Plasma Lipid Peroxidation Levels and Erythrocyte Superoxide Dismutase Activity in Patients with RA: The Relationship with Disease Activity Scores.

Dixit A¹*, Dhasmana DC, Kalra V², and Gupta P³.

¹Department of Pharmacology, U P Rural Institute of Medical Sciences and Research, Saifai, Etawah, India. ²Departments of Biochemistry, Himalayan Institute of Medical Sciences, Dehradun, India. ³Departments of Orthpaedics, Himalayan Institute of Medical Sciences, Dehradun, India.

ABSTRACT

To investigate the levels of lipid peroxidation and erythrocyte antioxidant status in patients with rheumatoid arthritis and to assess the relationship between disease activity scores and parameters of oxidative stress. The study included 30 rheumatoid arthritis (RA) patients and 10 healthy controls. Clinical and disease activity parameters were assessed in the patients. Erythrocyte levels of superoxide dismutase (SOD) and plasma levels of malondialdehyde (MDA) were investigated in both groups. In the present study, significantly increased lipid peroxidation, measured as plasma MDA, was demonstrated in RA patients as compared to controls (P<0.001). The activity of erythrocyte Sod was significantly reduced in RA patients (P<0.001). A negative correlation was observed between these two parameters in patient group. Meanwhile, positive correlations were observed between MDA and patient’s assessment of pain, patient’s global assessment of disease activity, and ESR. These results suggest presence of oxidative stress in RA patients as evidenced by enhanced lipid peroxidation and reduced antioxidant defence mechanism. The findings confirm the role of oxygen free radicals in pathogenesis of RA and that LPO markers can serve as potential markers of disease activity.

Keywords: rheumatoid arthritis, oxidative stress, malondialdehyde, superoxide dismutase

*Corresponding author
INTRODUCTION

Rheumatoid arthritis (RA) is a progressive, autoimmune, systemic disorder with a spectrum of clinical severity ranging from mild arthritis to crippling joint disorder. It is characterised by symmetric, chronic hypertrophic synovitis, leading to destruction of connective tissue and functional damage of cartilage and bone structures.[1] Although, there is persistent inflammation in joints, the pathophysiological basis of RA is not fully understood and it is suggested that there is elevated generation of free radicals in inflamed joints and impaired antioxidant systems in RA. [2]

Oxidative stress is an intense shift in the balance between prooxidants and antioxidants, created as a result of excess production of prooxidants or deficient antioxidants. Molecular oxygen, essential for the survival of all organisms is required for generation of aerobic energy by oxidative phosphorylation, a process occurring in mitochondria. Reactive oxygen species (ROS), are highly reactive oxygen metabolites produced as a by-product of this process which includes superoxide, hydrogen peroxide, and hydroxyl radicals, and their reactive products. These cytotoxic reactive oxygen species (ROS) are capable of causing damage to cell structures, including lipids and membranes, proteins, and DNA.[3] Among ROS, the superoxide anion (O$_2^-$) plays a pivotal role in inflammation, particularly in patients with inflammatory joint disease. [4] In recent years, increasing attention has drawn to the role of ROS in pathogenesis of RA.[5,6]

Multiple defence system, which includes intracellular enzymes (SOD, GPX, Catalase, GST, GSH) have evolved to protect cellular system from oxidative damage. Superoxide dismutase (SOD), enhances the otherwise slow spontaneous breakdown of superoxide anion (O$_2^-$) into hydrogen peroxide, thereby preventing the downstream formation of highly aggressive peroxynitrite and hydroxyl radical. Several studies suggest impaired enzymatic antioxidant system in RA and have reported different activities of SOD in patients of RA.[7,8] Lipid peroxidation (LPO) mediated by free radicals is considered to be major mechanism of cell membrane destruction and cell damage. Malondialdehyde (MDA), an end product of LPO is widely used as an indicator of oxidative stress. It is supposed that RA patients are more prone to lipid peroxidation as a result of reduced antioxidant systems. Increased level of MDA have been reported in serum and synovial fluid of RA patients. [9]

The purpose of the present study was to compare the status of antioxidant and lipid peroxidation in RA patients with that of healthy controls and evaluate the correlation between the parameters of oxidative stress and disease activity indices.

MATERIALS AND METHODS

Patients and Control

Patients for the study were selected from individuals attending the routine outpatient orthopaedic clinic at our institute. Thirty RA patients of either sex of ≥ 18 years of age, diagnosed according to American Rheumatism Association criteria and in functional class I, II, III by the criteria of American college of Rheumatology, who had not undergone any previous treatment for their arthritis, were included in the study.[10,11] The subjects were ranging in age 25-60 years. Ten age and sex matched healthy volunteers were also selected for the study simultaneously. Approval of institutional ethic committee and informed written consent of patients and healthy volunteers were obtained prior to their inclusion in the study. Individuals with concomitant rheumatic condition, diabetes, inflammatory bowel disease, hypertension, malignant disease, diseases of any origin which could cause oxidative stress or those receiving any disease modifying antirheumatic drug or oral corticosteroid given in last 6 months were excluded from the study. None of the participant was alcoholic, chronic smoker and using any vitamin supplementation or antioxidant drugs was also ensured. The complete clinical and personal history of the subjects was recorded. Complete count of tender joints (68 joints), swollen joints (66 joints), patients assessment of pain, patients global assessment of disease activity, and patients assessment of physical function using Modified Stanford Health Assessment Questionnaire was performed at baseline. [11] The assessment of pain and global assessment of disease activity was recorded on a visual analog scale (VAS; 0-100mm).
Estimation of SOD activity

SOD activity was determined in the hemolysate based on the inhibition of auto-oxidation of epinephrine to adrenochrome at pH 10.2.[12] Spontaneous oxidation of epinephrine leads to the generation of colored products. Superoxide anion is responsible for oxidation of epinephrine to adrenochrome. Superoxide dismutase acts as a potent inhibitor of epinephrine at elevated pH. The assay of SOD in terms of its ability to inhibit epinephrine autooxidation under specified condition was used. The auto-oxidation of epinephrine was followed in terms of production of adrenochrome which exhibits an absorption maximum at 480 nm.

RBC pack was collected in EDTA vial. The erythrocytes were washed four times with 3 ml of 0.9% NaCl solution and centrifuged for 10 minutes at 3000 rpm after each wash. The packs were stored at -80°C till estimation. Immediately before analysis RBC pack was mixed with 2 ml distilled water, mixed and left to stand at +4°C for 15 min. to prepare the hemolysate. This was centrifuged and clear supernatant was used for analysis. The reaction mixture contained of 0.3mM epinephrine, 0.1mM EDTA, in 0.5M Sodium bicarbonate buffer at pH 10.2 as substrate and a suitable aliquot of hemolysate. The reaction was started by adding substrate to assay system and increase in optical activity was observed for 4 minutes at 30°C on colorimeter. The amount of enzyme required to inhibit the rate of autooxidation of epinephrine by 50% was considered as 1 unit. The enzyme activity was expressed in units/mg Hb. The haemoglobin concentration was determined with the aid of Drabkin’s reagent, absorbance read at 540nm.

Estimation of malondialdehyde

For the quantitative evaluation of lipid peroxides, their transformation into a colored compound under the effect of thiobarbituric acid (TBA) was used.[13] The final product of fatty acids peroxidation reacts with TBA with the formation of a colored complex which was determined. The estimation of peroxidation was carried out by colorimetric determination of Thiobarbituric acid reactive substances (TBARS) at 535nm. Serum samples were collected in gel barrier vacutainer and stored at -20°C till estimation. A mixture of 250µL serum, 250µL TBA solution and 500µL orthophosphoric acid solution was taken in screwcapped vials and heated in boiling water bath for 30 min. The vials were cooled and chromogen extracted in 2ml butanol by vigorous mixing followed by centrifugation at 2000 rpm for 5 min. The absorbance of the butanol layer was read at 535nm. A similar method was used for standard and blank.

Hydrochloric acid, sodium bicarbonate, sodium carbonate, EDTA, orthophosphoric acid, n-butanol and trichloroacetic acid used were of analytical grade. L-epinephrine bitartarate, thiobarbituric acid and malondialdehyde standard prepared from 1,1,3,3 tetra ethoxy propane were obtained from Sigma Aldrich (St. Louis, MO, USA).

Statistical Analysis

The results were statistically analysed by Microsoft Excel statistic for Window program. To test the difference between controls and patients student t test was used for continous variable, and chi square for proportions. The results are expressed as mean±S.E and a two tailed P value <0.05 was considered as statistically significant.

RESULTS

There was no significant difference according to age between RA patients and healthy control subjects (P>0.05). The demographic data and laboratory findings of patients and control are shown in table1. The levels of C reactive protein, ESR, and total WBC count were significantly increased in rheumatoid arthritis patients. However, the mean level of haemoglobin in patients of RA was significantly decreased as compared to control subjects. The comparison of the oxidative stress parameters between RA patients and healthy controls as shown in table 1 demonstrates a significantly increased plasma levels of MDA in RA patients as compared to control subjects (P<0.001). The activity of SOD in erythrocytes was significantly decreased as compared to RA patients (P<0.001). Figure 1 depicts the clinical characteristics of RA patients. The mean swollen and tender joint count was 21.9 and 32.6 respectively with the morning stiffness of mean value 60.1±28.7 min. The mean value of patient’s assessment of pain on VAS (0-100mm) was 64.1mm.
Table1: Demographic and biochemical parameters in RA patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Control Subjects</th>
<th>RA patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>10</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Age (Mean ± SE)</td>
<td>40.7±3.2</td>
<td>46.67±2.18</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex (Female : Male)</td>
<td>7:3</td>
<td>23:7</td>
<td></td>
</tr>
<tr>
<td>Functional class in patients of rheumatoid arthritis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid Factor positive</td>
<td>Negative</td>
<td>22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C - reactive protein (mg/dl)</td>
<td>8.21±3.09</td>
<td>33.06±14.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/hr)</td>
<td>9.80±0.48</td>
<td>67.63±5.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.11±0.21</td>
<td>10.59±0.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total leukocyte count</td>
<td>5414±225</td>
<td>8301±398</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Erythrocyte SOD (U/mgHb)</td>
<td>7.02±0.58</td>
<td>3.43±0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma MDA (μ mol/L)</td>
<td>3.01±0.25</td>
<td>6.14±0.47</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

All the values are expressed as mean ±SE. SOD: Superoxide dismutase, MDA: Malondialdehyde

Figure 1: Clinical characteristics of RA patients. (VAS=Visual Analog Scale)

Figure 2: Correlation between plasma MDA and ESR in RA patients
Pearson correlation showed positive correlations between plasma MDA levels with ESR, patients assessment of pain on VAS scale and patients global assessment of disease activity as shown in figure 2 and 3 (r=0.35, P<0.05; r=0.4, P<0.05; and r=0.03, P<0.05 respectively). But, no particular correlation of MDA with swollen, tender joint count and physician global assessment was observed. Further, there was a negative correlation between erythrocyte SOD activity and plasma MDA levels in RA patients, shown in figure 4 (r=−0.37, P<0.05). However, there were no significant correlation between clinical parameters of disease activity and SOD enzyme activity.

**Figure 3: Correlation between plasma MDA and patient’s assessment of pain on VAS scale and patient’s global assessment of disease activity in RA patients**

![Figure 3](image)

**Figure 4: Correlation between plasma MDA and erythrocyte SOD in RA patients**

![Figure 4](image)

**DISCUSSION**

Under normal homeostatic conditions, production of endogenous ROS is balanced by the actions of cellular antioxidant defence systems to avoid oxidative stress. An imbalance between production of free radicals/reactive oxygen species and antioxidant defences leads to oxidative stress. Oxidative stress constitutes a serious pathophysiological factor for a wide variety of connective tissue disorders. Various lines of evidence suggest a role of oxidative stress in pathogenesis of RA.[14] The result of present study showed that oxidative stress in RA patients is higher than in healthy control subjects. Several studies have demonstrated increased oxidative enzyme activity along with decreased, increased or unchanged antioxidants level in RA sera[15] and evidences of lipid peroxidation; a “foot print” of ROS existence.[16].
In the present study the serum MDA was found to contain significantly high levels in RA patients than in control subjects. The pathogenesis of RA, a chronic relapsing immuno-inflammatory multisystem disease is multifactorial and recent research implicates oxygen free radicals as a mediator of tissue damage.[17] Additionally, phagocytic cells such as macrophages and neutrophils on activation undergo oxidative bursts producing highly toxic ROS which form an integral part of organisms’ defence against invading organisms.[18] It is known that activated inflammatory cells lead to production of reactive oxygen species in RA.[19] Increased ROS in turn cause peroxidation of polyunsaturated fatty acids, proteins and cause tissue damage. MDA, one of the final products of lipid peroxidation is significantly increased in RA patients in the present study. Our results are in agreement with other studies where increased lipid peroxidation in serum, plasma, and erythrocytes has been reported.[16,17,20-22] The increased ROS levels in RA create a prooxidant environment, as a result of impaired antioxidant defence system, leading to increased lipid peroxidation and MDA levels.[23] On the contrary, studies have also reported no change of MDA level in RA patients.[24,25] As reported by earlier studies and the present study, the increased level of MDA serve as indirect indicator of increased ROS formation as a consequence of depleted oxidative defence system. Further, it is presumed that lipid peroxidation caused as a result of increased generation of ROS may have a role in pathogenesis of RA.

Cells and tissues are protected from ROS induced damage by means of a variety of endogenous ROS scavenging proteins, enzymes, and chemical compounds. If these ROS are not scavenged by antioxidant mechanisms, these species being highly reactive may lead to widespread lipid, protein, and DNA damage in cells and tissues of patients with RA.[8] These systems consist of non-enzymatic antioxidants (Vit. A and E, beta-carotene, uric acid) and enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. Superoxide radical is the first product of molecular oxygen reduction, and SOD is the first step of defence in the intracellular antioxidant defence system. SOD, a catalyst for dismutation of superoxide radical into H2O2, protects cells and tissues from superoxide radicals and other peroxides.[26] In the present study, RA patients demonstrated significantly low erythrocyte SOD activity than in the control subjects. Our findings agree with Kartas et al [5] and Kızıltaş et al [27] who stated that antioxidant enzyme activity was found to be significantly lower in patients of RA as compared to controls. The decreased activity of SOD in RA patients is assumed to be as a result of degradation of these antioxidant enzymes by free radicals during the detoxifying process.[20] It is also possible that the lowered levels of SOD activity may be due to the inhibition of the enzyme by the hydrogen peroxide, which indicates high degree of superoxide anion production. [29] This suggests that SOD plays an important role in rheumatic process and in mitigating the oxidative stress. Nonetheless, there are confusing reports on erythrocytes activities in patients with RA, where some studies have reported that SOD level in erythrocytes of patients are not different from controls [6,8,20,28] and other reported increased activity of SOD in RA patients.[23] Chronic joint disease may deplete antioxidant defences whereas acute inflammation can upgrade them and this could have had an influence on the results of the present study. Another possibility is the drug naive status of the RA patients in present study, as it has been shown that low SOD levels in RA patients increased after administration of NSAIDs and reached normal levels.[30]

Phagocytic cells such as macrophages and neutrophils on activation undergo an oxidative burst that produces highly toxic ROS, designed to kill invading pathogens.[18] They also release other inflammatory mediators such as cytokines, CRP and RA factor into the cytoplasm to destroy phagocytosed material. Plasma and erythrocytes play an important role in regulating the oxidant reactions in surrounding medium, thereby preventing free radical mediated cytotoxicity. Our findings, suggested that the levels of erythrocyte SOD decreased in RA patients are consistent with previous studies. Circulating RBCs possess the ability to scavenge the ROS generated extracellularly by neutrophils and RBCs deficient in antioxidants are easily destroyed. The significantly decreased value of haemoglobin in RA patients observed in present study is supported by previous studies which reported that increased ROS production is indicative of RBC destruction in RA patients.[31] Inflammatory mediators such as CRP, RA factor, and ESR are sensitive inflammatory markers, reflecting the presence and activity of disease in RA. There was a statistically significant increase in CRP and ESR in patients with RA as compared to the control group, consistent with the findings of previous studies. [6]

The present study demonstrated significant correlation between clinical and laboratory parameters of activity in RA. MDA exhibited a direct correlation with patient’s assessment of pain and patient’s global assessment. This correlation between end products of lipid peroxidation and clinical parameters of disease activity makes it possible to use them as surrogate markers of disease activity. This also provides us clues about disease activity in RA patients. Additionally, a significant correlation was also observed between ESR, a...
serological marker of inflammation and MDA levels in RA patients of the present study. Our findings agree with that of Sarban et al [6] and support that MDA levels in RA patients may prove useful in predicting disease activity. Erythrocytes may be important in regulating oxidant reactions in the surrounding medium, thereby preventing free radical mediated cytotoxicity.[32] A negative correlation between serum MDA level and erythrocyte SOD activity was observed in the study, which makes clear that reduced level of antioxidant enzymes tilts the balance in favour of lipid peroxidation leading to tissue damage. However, the relationship of disease activity of RA with erythrocyte was not clear. There was a lack of correlation between SOD enzyme and clinical parameters of RA, possibly due to small population of study group which leads to lower statistical power. Therefore, studies including larger sample should be done in future.

CONCLUSION

In conclusion, in view of present findings, it is possible to conclude that there is an increased oxidative stress in the patients with RA as evidenced by enhanced lipid peroxidation with concomitant reduction in erythrocyte antioxidant defence mechanism. This compensatory change in the level of antioxidants may provide additional protection against LPO. The increased MDA levels, and its relationship with inflammatory marker ESR, have an importance as a potential marker for disease activity in RA. The increased oxidative stress in patients with RA probably depends on the inflammatory character of the disease. Thus, our results further strengthen the role of oxygen free radical in the pathogenesis of RA, and antioxidant treatment directed against ROS may be beneficial in the management of RA.

REFERENCES