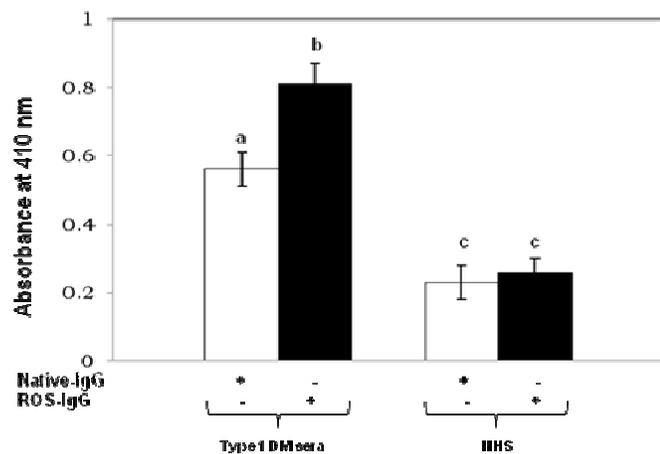


Figure 3. Direct binding ELISA of 1:100 diluted type 1 diabetes serum samples. The microtitre plates were individually coated with IgG and ROS-IgG (10 µg/ml). Normal human serum (NHS) samples used as negative controls. The numbers of diabetes serum samples were 24 and NHS samples were 22. Results are representative (mean ± SEM) of five independent experiments and differ without a common letter $P < 0.05$.

from sera of DM patients exhibiting strong binding to ROS-IgG was evaluated by competition ELISA using native and ROS-IgG as inhibitors. Results point to a higher reactivity of diabetes patient's autoantibodies (in sera 1, 2, 3, 4 and 5) towards ROS damaged IgG over native IgG (Figures 4). Similarly, rest of the sera also showed high percent inhibitions with ROS-IgG as compared to native IgG (Table 2). The average percent inhibition (\pm SD) in the binding of 24 type 1 DM sera to native and ROS-IgG were 27.8 ± 4.6 and 46.3 ± 8.3 , respectively. Maximum inhibitor concentration was 20 µg/ml in all the cases. The data reveals striking differences in the recognition of native and ROS-IgG by DM autoantibodies ($p < 0.001$). Our results are in full agreement to the view that oxidative damage of protein presents unique epitopes, which help to initiate autoimmunity.

DISCUSSION

Reactive Oxygen Species (ROS) play a key role in both normal biological functions and in the pathogenesis of certain human diseases. These are continuously generated in cells by cellular metabolism and by exogenous agents but increases in their steady states are thought to be responsible for a variety of pathological conditions, including cardiovascular diseases, aging and cancer (Kurien and Scofield, 2008). Excess generation of reactive oxygen species have the ability, either directly or indirectly, to damage proteins, DNA and other cell biomolecules (Rasheed, 2008; Scofield et al., 2005; Kurien and Scofield, 2008; Rasheed et al., 2006, 2008,

2009a; Rasheed and Ali, 2006; Rasheed et al., 2007a, b; Ahmad et al., 2009). Proteins are major targets for free radical attack - especially damaging is the hydroxyl radical (Rasheed, 2008; Rasheed et al., 2006, 2008; Rasheed and Ali, 2006; Rasheed et al., 2007, 2009). Protein oxidation, which results in functional disruption, is not random but appears to be associated with increased oxidation in specific proteins (Scofield et al., 2005; Kurien and Scofield, 2008). A number of studies indicate a strong role of protein oxidation as a primary cause of cellular dysfunction observed during aging and age-related neuro-degenerative diseases (Shacter, 2000; Hensley and Floyd, 2002) such as Alzheimer's disease, Parkinson's disease and in carcinogenesis (Tabner et al., 2001) and possibly some other protein conformational diseases. Inhibition of a wide array of enzyme activities has been reported (Arutyunova et al., 2003). Modification of structural protein can also lead to loss of function. For example, fibrinogen on exposure to ROS loses its ability to form a solid clot (Shacter et al., 1995). Oxidation of synovial fluid immunoglobulin causes aggregation and contributes to the etiology of rheumatoid arthritis (Chou, 2002). These disorders can be due to the formation of abnormal protein aggregates as a consequence of ROS damage. Increased levels of circulating antibodies and autoantibodies have been reported in the serum of patients with malignancies and other diseases including diabetes (Rasheed, 2008; Scofield et al., 2005; Kurien and Scofield, 2008; Rasheed et al., 2006, 2008; Rasheed and Ali, 2006; Rasheed et al., 2007, 2009) and it is now well accepted that ROS plays an important role in autoimmune diseases and in carcinogenesis and hydroxyl radical contributes to the structural changes that characterize the cancer and other diseases like phenotype (Kurien and Scofield, 2008).

In the present study, IgG was purified from normal human sera by protein-A Sepharose CL-4B affinity column, homogeneity of purified IgG was checked on SDS-PAGE. The affinity purified IgG was then modified by ROS, generated by the UV irradiation of hydrogen peroxide, resulting in extensive damage to IgG as evident by 45.4% UV hypochromicity at 280 nm. The observed hypochromicity could be due to the modification of chromophoric groups, modification of aromatic amino acid residues of IgG or due to structural alterations. The data of SDS-PAGE under non-reducing conditions reiterate our results, which indicate breakage of IgG upon ROS-modification as the band intensity was significantly decreased. Oxidation of tryptophan residues of IgG upon ROS modification was confirmed by the decrease in the fluorescence intensity after exciting the protein at 295 nm, this oxidative modification on tryptophan residues were further confirmed by exciting the protein at 280 nm. Increase in the ellipticity values of ROS-damaged IgG in far UV region of circular dichroic spectrum indicates a substantial loss of secondary structure of IgG. This study is the continuation of our previous study in which we

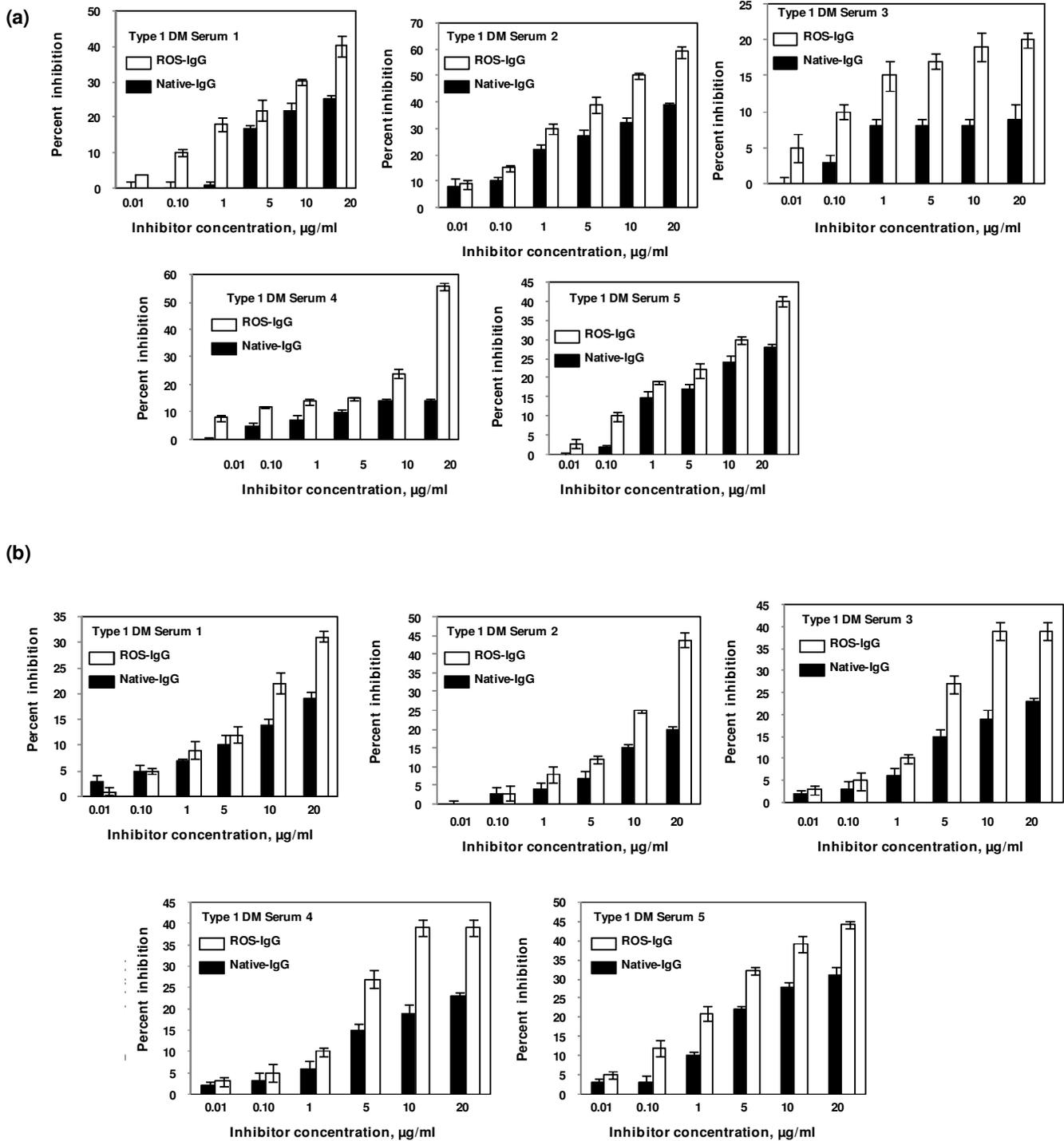


Figure 4. Competition ELISA of type 1 diabetes serum antibody from five patients (1, 2, 3, 4, 5). The inhibitors used were native IgG and ROS-IgG. The microtitre plates were coated with the ROS-IgG (a) and native IgG (b). Varying amounts of inhibitors (0 - 20 µg/ml) were allowed to interact with a constant amount of antiserum for 2 h and overnight at 4°C, the mixture was added to antigen-coated plates and the residual antibody level was detected by ELISA. Each histogram represents the mean ± SEM of three independent assays.

characterized the ROS modification of IgG (Rasheed, 2008). Protein carbonyl contents are actually the most

general indicator and by far the most commonly used biomarker of protein oxidation (Levine et al., 1994) . The

Table 2. Detection of antibodies against native and modified IgG in the sera of type 1 diabetes patients.

S. No.	Percent inhibition at 20 µg/ml	
	Native IgG	ROS-IgG*
1	28.4	38.3
2	34.5	55.1
3	15.1	24.7
4	30.3	48.4
5	33.4	39.8
6	29.4	54.7
7	28.7	53.6
8	24.8	45.3
9	23.4	38.4
10	33.9	42.8
11	28.1	39.1
12	34.2	64.8
13	24.9	57.4
14	30.7	37.2
15	29.0	49.0
16	27.2	43.8
17	28.1	47.3
18	30.2	43.1
19	20.4	51.4
20	27.2	52.6
21	20.8	42.0
22	24.6	48.3
23	31.3	45.6
24	28.6	50.8
Mean ± SD	27.8 ± 4.6	46.3 ± 8.3

*p<0.001 vs. native IgG.

oxidation of IgG was further evident by the significant increase ($p < 0.001$) in carbonyl contents. Despite the power of modern molecular approaches and persisting investigative efforts, type 1 diabetes remains an enigmatic disorder or the agent (or agents) triggering this autoimmune response remains to be identified (Park, 2007; Kawasaki et al., 2004; Phillips et al., 2004; Nazirogilu and Butterworth, 2005; Telci et al., 2000). Patients with diabetes have a diverse array of autoantibodies (Rasheed et al., 2008; Rasheed and Ali, 2006; Rasheed et al., 2009; Taplin and Barker, 2008), but the cellular and molecular mechanisms that are responsible for the production of autoantibodies in this disease and the way in which these antibodies participate in tissue destruction remain highly controversial (Taplin and Barker, 2008). Oxidative stress plays a central role in the onset of diabetes as well as in diabetes associated complications (Phillips et al., 2004). Various studies have shown that diabetes is associated with increased formation of free radicals and decrease in antioxidant potential. This leads to oxidative damage of cell components such as proteins, lipids and nucleic acid in both type 1 and type 2 DM (Nazirogilu and Butterworth,

2005). The role of oxidative protein damage in the pathogenesis of the diabetic state is being investigated extensively (Telci et al., 2000). Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function, whereas structural changes in protein are considered to be among the molecular mechanisms leading to diabetes and its complications (Telci et al., 2000; Taplin and Barker, 2008).

We and others (Rasheed, 2008; Scofield et al., 2005; Kurien and Scofield, 2008; Rasheed et al., 2006, 2008; 2009a, b; Rasheed and Ali, 2006; Rasheed et al., 2007, 2009) reported the presence of elevated levels of oxidized protein products, termed advanced oxidation protein products such as oxidized albumin, oxidized hemoglobin and oxidized immunoglobulin G in patients with various diseases. It has been well documented that IgG is quite vulnerable to ROS (Chou, 2002; Uesugi et al., 2000; Kleinveld et al., 1991; Rasheed, 2008). ROS-modified IgG was observed to be a potent antigenic stimulus inducing high titre antibodies in experimental animals as compared with native IgG (Rasheed, 2008). It was thought worthwhile to investigate the binding

characteristics of naturally occurring type 1 diabetes autoantibodies to ROS-damaged IgG so that the possible involvement of ROS-modified IgG in type 1 diabetes could be ascertained. Sera of 36 type 1 diabetes patients and 22 normal human subjects were collected for the present study. Of these, 66.7% diabetes sera showed preferentially high binding to ROS-IgG as compared to its native analogue as evident by direct binding ELISA. No appreciable binding was observed with the normal subjects. Competition ELISA reiterated the direct binding results, that the ROS-modified IgG was an effective inhibitor, showing substantial difference in the recognition of modified HSA over native IgG ($p < 0.05$). Autoantibodies from type 1 DM patients may show greater avidity rather than affinity towards ROS-IgG, as ROS caused extensive damage on IgG which may result in the generation of several epitopes. Thus by employing direct binding and competitive inhibition ELISA, the data clearly demonstrates a substantial increase in the recognition of ROS-modified IgG over native IgG by circulating type 1 diabetes autoantibodies. IgG is a major class of serum glycoprotein having 82 - 96% protein and 4 - 18% carbohydrate and it functions as a major effector molecule of the humoral responses in human. In this context, we expected that the characterization of oxidative status of serum IgG would provide alterations in the conformation and function of IgG which may result in modification of its biological properties. The oxidation of a protein typically results in an increase in carbonyl contents [19]. This increase is due to the oxidation of lys, arg, pro or other amino acid residues. In short, protein carbonyl groups are the biomarker of oxidative stress (Levine et al., 1994). In human plasma, all amino acids in the protein are susceptible to oxidative modification by oxidants such as hydroxyl radicals and hypochlorous acid (Levine et al., 1994). Present data showed IgG carbonyl contents were significantly ($p < 0.05$) increased *in-vitro* upon ROS modifications, these results were further confirmed by using ROS quenchers mannitol and DMSO, which inhibit protein-bound carbonyls generation significantly ($p < 0.05$). The above mentioned results suggest that in diabetes patients with increased oxidative stress, the oxidative modification of plasma proteins has been greatly enhanced. Since the abundant protein of plasma is IgG, it is likely to be extensively damaged and might be responsible for the pathological conditions associated with diabetes. These results suggest that IgG is continuously exposed to oxidative stress, so much so that alterations in its biological properties could result in the conformational changes of IgG.

CONCLUSIONS

The present article is the first report that demonstrates the presence of ROS-induced IgG damage in diabetes patients, which might play an active part in the

progression of disease. The present study further propose that, in addition to IgG in serum concentration, the quality of IgG molecules may be not only a crucial factor affecting its protective effects, but also a risk factor as a pro-oxidant in type 1 diabetes patients. We conclude that IgG after modification with ROS presents unique epitopes for the production of type 1 diabetes autoantibodies.

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REFERENCES

- Ahmad R, Rasheed Z, Ahsan H (2009). Biochemical and cellular toxicology of peroxynitrite: implications in cell death and autoimmune phenomenon. *Immunopharm. Immunotoxicol.*, 31:388-396.
- Arutyunova EI, Danshina PV, Domnina LV (2003). Oxidation of glyceraldehydes-3-phosphate dehydrogenase enhances its binding to nucleic acids. *Biochem. Biophys. Res. Commun.*, 307: 547-552.
- Carrard G, Bulteau AL, Petropoulos I, Friguet B (2002). Impairment of proteasome structure and function in aging. *Int. J. Biochem. Cell Biol.*, 34: 1461-1474.
- Chou C (2002). Binding of rheumatoid and lupus synovial fluids and sera-derived human IgG rheumatoid factor to degalactosylated IgG. *Arch. Med. Res.*, 33: 541-544.
- Hensley K, Floyd RA (2002). Reactive oxygen species and protein oxidation in aging: a look back, a look ahead. *Arch. Biochem. Biophys.*, 397: 377-383.
- Kawasaki E, Abiru N, Eguchi K (2004). Prevention of type 1 diabetes: from the view point of β cell damage. *Diabetes Re. Clin. Pract.*, 66: 27-32.
- Kleinveld HA, Sluiter W, Boonman AM, Swaak AJ, Hack CE, Koster JF (1991). Differential stimulation by oxygen-free-radical-altered immunoglobulin G of the production of superoxide and hydrogen peroxide by human polymorphonuclear leucocytes. *Clin. Sci.*, 80: 385-391.
- Kurien BT, Scofield RH (2008). Autoimmunity and oxidatively modified autoantigens. *Autoimmun. Rev.*, 7: 567-573.
- Laemmli KU (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- Levine RL, Williams J, Stadtman ER (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.*, 233: 346-357.
- Lowry OH, Rosenberg NJ, Farr AL (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
- Naziroglu M, Butterworth P (2005). Protective effect of moderate exercise with dietary vitamin C and E on blood antioxidative defense mechanism in rats with streptozotocin-induced diabetes. *Can. J. Appl. Physiol.*, 30: 172-185.
- Oakley BR, Kirsh DR, Morris NR (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analyt. Biochem.*, 105: 361-363.
- Park Y (2007). Functional evaluation of the type 1 diabetes (T1D) susceptibility candidate genes. *Diabetes Res. Clin. Pract.*, 77: 110-115.
- Phillips M, Cataneo RN, Cheema T, Greenberg J (2004). Increased breath biomarkers of oxidative stress in diabetes mellitus. *Clin. Chim. Acta*, 344: 189-194.
- Rasheed Z (2008). Hydroxyl radical damaged Immunoglobulin G in patients with rheumatoid arthritis: Biochemical and immunological

- studies. *Clin. Biochem.*, 2: 1-13.
- Rasheed Z, Kumar L, Abbas S (2009). Advanced glycation end-products damaged IgG, a target for circulating autoantibodies in patients with type 1 diabetes mellitus. *Open Glycosci.*, 2: 1-8.
- Rasheed Z, Khan MWA, Ali R (2006). Hydroxyl radical modification of human serum albumin generated cross reactive antibodies. *Autoimmunity*, 39: 479-488.
- Rasheed Z, Ali R (2006). Reactive oxygen species damaged human serum albumin in patients with type 1 diabetes mellitus: Biochemical and immunological studies. *Life Sci.*, 79: 2320-2328.
- Rasheed Z, Ahmad R, Rasheed N (2007). Reactive oxygen species damaged human serum albumin in patients with hepatocellular carcinoma. *J. Exp. Clin. Cancer Res.*, 26: 515-524.
- Rasheed Z, Ahmad R, Rasheed N (2007). Enhanced recognition of reactive oxygen species damaged human serum albumin by circulating systemic lupus erythematosus autoantibodies. *Autoimmunity*, 40: 512-520.
- Rasheed Z, Ahmad R, Ali R (2009). Structure and immunological function of oxidized albumin in lung cancer: Its potential role as a biomarker of elevated oxidative stress. *Br. J. Biomed. Sci.*, 66: 76-73.
- Rasheed Z, Ahmad R, Rasheed N (2008). Reactive oxygen species damaged hemoglobin presents unique epitopes for type 1 diabetes autoantibodies. *Inter. J. Bio. Chem.*, 2: 1-13.
- Shacter E, Williams JA, Levine RL (1995). Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. *Free Rad. Biol. Med.*, 18: 815-821.
- Shacter E (2000). Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.*, 32: 307-326.
- Scofield RH, Kurien BT, Granick S (2005). Modification of lupus-associated 60-kDA Roprotein with the lipid oxidation product 4-hydroxy-2-nonenal increases antigenicity and facilitates epitopes spreading. *Free Radic. Biol. Med.*, 38: 719-728.
- Tabner BJ, Turnbull S, El-Agnaf O (2001). Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. *Curr. Top. Med. Chem.*, 1: 507-517.
- Taplin CE, Barker JM (2008). Autoantibodies in type 1 diabetes. *Autoimmunity*, 41: 11-18.
- Telci A, Cakatay U, Salman S, Satman I, Sivas A (2000). Oxidative protein damage in early stage type 1 diabetic patients. *Diabetes Res. Clin. Prac.*, 50: 213-223.
- Uesugi M, Yoshida K, Jasin HE (2000). Inflammatory properties of IgG modified by oxygen radicals and peroxynitrite. *J. Immunol.*, 165: 6532-6537.