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Original Article

Free radical and antioxidant status in rheumatoid arthritis

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ABSTRACT

Introduction: Rheumatoid arthritis (RA) has long been categorized as a connective tissue disease and an autoimmune disease, but was not generally recognized, until recently, as a disease of oxidative stress. The present study attempted to gain an insight into the overall status of oxidative stress parameters in RA patients.

Objective: To assess the free radical and antioxidant status in RA patients.

Methods: Patients with RA satisfying the revised 1987 ACR classification criteria were included into group I ($n=60$). Group II ($n=60$) consisted of age and sex matched normal healthy controls. The free radical and antioxidant status of both groups were determined by a set of 5 parameters viz. serum nitrite, serum nitrate, plasma malondialdehyde, serum protein carbonyl and plasma superoxide dismutase.

Results: A total of 60 RA patients (M : 21; F : 39) with a mean ± SD age of 47.28 ± 11.72 years were included in the present study. The aforementioned parameters of free radical and antioxidant status were assayed and the results compared with those from 60 age and sex matched controls. All parameters were found to be significantly elevated in RA patients compared to the controls.

Conclusion: The findings suggest that oxidative stress generated in an inflamed joint can contribute to autoimmune phenomenon and connective tissue destruction in RA. New therapeutic protocols based on correcting oxidative stress levels may prove effective in restricting disease progression and limiting deformities.

Keywords: *Malondialdehyde, nitric oxide, protein carbonyl, rheumatoid arthritis, superoxide dismutase.*

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease of chronic polyarticular inflammation that leads to joint swelling, stiffness, deformity and loss of joint function with systemic manifestations.

The pathology of RA has been described in four basic stages. In the first stage, an unknown antigen reaches the synovial membrane and initiates a local immune response. In the second stage, a chronic synovial inflammation ensues with numerous cellular infiltrates and cytokines. In the third stage, 'pannus' develops eventuating in the final fourth stage of bone and cartilage destruction leading to irreversible joint damage and deformities. Overriding the chronic inflammation in synovial tissue is an acute inflammatory process in the synovial fluid with accumulation of inflammatory cells, predominantly polymorphonuclear leucocytes. These ingest immune complexes with the resultant production of free radicals (FR) and reactive oxygen species (ROS) via the NADPH oxidase system in the phenomenon termed 'respiratory burst'. Many of these inflammatory cells generate substantial quantities of nitrous oxide (NO), a free radical, in response to pro-inflammatory cytokines.[1]

Further, the volume of synovial fluid is increased significantly in inflamed joint and this response is believed to be another contributing factor in the generation of ROS and to its effects in the pathogenesis and persistence of rheumatoid synovitis.[2] Using Laser Doppler Flowmetry, it has been observed that exercise of an inflamed joint produced occlusion of the synovial capillary bed due to abnormal increase

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in pressure above the synovial capillary perfusion pressure. Moreover, a cessation of exercise brought about reperfusion of the synovial membrane. Thus movement of the inflamed joint provided the potential patho-physiological environment for the production of oxidative damage through free radical generation by hypoxic reperfusion injury.[3] Also, the combination of hypoxic and oxidative damage may decompartmentalise iron from ferritin, leaving it in a reactive form capable of stimulating $OH^{\bullet}$ formation. Thus, oxidative damage caused due to exercise of the inflamed joint (ischaemic reperfusion injury) has been documented to have a significant role in the pathogenesis of RA.[4] These increased FR, in turn, attacks vital cell components and macromolecules including lipids and proteins.[5] Malondialdehyde (MDA) and protein carbonyls are products of such oxidative damage of lipids and proteins respectively whose levels in biological fluids have been well documented to be used as a marker for oxidation mediated tissue damage.

Antioxidants are substances whose presence in relatively low concentration significantly delays or inhibits the rate of oxidative damage of target molecules. Superoxide dismutase (SOD) is an antioxidant enzyme and is the only enzyme whose substrate is a free radical. It forms the primary defense against oxidative stress catalyzing the reaction:

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

In the present study, serum levels of NO, a free radical, MDA and protein carbonyl, products of free-radical mediated tissue damage and SOD, the principal antioxidant enzyme were assayed in order to gain an insight into the overall status of oxidative stress parameters in RA.

MATERIALS AND METHODS

The subjects of the study were selected from patients attending the Orthopedics and Physical Medicine Departments of the R.L. Jalappa Hospital and Research Centre, Kolar and the MES Medical College, Perintalmanna. The subjects for the study were subdivided into 2 groups. Group I consisted of patients with RA (revised 1987 ACR criteria[6]). A total of 60 subjects were included in group I with a mean ± SD age of 47.28 ± 11.72 years and a sex distribution of M : F = 21 : 39. All subjects with clinical, biochemical or radiological evidence of any other overlapping rheumatological or immunological disorder were excluded from the study. All subjects suffering from any other chronic or acute illness were also excluded. Group II consisted of 60 age and sex matched normal healthy individuals. Informed consent was obtained from all subjects under study.

Experimental procedures

Venous blood was drawn from the subjects following 12–14 hours of overnight fasting. The following investigations were performed on fresh samples:

(a) Total nitrite (Griess reaction; modification of Granger et al.)[7]
(b) Total nitrate (reduction by nitrate reductase, then Griess reaction; modification of Granger et al.)[7]
(c) Plasma malondialdehyde (based on its reaction with thiobarbituric acid)[8]
(d) Protein carbonyl[9]
(e) Plasma superoxide dismutase (chemical system based on NADPH oxidation)[10]

RESULTS

The values of nitrite, nitrate, MDA, protein carbonyls and SOD of RA patients were compared with those from controls. It was observed that there was a significant elevation in all these parameters in RA cases when compared with the controls. These findings are documented in Table 1.

DISCUSSION

Nitrites and nitrates are derivatives of NO, which is generated in substantial quantities in the joint by chondrocytes, synovial fibroblasts and osteoblasts in response to pro-inflammatory cytokines.[11] In the present study, both serum nitrite and nitrate levels were found to be significantly elevated ($P < 0.001$) in patients with RA when compared to controls. This suggests that the arthritic joints in RA may be a potential source of nitrite and nitrate. It has been documented that formation of peroxynitrite and other radicals also occur through chemical reaction of NO. Similar elevation of nitrite and nitrates in RA has been reported by other workers. Nakamura et al. has further found serum NO levels to correlate significantly with morning stiffness, number of tender or swollen joints and CRP.[12] Clancy et al. has demonstrated that NO plays an important role in autoimmunity and inflammation. Probable mechanism of action of nitric oxide has been suggested, including its role in the cytotoxic mechanism of activated macrophages, inhibition

Table 1 Comparison of values of oxidative stress parameters and antioxidant levels between RA patients (group I) and controls (group II

Parameter	RA (group I)	Controls (group II)	Level of significance
Nitrite (μmol/l)	10.2±0.73	4.1±0.51	<0.001
Nitrate (μmol/l)	72.34±5.23	28.7±3.57	<0.001
MDA (nmol/ml)	16.26±2.8	6.86±1.7	<0.001
Protein carbonyl (nmol/mg)	1.38±0.18	1.29±0.15	<0.05
SOD (U/ml)	7.86±1.28	3.79±1.23	<0.001

of iron-sulphur centered enzymes and its antiproliferative effects.[13] Taysi et al. have proposed that widespread synovial inflammation might increase serum nitrate when synovial fluid cleared by the lymphatic system enters the systemic circulation and by equilibrium with the vascular compartment, within the synovium.[14] Another possible source of nitrate is the systemic vasculature and other cells in which the induction of nitric oxide synthesis has been shown. Potential sources of nitrite and nitrate, and therefore of NO include endothelial cells lining the synovial capillaries, infiltrating leucocytes and the resistant mesenchymal cells of the joint. Articular chondrocytes and synovial fibroblasts have been found to synthesize substantial amounts of NO in RA via the inducible form of NO synthase.[15] Furthermore, the neutrophils, lymphocytes, mast cells and especially macrophages are additional potential sources of NO in inflamed joints. This suggests increased endogenous NO synthesis in RA and NO and ROS production by the synovium and also by other tissues. These findings corroborate the elevated nitrite and nitrate levels demonstrated in the present study.

The lipid hydroperoxides and conjugated dienes that are formed as a result of oxidative damage of lipids, particularly polyunsaturated fatty acids (PUFAs), decompose to form numerous other products including alkanols, alkenals, hydroxyalkenal, volatile hydrocarbons and MDA. In the present study, the plasma MDA level was found to be significantly elevated ($P<0.001$) in RA patients in comparison to the controls. Other workers have reported similar findings and probable mechanism of this elevation of MDA in RA has been suggested. Kasama et al. have postulated that during hypoxia, glucose is increasingly used up. Thus, the glucose-deficient synovium may, during reperfusion, use lipolysis to maintain energy demands. In the process, some lipids may get oxidatively modified by FR and removed by macrophages. This foam cell accumulation has been evidenced in rheumatoid but not in normal connective tissue.[16] MDA has also been suggested to arise from cycloxygenase products. A simple non-enzymatic degradation of endoperoxides may form MDA. Further, it may also form as a by-product of thromboxane biosynthesis since the enzyme which converts PGH_2 to TXA_2 also generates the 17-hydroxy acid, heptahydroxyeicosatrienoic acid (HHT) with simultaneous generation of MDA. In fact, a stoichiometric correlation of products HHT, TXB_2 and MDA has also been reported.[17] These reports imply the basis of elevation of MDA observed in the present study.

Increased nitric oxide, as documented in subjects with RA, has further effects like oxidation of proteins, ultimately leading to increased protein carbonyl level. Lysine, arginine, proline and threonine residues of proteins are particularly sensitive to oxidation (including metal catalysed oxidation), leading in each case to the formation of protein carbonyls. Peptide carbonyl derivatives are obtained as fragmentation products of peptide bond cleavage reactions. Furthermore, carbonyl derivatives of proteins are also formed by the interaction of protein amino acid side chains with lipid peroxidation products like addition of 4-hydroxy-2-nonenal to lysine, histidine or cysteine residues. Also, glycation/glycoxidation reactions lead directly to carbonyl adducts and indirectly to the formation of *N*-carboxymethyl-lysine derivatives. The latter, by their strong chelating ability, are able to promote the generation of carbonyl groups by metal catalyzed reactions.[18]

In the presence of O_2, Fe(III) or Cu(II), and an appropriate electron donor, a number of enzymic and non-enzymic oxygen free radical generating systems are able to catalyze the oxidative modification of proteins. Whereas random modification of many different amino acid residues and extensive fragmentation occurs when proteins are exposed to oxygen radicals produced by high energy radiation, only one or a few amino acid residues are modified and relatively little peptide bond cleavage occurs when proteins are exposed to metal-catalyzed oxidation (MCO) systems.[19] MCO involves generation of H_2O_2 and reduction of Fe(III) or Cu(II) by a suitable electron donor like NADH, NADPH, ascorbate, mercaptanes, etc. Fe(II) and Cu(I) ions bind to specific metal binding sites on proteins and react with H_2O_2 to generate $OH^{\cdot}$. This highly reactive free radical attacks neighbouring amino acid residues, some of which are converted to carbonyl containing derivatives. These observations may explain increase in level of protein carbonyl found in subjects of RA. It has been estimated that the fraction of damaged proteins may be as high as

30% of the total in old animals.[20] Out of these, carbonyl derivatives account for only a fraction of the total oxidized amino acids. Thus, carbonyl levels represent only the tip of the iceberg of oxidative damage sustained by tissue proteins. The biological significance of these reactions has been highlighted by the demonstration that oxidative modification of proteins 'marks' them for degradation by proteases as also that protein oxidation contributes substantially to the intracellular pool of catalytically inactive or less active, thermo labile forms of enzymes which accumulate in cells during aging, oxidative stress and various pathological states including RA.[21]

In the present study, plasma SOD level in RA patients was found to be significantly elevated ($P < 0.001$) when compared to controls. This is corroborated by findings of other workers who have sought to explain the probable mechanism behind this elevation. Kasama et al. documented similar findings and suggested that the cytotoxicity resulting from increased ROS concentration in inflammatory synovial lesions may be effectively prevented by the body by induction of certain enzymes including SOD.[16] This view is supported by Davies in his review. He concludes that since oxidative stress levels may vary from time to time, organisms are able to adapt to such fluctuating stresses by inducing the synthesis of antioxidant enzymes. But despite these antioxidant mechanisms, oxidative damage remains an inescapable outcome of many disease processes, one among them being RA.[22]

Kucera et al. in their study 'free oxygen radicals and rheumatic diseases' suggest that patients with inflammation of the synovial membrane respond relatively frequently by a rise of indicators of antioxidant enzymes such as SOD. They are of the view that a defense reaction of the organism is obviously involved against the increased formation and action of free radicals in the inflamed articular lining. Furthermore, they postulate that SOD level could serve as an indicator for checking the progress of treatment in rheumatic diseases.[23] C'mien et al. have shown that patients of RA had a higher SOD and xanthine oxidase (XO) activities and higher MDA levels than non-rheumatoid controls. Thus, they have suggested that excessive free radical production through the xanthine oxidase system is the primary defect in RA, rather than an impaired antioxidant system. They have further suggested that the therapeutic use of XO enzyme inhibitors and some antioxidants may be beneficial in this regard.[24] Several studies[25–27] have documented such therapeutic use of antioxidants, principally SOD in RA with varying results.

The possibility that these changes seen in oxidative stress parameters in the serum of RA patients may be considered to be an accurate reflection of the synovial and articular changes has been suggested in several studies, which, after comparing the serum levels of markers of oxidative damage with their levels in synovial fluids and synovial tissues in RA, have concluded that the serum level of these markers may indeed be useful as a valid index for free radical mediated articular damage in RA. Thus, Sumii et al.[28] have suggested that serum SOD activity to be valid marker of articular destruction and repair and Mazetti et al.[29] after observing that serum levels of SOD in RA patients correlated positively with RA factor, a sensitive marker for disease activity in RA, have proposed SOD level in serum to be a marker of inflammatory activity in RA. Similarly, Chaturvedi et al.[30] and Dalle-Donne[5] have suggested serum level of MDA and protein carbonyls respectively to be valid biomarkers of free radical mediated tissue destruction in RA. Further, Taysi et al.[14] have sought to explain the basis of this correlation between serum markers and synovial inflammation. They proposed that abnormal level of these markers in synovial fluid might enter the systemic circulation by clearance through the lymphatic system as also by equilibriation with vascular compartment within the synovium. Several other studies[16,31,32] have documented the presence of abnormal levels of these oxidative stress parameters in synovial fluid, synovium and articular tissues of affected joints in RA, which were found in this study, to be similarly deranged in serum. This may provide additional indirect evidence that the changes seen in the serum may be considered to be an accurate reflection of the articular changes.

RA has long been categorized as an autoimmune connective tissue disease, but was not, until recently, recognized as a disease of oxidative stress. Anti-inflammatory therapies reduce acute inflammation with little if any overall effect on the disease course. In the present study, 5 parameters viz. serum nitrite and nitrate as a marker of free radical production, plasma MDA and protein carbonyl as a marker of free radical mediated tissue destruction and plasma SOD, an antioxidant enzyme were assayed to obtain a complete insight into oxidation mediated tissue damage in RA. The findings of the present study corroborate the evidence from other recent studies that oxidant stress generated within an inflamed joint can produce connective tissue destruction leading to joint and periarticular deformities in RA. These observations suggest that new therapeutic protocols based on correction of oxidative stress levels may prove effective in suppressing disease progression and limiting deformities.

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