The role of oxidative stress in inflammation in patients with juvenile rheumatoid arthritis

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Abstract

Objective: to determine the level of blood markers of cellular oxidative stress and of the antioxidant enzymatic system, establishing the oxidative profile in patients with juvenile rheumatoid arthritis.

Methods: a case-control study including 64 patients (46 female) with juvenile rheumatoid arthritis whose follow-up was carried out at the outpatient clinic of the Pediatric Rheumatology Service at Vall d’Hebron Hospital, Barcelona, Spain. Patients were divided into three subgroups based on the onset of symptoms within the first 6 months of the disease: polyarticular subgroup, oligoarticular subgroup, and systemic subgroup. The control group included 60 outpatients (38 female) being submitted to follow-up at the same hospital for noninflammatory diseases. We determined the plasmatic levels of malondialdehyde, lipoperoxide, hydroperoxide, carbonyl groups of proteins and of glutathione; we also determined the enzymatic activity of superoxide dismutase, glutathione peroxidase, and glutathione reductase.

Results: in comparison to controls, patients with juvenile rheumatoid arthritis presented high concentrations of lipid peroxidation products (determined by plasmatic levels of malondialdehyde, lipoperoxide, and hydroperoxide); oxidative damage of protein circulating in blood (determined by carbonyl content of plasmatic proteins); increase in the enzymatic activity of superoxide dismutase and glutathione peroxidase; and reduction in the activity of glutathione peroxidase and in the levels of glutathione peroxidase.

Conclusion: our results indicate the presence of molecular damage determined by oxygen free radicals in patients with juvenile rheumatoid arthritis. Activity of superoxide dismutase and alterations in the glutathione-redox enzymatic cycle confirm a decrease in the defense capacity of the cellular system against toxicity induced by oxidative stress in these patients.


Introduction

The formation of free radicals, which is secondary to the production of reactive oxygen species, is part of the physiological process of aerobic metabolism. In this manner, cellular metabolism produces free radicals in physiological conditions. These active radicals, in turn, can be very useful...
in acting, for example, as a defense mechanism controlled by molecular stimuli or signals against damage caused by microorganisms. Free radicals may also be generated by different cells in the organism when induced by exogenous sources (ionizing radiation, toxins, drugs, chemical products, environmental polluting agents, etc.), and by different pathological situations. In these cases, there may be different types of affected cell components.

Under normal circumstances, live organisms have antioxidant defense systems consisting of nonenzymatic antioxidants - including nonprotein antioxidants with low molecular weight (vitamins A and E, beta-carotene, uric acid, and so on) - and of enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-Red), which are capable of metabolizing oxygen free radicals. SOD functions as a catalyst in reactions that convert superoxide anions (O₂⁻) into hydrogen peroxide (H₂O₂), which is eliminated by catalase and glutathione peroxidase enzymes; moreover, the latter enzyme also inhibits other long-chain peroxides.

Oxidative damage to essential cell components caused by oxygen free radicals is generally considered a serious mechanism in the pathogenesis of many infirmities, such as cardiovascular diseases, atherosclerosis, diabetes mellitus, and inflammatory rheumatism. Increase in the levels of various biological indicators related to oxidative damage to cells, such as rupture of membranes by lipid peroxidation, rupture of DNA chains, and alteration in the structure or function of proteins, have been demonstrated in these situations. These modifications may be directly related to the development of inflammatory processes.

In juvenile rheumatoid arthritis (JRA), oxygen free radicals may be related to, for example, the pathogenesis of synovitis. Cells found in inflamed joints (macrophages, neutrophils, lymphocytes, and endothelial cells), once isolated and stimulated, are capable of producing oxygen free radicals. These radicals, in the presence of lipid, DNA, protein, carbohydrate, or proteoglycan molecules, can cause oxidative injury. In this sense, it is also well-known that there is a relation between oxygen free radicals and damage of articular cartilage. Hydroxyl radicals may affect the cartilage, hypochlorous acid may affect proteoglycans, and hydrogen peroxide (H₂O₂) may inhibit their synthesis. Intra-articular administration of H₂O₂ in female rats has induced the production of glucose oxidase enzyme, which may render severe articular lesions and necrosis of chondrocytes. The combination of increase in degradation with inhibition of synthesis favors the destruction of cartilage.

Oxygen free radicals are lipid peroxidation-inducing agents that cause the depletion of unsaturated fatty acids of the cell membrane, thus inducing loss of cell integrity and functional alteration of cell receptors and enzymes. Alteration in the composition of fatty acids of the cell membrane has been reported in different rheumatic diseases, such as Sjögren’s syndrome, JRA, and in rheumatoid arthritis. Peroxide and hydroxyl radicals induce peroxidation of polyunsaturated fatty acids of cell membranes that, for example, determine increase in cell rigidity and deformability in the erythrocyte, consequently increasing the susceptibility to hemolysis.

The objective of this study was to determine characteristics of blood markers of oxidative stress and of antioxidant enzymatic system in patients with JRA.

Patients and methods

Patients

We carried out a cross-sectional study of 64 patients, 46 females and 18 males, aged 1.6 to 28 years (age average at 12.1±5.4 years), and with JRA. Adult patients presented a common characteristic (n=5) of long evolution of the disease (varying from 120 to 312 months) and of being submitted to strict medical follow-up since their childhood at the Pediatric Rheumatology Service, Vall d’Hebron Hospital. Patients were divided into three subgroups according to different onset of symptoms within the first 6 months of the disease: polyarticular subgroup, oligoarticular subgroup, and systemic subgroup. The American Rheumatism Association criteria were followed.

Patients in each subgroup were classified according to the degree of disease activity, based on the evidence of symptoms and globular sedimentation rate at the moment of blood sample collection (Table 1).

The polyarticular subgroup included 17 patients (14 female), aged 2.6 to 28 years (age average at 5.6±0.9 years). The period of disease evolution varied from 13 to 312 months.

The oligoarticular subgroup included 35 patients (25 female), aged 1.6 to 24.5 years (age average at 11.2±5.1 years). The period of disease evolution varied from 11 to 151 months.

The systemic subgroup included 12 patients (7 female), aged 3.3 to 18 years (age average at 11.0±4.6 years). The period of disease evolution varied from 2 to 150 months.

Clinical data, disease activity rates, and treatment at the moment of blood sample collection were determined for all three subgroups.

The control group included 60 outpatients (38 female), aged 1.5 to 2.6 years. These outpatients were being submitted to follow-up at the same hospital for non-inflammatory diseases. Also, most control patients were being submitted to preoperative procedures for subsequent minor surgical procedure.

Blood samples

Venous blood samples were collected from fasting patients and controls as follows: 2 ml of blood with EDTA; 2 ml with heparin; and 2 ml without anticoagulant. Serum aliquots were obtained after centrifuging blood without
anticoagulant at 1,500 g and 4 °C for 10 minutes. Blood samples with EDTA and heparin were centrifuged under the same conditions, and aliquots of red blood cells and plasma were obtained. All aliquots were stored at -80 °C until analyses were carried out.

Markers of lipid and protein peroxidation

We determined levels of malondialdehyde (MDA), lipoperoxide (LPO), hydroperoxide (HPX), and of carbonyl groups in plasmatic proteins. MDA and LPO plasmatic levels were determined using spectrofluorometer after a reaction with diethylthiobarbituric acid.14,15 Plasmatic HPX were quantified using PIERCE spectrophotometric assay (PeroXOquant™ Quantitative Peroxide Assay), according to the method described by Nourooz-Zadeh et al.16 Carbonyl groups of plasmatic proteins were measured according to method described by Levine et al.17

Markers of enzymatic antioxidant system activity

We determined the activity of SOD, GSH-Px, and GSH-Red enzymes, and also the levels of glutathione (GSH) using whole blood samples. Enzyme activity of SOD in red blood cells was determined by the spectrophotometric assay, as described by Nebot et al.18 Activity of GSH-Px in red blood cells was quantified by the colorimetric assay, as described by Paglia and Valentine.19 Activity of GSH-Red in plasma was determined using the spectrophotometric assay, as described by Goldberg and Spooner.20 GSH was quantified using the colorimetric assay with Bioxitec™ GSH-400 reactive kit (Oxis Internation Inc., Portland, USA).

Statistical analysis

Results were presented in average value ± standard error for the whole group of patients, both for the clinical subgroups and for the control group. Results were compared by simple linear regression and analysis of variance (ANOVA), using the statistical software Stat View II™ (Abacus Concepts, Berkeley, USA). Confidence level was 95%, and differences were considered statistically significant when P<0.05.

Results

Tables 2 and 3 present results of protein and lipid peroxidation markers and of activity of the antioxidant defense system observed in the whole group of patients with JRA, both in the clinical subgroups and in the controls.

MDA plasmatic levels were significantly higher in both the whole group of patients with JRA and in clinical subgroups than those of controls (Table 2). Clinical subgroups presented MDA values significantly higher than those of controls and progressively higher in polyarticular, oligoarticular, and systemic subgroups (Figure 1).

Plasmatic LPO were higher among JRA patients, but the difference was not significantly higher when compared to controls. The systemic subgroup presented significantly higher values when compared to the oligoarticular subgroup (P<0.02) and to the controls (P<0.01) (Table 2).

Average plasmatic levels of HPX among patients with JRA were higher than those among controls. The polyarticular subgroup presented significantly higher values when compared to the oligoarticular subgroup (P<0.02) and to the controls (P<0.01) (Table 2). The systemic JRA subgroup presented the highest average levels, and differences were significant when compared to the polyarticular and the oligoarticular subgroups and to controls (P<0.02).

Plasmatic protein levels of carbonyl groups in the whole group of patients with JRA and in clinical subgroups were higher when compared to controls (Figure 2). Differences were statistically significant in comparing JRA patients (P<0.0001), oligoarticular subgroup (P<0.0001), systemic subgroup (P<0.0001), and polyarticular subgroup (P<0.01) to the control group (Table 2). The highest levels of plasmatic protein of carbonyl groups in JRA were observed among

<table>
<thead>
<tr>
<th>Degree of activity</th>
<th>Classification</th>
<th>Symptomatology</th>
<th>GSR</th>
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</thead>
<tbody>
<tr>
<td>Active with treatment</td>
<td>A₁</td>
<td>Present</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>A₂</td>
<td>Present</td>
<td>Between 20 and 40</td>
</tr>
<tr>
<td></td>
<td>A₃</td>
<td>Present</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Inactive with treatment</td>
<td>B</td>
<td>Absent</td>
<td>Normal</td>
</tr>
<tr>
<td>Inactive without treatment</td>
<td>C</td>
<td>Absent</td>
<td>Normal</td>
</tr>
</tbody>
</table>

GSR: Globular sedimentation rate
systemic patients. Differences between subgroups of patients were not significant.

Enzymatic activity of erythrocyte SOD was higher in the whole group of patients with JRA and in subgroups of patients when compared to controls. Differences were statistically significant when comparing JRA patients (P<0.0001), polyarticular subgroup (P<0.05), oligoarticular subgroup (P<0.0001), and systemic subgroup (P<0.001) to the control group (Table 3). Differences between subgroups of patients were not significant.

Average values of blood GSH were significantly lower in the whole group of JRA patients and in the polyarticular and the systemic subgroups when compared to controls (Table 3). Average GSH concentration in the oligoarticular subgroup was slightly lower than that of controls; this difference, however, was not statistically significant. Average GSH concentration in the systemic subgroup presented lower values in relation to those of controls and other subgroups. Comparison between polyarticular, oligoarticular, and systemic subgroups of patients indicated a significant difference between polyarticular and systemic subgroups (P<0.02).

Table 2 - Markers of protein and lipid peroxidation in JRA patients, in clinical subgroups, and in controls

<table>
<thead>
<tr>
<th></th>
<th>JRA (n=64)</th>
<th>Polyarticular (n=17)</th>
<th>Oligoarticular (n=35)</th>
<th>Systemic (n=12)</th>
<th>Controls (n=60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (M/L)</td>
<td>0.72±0.05</td>
<td>0.62±0.04</td>
<td>0.74±0.07</td>
<td>0.77±0.13</td>
<td>0.33±0.008</td>
</tr>
<tr>
<td>LPO (µ L/M)</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>HPX (µ M/L)</td>
<td>6.04±0.83</td>
<td>4.75±1.04</td>
<td>5.40±1.03</td>
<td>9.75±2.78</td>
<td>4.76±0.53</td>
</tr>
<tr>
<td>CG (nmol/g)</td>
<td>0.68±0.05</td>
<td>0.60±0.08</td>
<td>0.67±0.06</td>
<td>0.83±0.15</td>
<td>0.36±0.03</td>
</tr>
</tbody>
</table>

JRA: juvenile rheumatoid arthritis; MDA: malondialdehyde; LPO: lipoperoxides; HPX: hydroperoxides; CG: protein carbonyl groups; NS: nonsignificant, significance (P) in relation to control group.

Figure 1 - Concentrations of MDA in patients divided according to the type of JRA and in controls. The histograms indicate mean values, and the gray areas indicate the standard error. The level of significance of the polyarticular, oligoarticular, and systemic subgroups, in comparison to controls, was P<0.0001

Figure 2 - Concentrations of plasma carbonyl groups of plasmatic proteins in patients divided according to the type of JRA and in controls. The histograms indicate mean values, and the gray areas indicate the standard error. The level of significance was P<0.01 for the difference between polyarticular subgroup and controls, and P<0.0001 for the difference in oligoarticular and systemic subgroups in comparison to controls.
Enzymatic activity of erythrocyte GSH-Px was lower in the whole group of patients with JRA and in subgroups of patients when compared to controls (Figure 3); these differences were statistically significant (P<0.0001). Differences between subgroups of patients were not significant (Table 3).

The enzymatic activity of plasmatic GSH-Red was higher in the whole group of patients with JRA and in subgroups of patients when compared to controls; in this case, patients in the systemic subgroup presented the highest values. Differences were statistically significant when comparing JRA patients (P<0.0001), polyarticular subgroup (P<0.01), oligoarticular subgroup (P<0.01), and systemic subgroup (P<0.0001) to the control group (Table 3).

**Discussion**

Our results aimed at determining the products of lipid and protein peroxidation in organic fluids or cells.

The MDA is a product generated during enzyme oxygenation of arachidonic acid, and it is a product of the oxidative degradation of lipids. JRA patients presented concentrations of plasma MDA significantly higher than those of controls. Our results are similar to those found by Bernacka et al., in which serum MDA levels were higher among patients with rheumatoid arthritis; this suggests that lipid peroxidation is increased among patients with inflammatory joint disease. Similar results were described in the synovial fluid. Identification of products of lipid peroxidation in the synovial fluid and in the serum of patients with rheumatoid arthritis has been recognized as an indirect evidence of the effect of free radicals on the pathogenesis of this disease.

By dividing patients according to type of JRA, it was possible to verify significant differences in comparing the three subgroups to controls. We observed that the subgroup of patients with systemic JRA, which is considered the most severe type of JRA, presented higher levels of MDA. In this group, we also observed the highest recurrence of acute episodes and of patients who had been submitted to short-term treatment, which could explain the increased oxidative stress. Our observations are in agreement with results found by Meres et al. and Imadaya et al., which described an increase in the MDA concentration in adult patients with rheumatoid arthritis.

In our group of patients, concentrations of plasma LPO, which are also a product of the lipid peroxidation process, were higher in comparison to controls. This difference, however, was not statistically significant. Yet, by dividing

![Figure 3](image-url)

**Figure 3** - Concentrations of MDA in patients divided according to the type of JRA and in controls. The histograms indicate mean values, and the highlighted areas indicate the standard error. The level of significance of the polyarticular, oligoarticular, and systemic subgroups, in comparison to controls, was P<0.0001.

<table>
<thead>
<tr>
<th>Table 3 - Markers of antioxidant defense system activity in JRA patients, in clinical subgroups, and in controls</th>
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</thead>
<tbody>
<tr>
<td><strong>JRA</strong></td>
</tr>
<tr>
<td>(n=64)</td>
</tr>
<tr>
<td>SOD (U/ml)</td>
</tr>
<tr>
<td>GSH (mM)</td>
</tr>
<tr>
<td>GSH-Px (U/g Hb)</td>
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<td>GSH-Red (U/L)</td>
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JRA: juvenile rheumatoid arthritis; SOD: superoxide dismutase; GSH: glutathione; GSH-Px: glutathione peroxidase; GSH-Red: glutathione reductase; NS: nonsignificant, significance (P) in relation to control group.
patients into subgroups according to type of disease, it was possible to demonstrate a significant difference when comparing concentrations of plasma LPO in systemic patients to oligoarticular patients and to controls. Findings of higher LPO concentration among systemic patients are consistent with those of higher MDA concentration among these same patients.

The reaction of oxygen with unsaturated lipids can generate numerous compounds, such as HPX, which are the first products of the reaction. According to Nourooz-Zadeh et al., high HPX concentrations in plasma are a direct evidence of the presence of oxidative stress.

In the whole group of patients with JRA, plasmatic levels of HPX were higher in comparison to controls, but differences were not statistically significant. The subgroup of systemic patients presented a significant increase in HPX concentration in comparison to other subgroups of patients and to controls. This may be explained by the severity that is peculiar to systemic JRA - this subgroup presents the most important increases in lipid peroxidation.

The oxidation of proteins is typically characterized by the introduction of carbonyl groups into side-chain proteins. The quantification of protein-bound carbonyl groups allows us to carry out assays aiming at assessing oxidatively modified proteins. Several studies have described increase in the levels of carbonyl groups related to ischemia-reperfusion phenomena, correction of hyponatremia, cataract, synovial fluid in patients with rheumatoid arthritis, aging, and cells exposed to active neutrophils.

Long-term exposure of proteins to free radicals may lead to spontaneous modifications. These complex reactions result in accumulation and degradation of damaged proteins. Oxidative modification of proteins has been described as a protein marker mechanism in phenomena of destruction and replacement of proteins. Starke-Reed & Oliver suggest that oxidative stress may render proteases susceptible to proteolysis. It is possible that the increased oxidation of proteins in the synovial fluid of patients with arthritis is related to the high proteolysis in the articulation of these patients.

In relation to protein carbonyl groups, which indicate the direct effect of protein oxidation, our results presented significantly higher levels of such structures in plasma in the whole group of patients with JRA, and in all three subgroups, in comparison to controls. The highest carbonyl group plasmatic concentrations were observed in systemic patients.

Increased carbonyl content of proteins groups may destabilize or inhibit biological activity. Other studies have shown that the concentration of oxidized proteins increases with age, with exposure (laboratory animals) to oxygen at 100%, and with the reduction of enzyme biological activity, resulting in an increase in cellular lability.

The superoxide radical is the first product of molecular oxygen reduction. In addition to its natural toxicity, it is an important source of hydroperoxides and other reactive free radicals. The activity of superoxide dismutase (SOD), a catalyst for dismutation of superoxide radicals into \( \text{H}_2\text{O}_2 \) and into molecular oxygen, protects cells and tissues from superoxide radicals and other peroxides, such as lipid peroxides in vivo. These enzymes represent the first line of defense against superoxide radicals, and their production is rapidly induced under certain circumstances, such as exposure to oxidative stress.

Different authors have observed that oxidative stress induces the activity of SOD in leukocytes and erythrocytes. Theoretically, since there is no protein synthesis in erythrocytes, there should not be any need for induction of the enzymatic activity. However, in erythrocyte-precursor cells, induction of SOD may occur following the oxidative process. This may explain the increase in erythrocyte SOD activity observed in our study when comparing the whole group of patients, and subgroups of patients, with controls. Since SOD represents the first line of defense in the intracellular antioxidant defense system, and since its activity may be increased in order to compensate excessive production of superoxide radicals, the increase in enzyme activity suggests an adaptive response of patients against possible damages caused by oxygen free radicals.

The glutathione redox enzymatic cycle represents the most important intracellular defense against toxicity induced by oxygen free radicals. The cycle includes glutathione (GSH), and the enzymes glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-Red). The GSH-Px enzyme uses GSH as a substratum in reactions that catalyze reduction of \( \text{H}_2\text{O}_2 \), of fatty acids, and organic hydroperoxides into water and hydroxylated fatty acids. During the reduction of peroxides, oxidized GSH is produced. The GSH-Red enzyme reduces oxidized GSH, thus regenerating GSH. Under oxidative stress, there is an excess glutathione redox cycle, and thus an increase in the concentration of oxidized GSH. As a consequence, excess GSH is eliminated through the bile.

The significant reduction of erythrocyte GSH-Px activity in the whole group of patients and in subgroups of patients when compared to controls could be explained by the reduction of GSH found in arthritical patients, since GSH is a substratum and a cofactor of GSH-Px. Also, lower levels of GSH result in lower activity of GSH-Px, which, in turn, may increase vulnerability to oxidative stress.

In addition to reduction of available GSH, the reduction of GSH-Px activity may also be caused by the process of enzyme inactivation. The enzyme itself may be inactive under conditions of intense oxidative stress, which contributes to low GSH-Px activity.

Glutathione reductase plays an important role as an intracellular antioxidant in order to maintain a high GSH/oxidized GSH ratio, which is a fundamental condition for...
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protection against oxidative damage. Enzyme activity of plasmatic GSH-Red was significantly higher in the whole group of patients and in subgroups of patients when compared to controls. This result suggests an adaptive response in patients facing an increase in oxidative stress, and could be the consequence of a process of enzyme induction.

In conclusion, we found high concentrations of products derived from lipid peroxidation while assessing plasmatic levels of MDA, HPX, LPO, and the oxidative damage of circulating protein according to carbonyl content of plasmatic protein. Our results suggest the association of cellular damage caused by oxygen free radicals with the pathogenesis of JRA. The increased activity of the antioxidant SOD enzyme, and the modifications observed in the glutathione redox cycle, which includes GSH, and GSH-Px and GSH-Red enzymes, assessed as a whole, confirm a reduction of intracellular defense protection against toxicity induced by oxidative stress in these patients. Patients with systemic JRA presented highly marked modifications related to the presence of systemic oxidative stress, characterized by intense lipid and protein peroxidation and reduced antioxidant defense system.

References


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