Original Article

Evaluation of levels of oxidative stress as a potential biomarker in patients with rheumatoid arthritis

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Abstract

Objectives: One of the most prevalent autoimmune disease globally, rheumatoid arthritis (RA) is caused by interplay of multiple inflammatory mediators in specific joints. Altered redox balance is one of the key factors in pathophysiology of RA. This study aims to find whether oxidative stress in peripheral blood neutrophil correlates with the disease activity and disability associated with it. **Methods:** Ten healthy controls and 29 RA patients with moderate to severe disease activity (DAS28 score >3.2) were recruited and reactive oxygen species (ROS) level in peripheral blood neutrophil was measured using flow cytometry at baseline visit and after 6 months follow-up. Functional status of RA patients was measured using Health Assessment Questionnaire Disability Index (HAQ-DI). **Results:** RA patients showed significantly higher level of ROS in compared to healthy control. DAS28 correlated well with ROS at baseline visit (Pearson's r = +0.63) as well as follow-up visit (Pearson's r = +0.75). HAQ-DI showed weak positive correlation at baseline visit (Pearson's r = 0.1) but it was negative at follow-up visit (Pearson's r = -0.19). **Conclusions:** Oxidative stress mirrors the disease activity in RA and can be considered as a biomarker, but it is not related with functional ability of the patients.

Keywords: DAS28, HAQ-DI, oxidative stress, reactive oxygen species, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects almost 1% of global population. Several joints especially small joints of hands and feet and large joints like elbow, shoulder, and knees are commonly affected along with some extra-articular manifestations. Morning stiffness for more than 30 min, pain and swelling and gradual progression to deformity are the characteristic features of joint involvement in RA.[1]

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Etiology of RA is yet to be fully established. The pro-inflammatory cytokines TNF-α and IL-1β are important mediators of synovial inflammation and subsequent bone erosion. These pro-inflammatory cytokines play an important role by stimulating cells to produce collagenase, other neutral proteases and proteolytic enzymes that degrade the cartilage locally and also inhibit synthesis of new matrix molecules.[2] The inflammation is orchestrated by lymphocytes, macrophages, and neutrophils.[3,4]

Several studies have already proved the occurrence of oxidative stress in RA patients.[5-7] In a cross-sectional study, we have already found significant rise of reactive oxygen species (ROS) locally as well as systemically.[8] There is increased ROS production

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in peripheral blood neutrophil and the level correlated with disease activity in RA patients. Somatic mutation, defect in DNA damage repair pathways, oxidative modification of auto-antigens, alteration of cell signalling are the possible mechanisms of ROS-mediated cell injury in the pathogenesis of RA.[9-11]

But there are still very few publications reporting a longitudinal study regarding improvement of redox homeostasis with disease-modifying antirheumatic drug (DMARD) therapy. This study was undertaken to evaluate ROS levels in peripheral blood (neutrophils) in patients with high disease activity score (DAS28) and find any correlation between disease activity (measured by Disease Activity Score 28 - DAS28) with ROS levels at baseline and after 6 months of therapy. We also tried to establish a correlation between functional disability in RA patient (as measured by Health Assessment Questionnaire Disability Index – HAQ-DI) and ROS level in peripheral blood neutrophil.

Materials and Methods

Study Population

Study population were recruited from outpatient clinic of Department of Rheumatology, Institute of Post-Graduate Medical Education and Research, Kolkata, India. The study protocol followed the principles expressed in the Helsinki Declaration of 1983 and received prior approval from Institutional Ethics Committee (IPGME&R Research Oversight Committee, 244 AJC Bose Road, Kolkata- 700020, India, Memo No. Inst/IEC/70 dated 05.02.2013). Informed consents were obtained from all participants. The study population included adult (age >18 years) patients with RA, who fulfilled "The 2010 ACR-EULAR classification criteria for rheumatoid arthritis" and ESR-based DAS28 score >3.2 (moderate to severe disease activity).[12,13] Patients suffering from any other autoimmune disorder, neurologic disorder, or arthropathies were excluded from the study. Age- and sex-matched healthy volunteers were recruited from the faculty and students at our institute.

Sample size

Previous studies have shown the correlation between ROS and DAS28 varies between 0.5 and 0.7.[6,14] Assuming a sample correlation coefficient of 0.5, Type-I error of 0.05, and power of 80%, required sample size was 29 subjects. We calculated the sample size using Fisher's arctanh transformation.[15]

Study design

This is a longitudinal observational study. Patients were screened and recruited according to inclusion and exclusion criteria and blood sample was collected to measure the ROS level in Neutrophil at baseline visit. After 6 months, they were evaluated clinically, and ROS level was measured again.

Disease activity assessment

Disease activity status in RA patients was evaluated by DAS28 scoring. This is calculated by number of tender and swollen joint count among 28 joints, ESR level, and a Visual Analogue Scale of pain by the patient himself.[13,16] The degree of disease activity can be classified as high (DAS28 > 5.1), moderate (3.2 < DAS28 ≤ 5.1), or low (DAS28 ≤ 3.2).

Physical functionality assessment

The Health Assessment Questionnaire Disability Index (HAQ-DI) indicates the level of respondent's functional ability. It is a sensitive predictor of impending disability and associated costs.[17] It assesses a patient's abilities during last 1 week using their usual equipment. There are 20 questions covering eight areas of functioning—dressing, eating, and similar activities. For each item, there is a four-level Likert scale from 0 to 3 denoting "no difficulty" to "unable to do". HAQ-DI score is calculated as mean of the highest component score in individual category.[18]

Reagents

All reagents were obtained from Sigma Aldrich (St. Louis, MO, USA).

Isolation of neutrophils

Neutrophils were isolated from peripheral blood using HiSep 1077 and GranuloSep GSM 1119. Initially, a double gradient (Percoll density gradient) is formed by layering an equal volume of HiSep 1077 over GranuloSep GSM 1119. Blood is carefully layered on to the upper HiSep 1077 medium and centrifuged (3,000 rpm for 30 min.) at room temperature. Neutrophils present at the 1077/1119 interphase layer were isolated, washed twice in RPMI 1,640 medium, centrifuged (1,600 rpm for 10 min.), and suspended in RPMI 1640. We checked cell viability using trypan blue (>95%).

Flow cytometric principle for measurement of reactive oxygen species (ROS)

2' 7' Dihydrodichlorofluorescein Diacetate (H_2DCFDA) is a non-fluorescent dye that freely enters the cell. It undergoes hydrolytic cleavage by intracellular esterases, H_2DCF is produced which is non-fluorescent but impermeable and hence retained within the cell. H_2DCF is then oxidized by intracellular oxidants to the highly fluorescent DCF which can be measured on a Flow Cytometer, as fluorescence is directly proportional to the intracellular ROS generation.[19]

Intracellular fluorescence from the Cells (10^6) were monitored on a Flow Cytometer (FACS Calibur, Becton Dickenson, San Jose, CA, USA) equipped with an argon ion laser (15 mW) tuned to 488 nm. Fluorescence of DCF was captured in FL 1 Channel with 530/30 - nm band pass filter. Fluorescence was measured in the log mode and expressed as Geometric Mean Fluorescence Channel (GMFC). Analysis was performed using BD Cell Quest Pro Software on 5,000 gated events.

Measurement of ROS in neutrophils

To measure baseline intracellular ROS, neutrophils (1×10^6/ml)

isolated from peripheral blood of RA patients were incubated for 30 min. at 37°C with 0.25 μM H2DCFDA. Fluorescence of 5,000 cells was acquired by flow cytometry using a FSC versus SSC plot to gate the population and a FL 1 histogram to calculate the fluorescence. Finally, analysis was made by BD Cell Quest Pro software to detect GMFC.

Statistical analysis

The study specific data was collected in a case record form (CRF). The data from the CRF and GMFC values were transcribed onto an excel database and analyzed by R version 3.5.1 and R Studio version 1.0.136 (R foundation) statistical software (Language). Comparison between control group and RA was done using Student's Unpaired *t*-test. Paired data from baseline and follow-up visit was calculated using Wilcoxon signed rank test and correlation coefficient was reported as Pearson's *r*. p-value < 0.05 was considered as statistically significant.

Results

Study population

The study population included 29 patients of RA who have moderate to severe disease activity (DAS28 > 3.2) and 10 healthy controls [Figure 1]. Baseline profile of the subjects are summarized in Table 1. Evidently most of the patients were female and more than 40 years old (Mean = 43.3, SD = 12.2). Rheumatoid factor positive cases were 86.20% and 62.1% were Anti-CCP positive. Mean duration of disease was approximately 10 years. None of the patients were on antioxidant therapy.

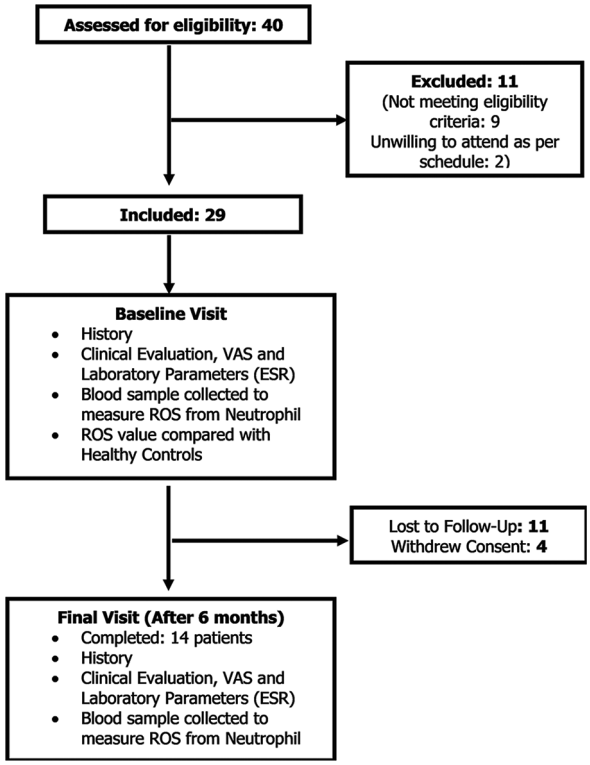

Figure 1: Study Design and Flow of study participants

Higher reactive oxygen species (ROS) level in neutrophils of RA patients

The baseline ROS level (expressed as GMFC) was found to be higher in RA patients (n = 29, Mean = 609, SD = 392) than healthy controls (n = 10, Mean = 205.0, SD = 64.9) and it was statistically significant (P value < 0.001, Student's Unpaired t-test). Figure 2 shows the comparison between the groups.

Changes of parameters from baseline to follow-up visit

The patients were treated with multiple DMARD during the study period of 6 months. Table 2 shows the levels of DAS28, ROS, and HAQ-DI from baseline to follow-up visit. There is reduction of all the parameters but none of them were statistically significant.

Correlation between disease activity and ROS in neutrophil

At baseline visit, DAS28 was found to be positively correlated with ROS level in neutrophils (Pearson's r = 0.63, P value < 0.001) [Figure 3a]. This positive correlation was even stronger in follow-up visit (Pearson's r = 0.75, P value = 0.003) [Figure 3b].

Correlation between physical functional status and ROS in neutrophil

Correlation between Physical functional ability, that is, HAQ-DI and ROS was weakly positive at baseline visit (Pearson's r = 0.1, P value = 0.61) [Figure 3c]. This was reversed in follow-up visit into weak negative correlation (Pearson's r = -0.19, P value = 0.52) [Figure 3d].

Discussion

Generation of lipid peroxides and ROS may play a significant role in the pathogenesis of RA. Studies have shown multidirectional

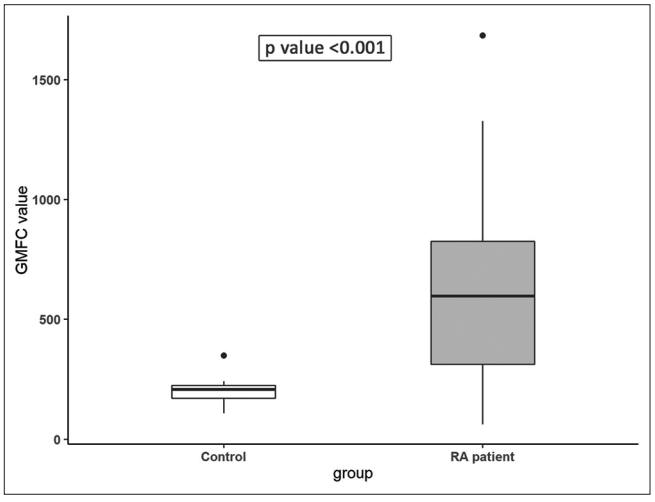

Figure 2: Boxplot showing ROS generation (GMFC) in Neutrophils was higher in RA patients compared to healthy controls

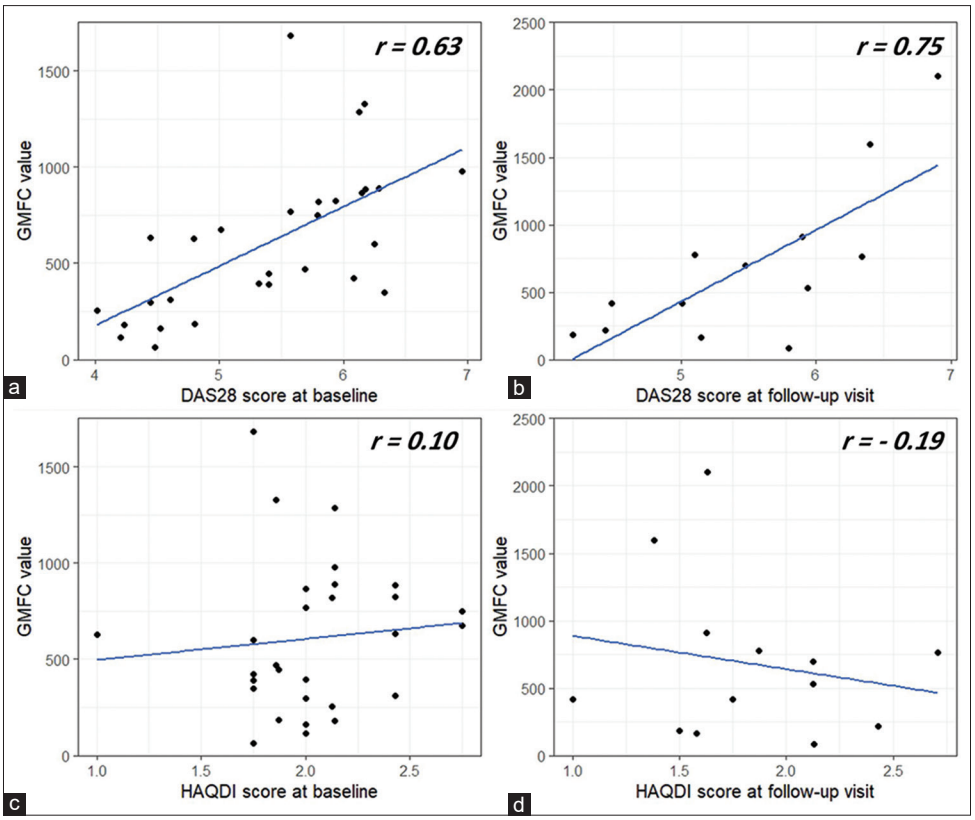

Figure 3: Correlation of ROS with DAS28 and HAQ-DI at baseline visit (a and c) and follow-up visit (b and d)

Table 1: Baseline Parameters of study participants

	Control *n*=10	RA patient *n*=29	*P*
Age - Years (Mean, SD)	38.3 (13.2)	43.3 (12.2)	0.312
Sex: Number (Percent)			1.000
Male	2 (20.0%)	7 (24.1%)	
Female	8 (80.0%)	22 (75.9%)	
Disease duration - Months (Mean, SD)	-	115 (82.0)	
Morning Stiffness duration - Minutes (Mean, SD)	-	51.7 (35.0)	
Anti-CCP Antibody - Positive: Number (Percent)	-	18 (62.1%)	
Rheumatoid Factor- Positive: Number (Percent)	-	25 (86.2%)	
WBC count per cubic millimeter (Mean, SD)	6830 (1941)	7550 (2764)	0.378
ESR - mm in 1st h (Mean, SD)	30.8 (13.1)	57.0 (25.7)	<0.001
DAS28 Score (Mean, SD)	-	5.40 (0.80)	
Baseline ROS level (Mean, SD)	205 (64.9)	609 (392)	<0.001
HAQDI score (Mean, SD)	-	2.03 (0.35)	
Patient already on DMARD: Number (Percent)	-	26 (89.65)	
Methotrexate: Number (Percent)	-	25 (86.20)	
Hydroxychloroquine: Number (Percent)	-	10 (34.48)	
Leflunomide: Number (Percent)	-	2 (86.20)	
Sulfasalazine: Number (Percent)	-	10 (34.48)	
Glucocorticoid: Number (Percent)	-	7 (24.13)	

P value in the last column is from intergroup comparison by Chi-square test for gender, Student's unpaired *t*-test for rest of the parameters. SD=Standard deviation

alteration in the antioxidant defence system and increased lipid peroxidation in RA patients.[20,21] We found higher oxidative stress in neutrophils in RA patients compared to healthy controls (*P* value < 0.001). A cross-sectional study in our hospital showed raised level of ROS from blood neutrophils positively correlates with ROS level detected in the neutrophils sourced from synovial infiltrate as well as DAS28 score (r = 0.65, CI = 0.03–0.908).[14] In this study also, we found positive correlation between disease activity and ROS generated in peripheral blood neutrophils. After 6 months follow-up, this correlation was even stronger. There was also reduction of ROS level in the follow-up visit in alignment with reduction of DAS28 and HAQ-DI.

Table 2: Changes of DAS28 Score, ROS level and HAQDI Score in RA patients (*n*=14)

	Baseline visit	Follow up visit	*P*
DAS28 Score (Median, IQR)	5.680 (0.682)	5.315 (1.272)	0.193
ROS level (Median, IQR)	794.83 (326.375)	532.0 (563.4)	0.375
HAQDI Score (Median, IQR)	2.062 (0.495)	1.690 (0.605)	0.167

P value in the last column is from intergroup comparison by Wilcoxon signed rank test.
IQR=Interquartile range

We could not find any literature where correlation between functional status of patient and oxidative stress has been explored. In our study, we could not find any significant correlation between HAQDI and ROS generated in peripheral blood neutrophil. This is probably because during the course of the disease, inflammation gradually declines, and deformities appear. So, a functionally impaired patient with high HAQDI does not necessarily show high DAS28 score or high systemic oxidative stress.

Preclinical studies showed promising results with enzymatic antioxidants, superoxide dismutase in treatment of RA.[22] Antioxidants along with conventional therapy in patients with RA increases the concentration of post-treatment antioxidant level, decreases the malondialdehyde (MDA- marker of oxidative stress).[23] An open pilot study found significant decrease in swollen and tender joint counts and overall improvement of general health with 10 weeks antioxidant add-on therapy.[24] However, on the contrary, certain studies found no improvement in systemic antioxidant status and RA disease activity after antioxidant supplementation. A placebo controlled randomized controlled trial reported vitamin-E supplements (Dose - 600 IU alternate day) is not associated with a significant reduction in the risk of developing RA.[25] Another study showed antioxidant rich Mediterranean diet intervention did not change the levels of plasma antioxidants and urine MDA.[26] In a review article, Bala *et al.* highlighted recent studies describing the connections between RA and free radicals, and the approach of different anti-oxidant strategies with success probabilities.[27] To conclude, the efficacy of antioxidant supplementation is still controversial. Appropriate dose, dosing frequency, and duration of therapy need to be optimized before making it a routine practice as an adjunct to the conventional therapy.

One of the major limitations of the study was inability to analyze effects of different DMARDs on ROS level of individual patients because of inadequate sample. High fraction of dropouts also reduced the power of the study.

Taken together, this study supported the hypothesis that patients with RA suffer from increased oxidative stress especially in neutrophils and as ROS correlated with clinical parameters like DAS28 and HAQDI, monitoring levels of ROS might be useful for monitoring active status of the disease as well as future target of drug therapy.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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