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Biomarkers of Oxidative Stress and Inflammation in Patients with Rheumatoid Arthritis

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Abstract: Rheumatoid arthritis (RA) is a chronic, autoimmune, systemic disease, characterized by polyarthritis, erosive synovitis and sometimes shows multi system involvement. Oxidative damage induced by reactive species has been related to the pathophysiology of RA. The inflammation in the joints leads to joint damage and thus influences the quality of life in these patients. This work was undertaken to determine oxidative stress markers in patients with rheumatoid arthritis and to observe its correlation with inflammatory markers. Malondialdehyde (MAD) and Nitric oxide (NO) are estimated as the markers of oxidative stress and High sensitivity C-reactive protein (hsCRP) is measured as a marker of inflammation. A cross-sectional study in rheumatoid arthritis patients and healthy controls was done. We included 40 rheumatoid arthritis patients and 40 healthy controls. MDA, NO and hsCRP are measured in all the subjects. Statistical analyses were done using SPSS statistical software version 17.0. MDA, NO and hsCRP levels were increased in RA patients when compared to controls ($p<0.001$) and a positive correlation between the inflammatory marker and oxidative stress markers was also observed in these patients. The value of R is 0.7277 for MDA and hsCRP where as R value is 0.6153 for NO and hsCRP. The findings of present study support the concept of oxidative stress leading to tissue damage and inflammation. The positive correlation between inflammation and oxidative stress in these patients indicate the importance of correction of oxidative stress along with inflammation, further large and well controlled studies are needed to establish the role of anti oxidants in treatment protocols of RA patients.
Keywords: C-Reactive protein, Inflammation, Malondialdehyde, Nitric Oxide, Rheumatoid Arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, auto immune disease. There is symmetrical arthritis which involves both small and large joints. It typically presents as inflammation, pain, stiffness, loss of function and finally leads to joint damage and bone destruction. There is significant mortality and morbidity in affected individuals as it is not confined to joints that are involved, it progresses to become a systemic disease and multi organ involvement is seen [1]. Approximately 1 to 2% of general population worldwide are affected by RA where as incidence in India is 0.75% and prevalence is quite similar to that of developed countries [2]. Reactive oxygen species [ROS] and reactive nitrogen species (RNS) are considered to be mediators and effectors of cartilage damage in RA where ROS are especially involved in extra cellular degrading activity and contribute to the pathogenesis of arthritis [3]. ROS are the radicals generated in the living systems; they are oxygen-derived radicals which include superoxide, peroxyl, perhydroxyl and hydroxyl radicals. Hydrogen peroxide and singlet oxygen are non-free radical species that are easily converted into free radicals. RNS represents nitric oxide, nitrogen dioxide and peroxynitrite. All these species contain one or more unpaired electrons in their outer orbital shell and so they are called as free radicals [4]. Lipid peroxidation mediated by free radicals is considered to be major mechanism of cell membrane destruction and cell damage. Uncontrolled production of these free radicals may play an important role in the pathogenesis of RA [5].

As these free radicals are highly reactive, it is difficult to measure their presence as such. So, it is better to measure their effects on various lipids, proteins and nucleic acids. Oxidative damage of lipids gives several by-products including alkanols, alkenals, hydroxyalkenal, volatile hydrocarbons and MDA. NO spontaneously oxidises to its inactive stable end products nitrite and nitrate [6]. In this study MDA is measured as level of reactive oxygen radicals and nitrate, the stable product of nitric oxide are estimated.

C - reactive protein is a general marker of systemic inflammation, measurement of serum CRP concentration as a measure of disease activity is being practiced since a long time. High sensitivity CRP (hsCRP) testing has been recommended to measure low disease activity in RA which is associated with poor long term outcome, since routine CRP testing may not detect the low grade systemic inflammation that is commonly observed in RA [7]. In this study we estimated hsCRP as the marker of inflammation and disease activity and correlated hsCRP with oxidative stress markers.

MATERIALS AND METHODS

A cross-sectional study was done in which 40 clinically diagnosed female patients with active RA between 30 and 60 years of age, who were not on methotrexate were chosen. An equal number of age, sex and socioeconomic status matched healthy volunteers served as control group. Informed and written consent was taken from all subjects who participated in the study after explaining the objectives of the study completely. Complete clinical and personal history was recorded. Institutional ethical committee approval was taken before starting this study.

The inclusion criterion for cases is based on Revised American Rheumatology association criteria 1987 for the diagnosis of rheumatoid arthritis [8]. Patients with other forms of arthritis, Diabetes, Hypertension, Liver and kidney disorder, recent fever, active infection, patients using anti inflammatory and anti oxidants which can alter the study parameters and those who does not give consent are excluded from this study. None of the subjects are alcoholic or smokers. Patients who take alternate medicines like ayurveda and homeopathy were also excluded.

After making the subjects comfortable, under strict aseptic precautions 3ml of venous blood was collected from anticubital vein into 2 tubes, 2ml in tube containing anti coagulant (Di Sodium EDTA) and 1ml in plain tube. Plasma was immediately separated from 2ml tube and 1ml of plain sample was allowed to stand for 30 mins and serum separated by centrifugation at 3000 rpm for 10mins.

Free malondialdehyde, as a measure of lipid peroxidation, was measured using thiobarbituric acid reactive substance (TBARS) method after precipitating the proteins with trichloroacetic acid [9]. Nitrate, the stable product of nitric oxide is reduced to nitrite by kinetic cadmium reduction method after deproteinisation of sample by Somogyireagent. The nitrite produced is determined by diazotization of sulphanilamide and coupling with Naphthalenediamine [10]. All reagents used were of analytical reagent grade. Obtained from sigma chemicals, St.Louis, MO. hsCRP is measured using turbidimetric immunoassay where serum C -reactive protein causes agglutination of the latex particles coated with anti human C -reactive protein.

Statistical analysis

The data collected was entered in excel sheet (Microsoft office excel 2007) and analysed by Fisher's exact test using SPSS statistics 17.0 software. Statistical significance analysis was done between cases and controls, for all the markers and Pearson correlation coefficient is used to measure the strength of a linear association between oxidative and inflammatory markers. The data was expressed as mean ± standard deviation. $P \leq 0.05$ is considered as significant.

RESULTS

Table-1 shows mean ± standard deviation of age, duration of disease and no of samples in each group. Table-2 shows values of Malondialdehyde (MDA); Nitric oxide (NO); high sensitivity C-reactive protein (hsCRP) level in controls and in patients with rheumatoid arthritis. The levels of MDA, NO, and hsCRP are significantly increased in patients with RA when compared with controls ($p<0.001$). Figure-1 shows scattered plot of hsCRP and MDA among rheumatoid arthritis patients. The results indicate that plasma MDA was positively correlated with hsCRP in RA patients. The value of R is 0.7277. Figure-2 shows scattered plot of hsCRP and NO levels among rheumatoid arthritis patients. The results indicate that plasma NO was positively correlated with hsCRP level in RA patients. The value of R is 0.6153. This is a moderate positive correlation, which means there is a tendency for high X variable scores go with high Y variable scores (and vice versa).

Table-1: Mean ± standard deviation of age, duration of disease and no of samples in each group

	Controls	RA Patients
Total no of samples	40	40
Age in years(mean ± SD)	44.30±8.16	48.02±6.92
Duration of disease(years)	---	4.62±5.33

Table-2: Malondialdehyde (MDA); Nitric oxide (NO); High sensitivity C-reactive protein (hsCRP); in controls and in patients with rheumatoid arthritis.

Parameters	Controls	RA Patients	P value	Significance
MDA(µmol/L)	1.36+/-0.47	4.81+/-0.65	<0.001*	HS
NO(µmol/L)	37.11+/- 5.08	61.13+/-9.10	<0.001*	HS
hsCRP(mg/L)	0.76+/-0.32	2.35+/-1.44	<0.001*	HS

Values are expressed as mean ± standard deviation
* P < 0.001 compared to controls; HS highly significant.

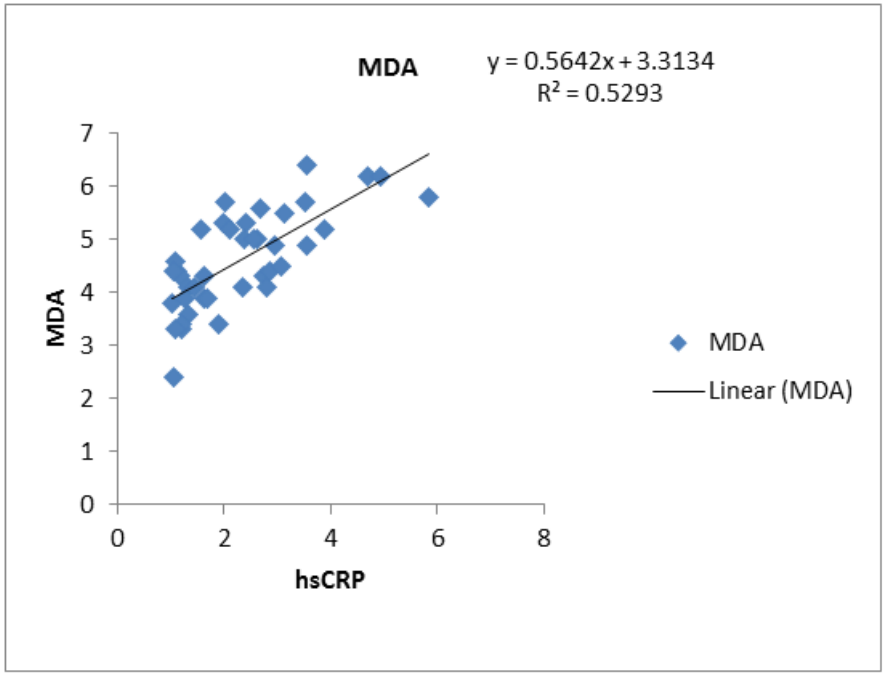

Fig-1: Scattered plot of hsCRP and MDA among rheumatoid arthritis patients.
MDA- Malondialdehyde; hsCRP- High sensitivity C-reactive protein

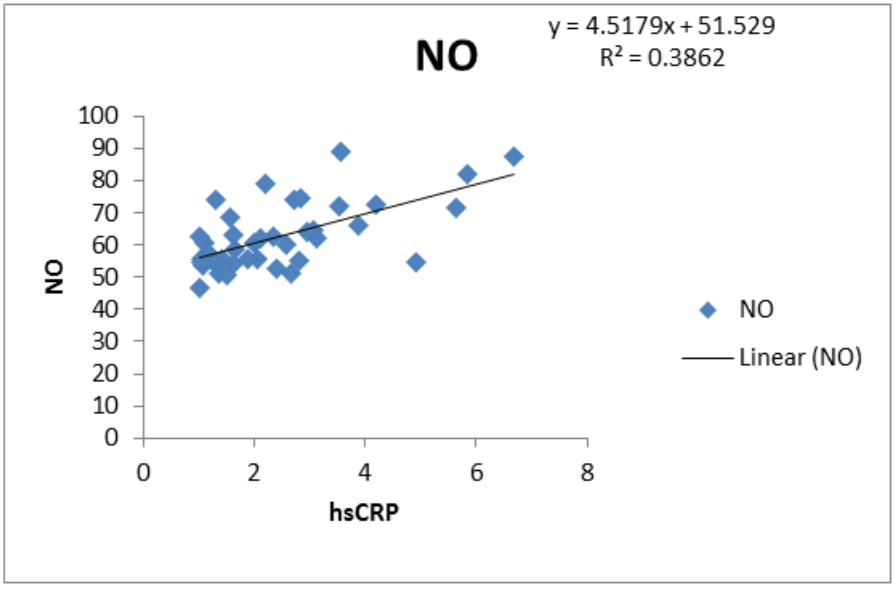

Fig-2: Scattered plot of hsCRP and NO among rheumatoid arthritis patients.
NO- Nitric oxide; hsCRP- High sensitivity C reactive protein

DISCUSSION

RA is a most common autoimmune inflammatory disease which starts with decreased tolerance to modified self antigens. Environmental and genetic factors are thought to cause inappropriate immunomodulation and result in inflammation, which leads to synovial damage in these patients [11]. The reactive oxygen species also play a major role in this process. Tissue injuries due to oxidative stress have been implicated in the pathogenesis of RA [12]. ROS are generated by stimulation of inflammatory cytokines such as tumour necrosis factor (TNF) or Vascular endothelial growth factor (VEGF), these free radicals are enormously produced at the site of inflammation. They play an important role as second messengers in inflammatory and immunological cellular response in RA [13]. Free radicals on the other hand can degrade joint cartilage directly by attacking its proteoglycan and inhibiting its synthesis [14]. It has been reported that lubrication property of synovial fluid is lost due to depolymerisation of hyaluronic acid on exposure to superoxide and hydrogen peroxide [15]. At the site of inflammation, lipid peroxides that are generated in the tissue diffuse into blood, these are estimated in serum or plasma, which intern reflects the severity of the tissue damage [16].

In this study we observed a statistically significant increase in MDA levels ($p<0.001$) in patients with RA when compared with controls and when we observed a significant positive correlation between hsCRP and MDA in RA patients. ($R=0.7277$). In RA, activated macrophages and neutrophils release oxidants in high concentration which acts on lipids, proteins and DNA, as a result unsaturated fatty acids undergo lipid peroxidation and MDA is released [17]. This reacts with lysine residue in protein to produce immunogenic molecules which increases inflammation [18]. The correlation of MDA and hsCRP suggests that free radicals play a major role in pathogenesis and severity of inflammatory arthropathy.

Similar studies were done by F. Kartas *et al*., [19], K Bhowmick *et al*., [20], S.D Walwadkar *et al*., [18]. They observed significant higher MDA levels in cases than controls. This increase in MDA in RA patients can be explained on basis of increased lipolysis due to hypoxia reperfusion in inflamed joints. Some lipids are oxidatively modified by free radicals and are removed by macrophages [21].

In this study we observed a statistically significant increase in NO levels ($p<0.001$) in patients with RA when compared with controls and when we observed a significant positive correlation between hsCRP and NO in RA patients. ($R=0.6153$). The cause of increase is not clear, probably it could be due to increased secretion of NO from cells like neutrophils, lymphocytes, mast cells and macrophages in inflamed joints which leads to increased diffusion of NO into vascular compartment. It has been found that, the articular cartilage and synovial fibroblasts synthesise substantial amounts of NO, suggesting joints as potential source of NO [18]. The other cause could be production of NO by systemic vasculature and other cells [22]. This might also be due to enhanced activity of nitric oxide synthase enzyme which is an enzyme responsible for production of NO [23]. NO leads to tissue damage and peri-articular bone loss, after converting into peroxylnitrite radical ($ONOO^{\cdot}$). This also affects the physiological process within joints including modulation of interleukin-1(IL-1) induced bone resorption and cartilage metabolism. This radical can further converted into cytotoxic hydroxyl radicals ($OH^{\cdot}$) and nitronium ions ($NO2+$) [24]. These findings suggest that the cytotoxic and cytostatic effects of NO results in inflammation in RA patients which substantiates our results of correlation.

Similar studies were done by Triveni *et al*., [25] and Aida A Mohmoud *et al*., [26] where they observed significant increase in NO levels in patients with RA when compared to controls. In another study done by Ersoy *et al*., [27] they observed significant correlation between NO levels and disease activity. So, observations from the present study are in agreement to other similar published studies.

CONCLUSION

Biochemical alterations in our study reflect the pathogenesis of RA and support the concept of oxidative stress leading to tissue damage and inflammation, which is evidenced by increased MDA and NO levels. This study also indicates a strong relationship between oxidative stress and inflammatory markers in RA. Further long-term, in-depth studies about this aspect may help in therapeutic management of RA patients, which may involve correction of inflammation along with correction of oxidative stress with exogenous antioxidants supplementation and dietary modifications.

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