Determination of oxidant stress in plasma of rheumatoid arthritis and primary osteoarthritis patients

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Determination of oxidant stress in plasma of rheumatoid arthritis (RA) and primary osteoarthritis (POA) patients is important in understanding the pathogenesis of these diseases. In this study, we examined the relationship between oxidant stress and inflammation by measuring protein carbonyl content, thiol levels and plasma protein fractions as the oxidation markers and erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) tests as inflammation markers. Protein carbonyls content was higher in RA and POA patients, as compared to controls ($p<0.0001$), while the plasma thiol levels in both groups of patients were significantly lower than controls ($p<0.0001$). Increased levels of proteins under 40 kDa molecular mass were detected in the RA and POA patients compared to that of controls ($p<0.0001$) both in HPLC and SDS-PAGE analysis. Total protein concentration in plasma of RA patients was higher than the controls ($p<0.0001$), while in POA patients was lower than that of controls ($p<0.0001$). ESR and CRP levels were higher in both the patient groups than the normal group ($p<0.0001$). These results suggested that alterations in the oxidant stress markers could be the cause of inflammation in these diseases. Thus, while working for RA/POA treatment strategies, consideration of the relationship between oxidant stress and inflammation would be worth evaluating.

**Keywords:** Oxidative stress, Inflammation, Rheumatoid arthritis, Primary osteoarthritis, Biochemical markers

Rheumatoid arthritis (RA) is a disabling autoimmune disease characterized by chronic inflammation of the joints\textsuperscript{1}. For the majority of cases, the synovitis leads to permanent damage of the articular cartilage and bone\textsuperscript{2}. RA affects about 1 in 50 people worldwide and is three-times more common in females than males. It is most common in young and middle-aged adults but can also affect children and the elderly. Osteoarthritis (OA) is a major cause of morbidity, disability and loss of function in the general population. Also, OA is a common, but serious problem whose symptoms include pain and joint swelling\textsuperscript{3,4}. OA results from the pathological imbalance of degradative and reparative processes\textsuperscript{5} and affects the entire joint structure. The cartilage, synovium and bone can be major sites for production of cytokines/mediators, which can be classically associated with inflammation and even-tually promote progressive joint destruction\textsuperscript{6,7}.

Reactive oxygen species (ROS) produced during the course of cellular oxidative phosphorylation, and by activated phagocytic cells during oxidative burst, if exceeds the physiological buffering capacity can result in oxidative stress. ROS play major role(s) in the generation of acute and chronic inflammatory diseases\textsuperscript{8}. In patients with inflammatory joint diseases, especially in RA, the role of ROS has been implicated in the pathogenesis of acute and chronic inflammatory synovitis\textsuperscript{9}. It is well known that proteins, the major macromolecular components of human blood plasma are among the main targets of oxidation\textsuperscript{10}. Inflammation is the body’s immediate response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals or physical injury\textsuperscript{11}. The excessive production of ROS can damage proteins, lipids, nucleic acids, and matrix components\textsuperscript{1,12}. Modification of plasma proteins by oxidation can alter protein structure and impair biological functions, leading to cell death\textsuperscript{13}.

Autoimmune diseases are characterized by an immune response against the body’s own tissues.
Plasma proteins, DNA and lipids of patients with autoimmune diseases may be more vulnerable to ROS than those from healthy individuals. This may result in more damage to DNA and higher susceptibility to killing by free radicals\textsuperscript{14}. Evidence of oxidative damage to cartilage, extracellular collagen, and intracellular DNA has also been demonstrated\textsuperscript{15}. Reactive oxygen and nitrogen species directly damage DNA and impair DNA repair mechanisms. This damage can occur in the form of DNA strand breakage or individual nucleotide base damage. DNA reaction products, in particular 8-oxo-7-hydrodeoxyguanosine formed by the reaction of hydroxyl radicals (OH) with deoxyguanosine are elevated in leukocytes and sera of patients with RA\textsuperscript{16,17}. Detection of oxidative stress and low antioxidant concentrations in the plasma of RA and POA patients suggest the involvement of free radicals in inflammatory processes\textsuperscript{18}. Assessment of such biochemical markers as protein carbonyl content is considered as one of the best possible ways to monitor inflammation\textsuperscript{19}. Protein thiol groups may scavenge oxidants, thus sparing antioxidants and/or cellular constituents from attacks. Their measurement in serum provides an indirect reflection of the anti-oxidative defence\textsuperscript{20}. However, studies examining lipid peroxidation and intracellular/extracellular antioxidant potential in the plasma of RA patients compared with the healthy subjects have not always produced uniform results\textsuperscript{20-22}.

In this study, we have examined the relationship between oxidant stress and inflammation by measuring protein carbonyl content, thiol levels and plasma protein fractions as the oxidation markers and erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) tests as inflammation markers.

**Materials and Methods**

**Reagents**

Bovine serum albumin (BSA), trichloro-acetic acid (TCA), phosphate buffer, di-nitro phenyl hydrazene (DNPH), EDTA, 5,5’-dithiobis 2-nitro-benzoic acid (DTNB), and other general chemicals/reagents were purchased from Sigma Chemical Co., Inc. (St. Louis, MO, USA). Ethanol and ethyl acetate were obtained from Merck (Darmstadt, Germany).

**Study subjects and specimens**

Twenty RA patients with mean age of 48 ± 8 yrs and a disease duration of 11 ± 7 yrs and 20 POA patients with mean age of 52 ± 4 yrs and a disease duration of 9 ± 6 yrs were included in the study. The mean age of healthy control subjects (n = 20) was 25 ± 5 yrs. Written informed consents, as approved by the Ethics Committee of Istanbul University Medical Faculty, Istanbul were obtained for individual cases. None of the controls had any symptoms of inflammatory diseases.

The RA was defined based on the American College of Rheumatology (ACR) diagnostic criteria for clinical remission of RA\textsuperscript{23}. Patients with POA of the knee fulfilled the ACR criteria for knee OA\textsuperscript{4}. The POA patients were in pre-operative period of knee arthroplasty. All patients had swollen inflamed knee joint. Uncontrolled medical conditions such as missing smoking data, diabetes, cancer, using antioxidants and having cardiovascular diseases, breast feeding and pregnant women were excluded from the study. The patients were under treatment with disease modifying anti-rheumatic drugs including sulfasalazine, methotrexate and non-steroidal anti-inflammatory drugs (NSAID).

Blood was collected in 0.12 M trisodium citrate (9:1 ratio, v/v) through venapuncture from all subjects. Normal control bloods were collected from participants who reported to have no history of RA and POA ever. To obtain plasma, the blood samples were centrifuged at 3,000 rpm for 10 min at 4°C and supernatants were aliquoted for storage at -80°C until further use.

**Assay procedures**

**Determination of carbonyl levels**

Protein carbonyls were estimated using the method of Levine et al\textsuperscript{24} with slight modifications. Briefly, 0.5 mL plasma (1 mg/mL) were treated with 0.5 mL TCA (20% solution) at room temperature for 10 min, and centrifuged at 3,000 rpm for 10 min. The pellet was treated with 0.5 mL of 10 mM DNPH in 2 M HCl, or with 0.5 mL of 2 M HCl alone for the blank. Samples were incubated for 30 min at room temperature in the dark, and then treated with 20% TCA, and centrifuged at 3,000 rpm for 10 min. The pellet was washed three times in ethanol/ethyl acetate (v/v); and 1.5 mL of 1 M NaOH was added to the pellet followed by an incubation at 37°C for 15 min. Carbonyl concentrations were determined utilizing molar absorption coefficient of $\varepsilon_{370} = 22,000 \text{ M}^{-1}\text{cm}^{-1}$ using a Shimadzu UV-spectrophotometer and expressed as nanomoles of carbonyls per mg protein.

**Thiol determination**

Thiol determination was based on the thiol/disulfide reaction of thiol and Ellman’s reagent,
DTNB$^{25}$. Briefly, 50 µL of the sample mixed with 1 mL 0.1 M Tris, 10 mM EDTA, pH 8.2 constituting the blank reaction, was assessed at 412 nm. The addition of 40 µL 10 mM DTNB in methanol triggered the reaction and absorption at 412 nm was measured after stable colour formation (within 1-3 min). The concentrations of thiol groups were calculated using a molar extinction coefficient of 13,600 M$^{-1}$cm$^{-1}$ using a Shimadzu UV-spectrophotometer and expressed as µmol/L.

HPLC analysis

Plasma low molecular mass protein fractions were detected on HPLC after 50-fold dilution of specimens on an Agilent 1,100 Series HPLC system equipped with G1315A model of diode array detector. Protein-Pack-125 column was equilibrated with 0.1 M phosphate buffer (NaH$_2$PO$_4$/Na$_2$HPO$_4$), pH 7.4. Samples were eluted at a flow rate of 1 mL/min and detected by their absorbance at 280 nm, and the peak areas were recorded. Molecular mass (in kDa) of protein standards were: myosin = 205; β-galactosidase = 116; phosphorylase b = 97; albumin bovine = 66; albumin egg = 45; and carbonic anhydrase = 29. All samples were assayed at least in duplicate.

SDS-PAGE analysis

Each sample was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% gel crosslinking as described by Laemmli$^{26}$. The protein bands were visualized with the use of Coomassie brilliant blue. In order to determine the plasma proteins (such as albumin, immunoglobulins), densitometric assay was performed (Helena Junior 24, Model 700). Bands of plasma proteins were scanned and analyzed for their band surface (cm$^2$) using planimetry. The data were expressed as the percent protein area.

Assay of protein, CRP and ESR levels

Total protein content in plasma was measured by Lowry et al$^{27}$ using BSA as the standard. Plasma C-reactive protein (CRP) level in each specimen (in mg/L) was quantified by a nephelometric assay$^{28}$. Erythrocyte sedimentation rate (ESR) was determined as described elsewhere$^{29}$.

Statistical analyses

All categorical measurements were reported as numbers with percentages. The continuous variables were expressed as mean ± standard deviation (SD). Comparisons between the controls vs. RA and POA patients values were performed by ANOVA (post-hoc Tukey’s test). Statistical significance was expressed when the achieved $p$ values were <0.05.

Results

In this study, we determined the levels of protein oxidation in plasma from the RA and POA patients and the results were compared with normal subjects’ plasma. Plasma protein carbonyl content, thiol levels, total protein levels, HPLC analyses, protein peak numbers and peak areas were determined. The levels of plasma protein carbonyls were significantly higher in both RA and POA patients than their controls, while thiols were significantly lower in RA and POA patients than the controls (Table 1). Overall, the concentration of thiols was relatively lower in the POA patients group, as compared to the RA group, and the inter-group analysis revealed statistical significance. Thus, our data demonstrated a higher level of oxidative stress in patients with RA and POA, as evidenced by increased protein-bound carbonyl concentrations in conjunction with the decreased serum protein thiol levels.

On the other hand, total protein level was higher in the RA patients, but was lower in POA patients than their controls (Table 1). The CRP and ESR levels were also significantly higher in the samples from patients with RA and POA, as compared to their controls (Table 1). Protein carbonyl content negatively correlated with thiol levels in both the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>RA</th>
<th>POA</th>
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<tbody>
<tr>
<td>Carboxyl (nm/mg of Protein)</td>
<td>0.68 ± 0.12</td>
<td>1.66 ± 0.10$^{*}$</td>
<td>2.54 ± 0.63$^{*}$</td>
</tr>
<tr>
<td>Thiol (µmol/L)</td>
<td>355.74 ± 46.31</td>
<td>246.96 ± 32.27$^{*}$</td>
<td>136.71 ± 61.12$^{*}$</td>
</tr>
<tr>
<td>Protein (mg/mL)</td>
<td>6.85 ± 0.06</td>
<td>7.37 ± 0.25$^{*}$</td>
<td>6.50 ± 0.27$^{*}$</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.95 ± 0.76</td>
<td>6.35 ± 1.09$^{*}$</td>
<td>7.30 ± 1.45$^{*}$</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>9.46 ± 2.66</td>
<td>14.60 ± 2.11$^{*}$</td>
<td>15.45 ± 2.72$^{*}$</td>
</tr>
</tbody>
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Abbreviations: RA = rheumatoid arthritis; POA = primary osteoarthritis; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

Statistical significance: $^*$ = $p<0.001$, when compared with control values; $^\dagger$ = $p<0.0001$, the inter-group comparison for protein content and carbonyl content; $^\ddagger$ = $p<0.01$, the inter-group comparison for CRP and ESR values.
controls and the patient groups (both RA and POA patients) and were associated with the CRP and ESR changes in RA and POA patients. Also, thiol levels were inversely correlated with the disease activity for the RA and POA patients. Earlier, elevated protein oxidation in joints with inflammation and impaired antioxidant system has been shown in patients with RA\textsuperscript{1,23}.

The results obtained from the HPLC analyses of plasma proteins are summarized in Table 2 and the chromatographic elution profiles of representative control and RA/POA patients are own in Fig. 1. Protein peak areas were unchanged in the high molecular mass plasma proteins in the RA patients as compared to the controls, but protein peak areas at retention time (Rt): 11 min (under 40 kDa) in this group were significantly higher than the controls (Figs 1A and B). Also, in the POA group of patients, proteins peak areas (under 40 kDa) were found to be significantly increased (Table 2). Furthermore, the results revealed that RA patients had almost two-fold increase in the protein peak areas as compared to the POA group. It was, however, unclear as to why the RA patients were more vulnerable to the induction of oxidative insult than the POA patients. Presumably, this might be due to a higher level of inflammation in this disease, which generates oxygen radicals as by-products.

In order to determine as to which serum protein fractions contained carbonyl and expressed albumin (66 kDa), light-chain (20 kDa) and heavy-chain (45 kDa) immunoglobulins (Igs), densitometric assay was performed after the SDS-PAGE analysis of the RA and POA samples, compared with the control samples, suggesting that slightly higher concentration of albumin was present in POA plasma, as compared with RA (5%, \(p<0.05\)) (Figs 2A and B). Albumin levels, however, were higher in the RA and POA samples as compared to the controls (35%, \(p<0.001\)). Interestingly,
Igs levels with the low molecular mass (29 kDa) were significantly higher in RA and POA samples than the controls (400%, $p<0.0001$), but of 45 kDa were the same in patients and control plasmas.

Furthermore, our results as shown by the HPLC analyses revealed that the RA patients have almost two-fold increase in the plasma protein peak areas as compared to the POA plasma groups. It is, however, unclear as to why the RA patients are more vulnerable to the induction of oxidative insult than the POA patients. Presumably, a higher level of inflammation in this disease, which generates oxygen radicals as by-products may provide possible explanations.

**Discussion**

Previous investigations suggest that the reduction of the damage caused by oxidative stress participates in several processes ranging from simple electron donation (GSH) and anti-oxidant catalysis in regulation as part of complex biochemical signaling and antioxidant response. In the present study, significantly lower serum thiol concentrations were observed in RA and especially in POA patients than in controls, providing evidence for antioxidant protection in these diseases. We suggest that this patient population is at considerable risk of tissue oxidant injury because of the impaired plasma antioxidant defences. We also observed an important negative correlation between the disease activity and thiol concentrations. This suggests an inter-dependent relationship between the disease activity and the intensity of free radical reactions. We provide evidence that such plasma proteins, particularly albumin and Igs are oxidatively modified by an increased protein peak areas. Hence, our results confirm the role of free radical reactions in the pathogenesis of RA and POA, acting as messengers that formation of oxidized plasma proteins are associated with local inflammatory responses.

These findings support the hypothesis that proteins may be oxidized during acute inflammation, leading to decreased functional activity. *In vitro* studies have shown that the action of ROS on proteins results in the formation of carbonyl groups. The structure and activity of oxidized proteins change profoundly when compared with their native forms. Therefore, evaluation of the carbonyl group content in plasma proteins is an acknowledged marker for the intensity of free radical reactivity. Evidence for increased production of reactive oxygen species in RA patients includes elevated levels of lipid peroxidation products, degradation of hyaluronic acid by free radicals, decreased levels of ascorbic acid in serum and synovial fluids, and increased breath pentane excretion. Moreover, the levels of thioredoxin and sialic acid, which are markers of oxidative stress are significantly higher in synovial fluids from RA patients compared to other forms of arthritis.

Previous studies have also demonstrated elevated levels of protein oxidation products in a number of pathologic conditions including atherosclerosis, diabetes mellitus, neurodegenerative syndromes, and systemic sclerosis. In the present study, we examined the protein carbonyl and thiol contents for oxidative stress intensity in patients with RA and POA. Earlier studies from our laboratory have shown that oxidized fibrinogen binds less effectively to platelet glycoprotein (GP) IIb/IIIa receptors and to whole platelets than native fibrinogen, and oxidized plasma proteins upon treatment with chloramin-T as the oxidized agent had the highest levels of carbonyl content.
Earlier studies have shown that the oxidation of proteins can modulate some of the biochemical properties such as enzyme activities, modification of transcription factors, and susceptibility to proteolytic degradation. Since cardiovascular disease is one of the principal causes of both morbidity and mortality in patients with RA, it is fair to implicate that inflammation and subsequent cardiovascular risk(s) may be linked as a result of the oxidative stress.

In conclusion, both RA and POA are inflammatory diseases and plasma proteins of these patients are susceptible to oxidative stress. Hence, the increase in oxidative stress could be accepted as the possible reason affecting the intensity of these diseases. Therefore, the positive correlative determination between the increased oxidative stress and inflammation intensity could contribute to the understanding of the pathogenesis involved in RA and POA disease processes.

References