APJMP 2016; Vol.1 (01): pp. 2-10 **Original Research Article**

ASIAN PACIFIC JOURNAL OF MEDICAL & PHARMACEUTICAL SCIENCES

Available online at http://ainstin.com

Received: 11-05-2016
Revised: 04-08-2016
Accepted: 10-08-2016

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ANTIOXIDANT DEFENCE AND DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS PATIENTS

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ABSTRACT

Oxidative stress is implicated in pathogenesis of rheumatoid arthritis and vitamin D deficiency is associated with severity of rheumatoid arthritis (RA).With this background the levels of superoxide dismutase, catalase and glutathione reductase and thiobarbituric acid reactive substances and vitamin D levels were analyzed.Twenty healthy subjects, twenty four Rheumatoid arthritis patients on methotrexate (MTX) monotherapy and fourteen patients who had not taken any pharmacological treatment, were recruited in the study. The oxidative stress was found to be high in Rheumatoid arthritis patients both naïve and those on methotrexate therapy, when compared to healthy controls. Antioxidant defense was found to be less in naïve patients and the disease activity score was found to be worsened in both treatment naïve and Methotrexate treated patients. Nutritional enrichment or antioxidant supplementation along with conventional treatment could improve the functional status in naïve Rheumatoid arthritis patients, whereas increment of vitamin D levels could improve the therapeutic outcome for patients on monotherapy with methotrexate.

Keywords*:* Oxidative stress, Rheumatoid arthritis, vitamin D, Methotrexate.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disorder characterized by synovial destruction [1]. In RA the synovium is continuously under stress, which activates multiple pathways leading to its damage. Among the different kinds of stress, oxidative stress plays a major role in activating multiple pathways of of antigens, leading to autogenicity.

In RA the shift of oxidant/antioxidant balance causes activation of antigen presenting cells (APC) such as dentritic cells (DCs) which trigger the immune reactions. APC causes differentiation of T helper cells (Th) Th0 which transform to Th1 and Th2, called as polarized cells. This polarized T lymphocytes generate proinflammatory cytokines such as tumor necrosis factor (TNFα), and several interleukins (IL-1, IL-6 etc) which further intensify the inflammatory reaction [1]. It has been reported that oxidative stress can also induce mutations in p53 tumor suppressor gene which could self-perpetuate the cascade of immunological events [2, 3]. The excessive oxidative metabolism and continuous oxidative stress can damage proteins, lipids, nucleic acids and matrix components, which contribute to RA pathogenesis.

Vitamin D has immunoregulatory properties and has a role in controlling RA. Vitamin D receptors are expressed in immune cells and antigen-presenting cells. Vitamin D deficiency is common in rheumatoid arthritis and has an inverse correlation with disease activity [4]. The impact of oxidative stress and role of vitamin D has been represented in Fig 1 & 2.

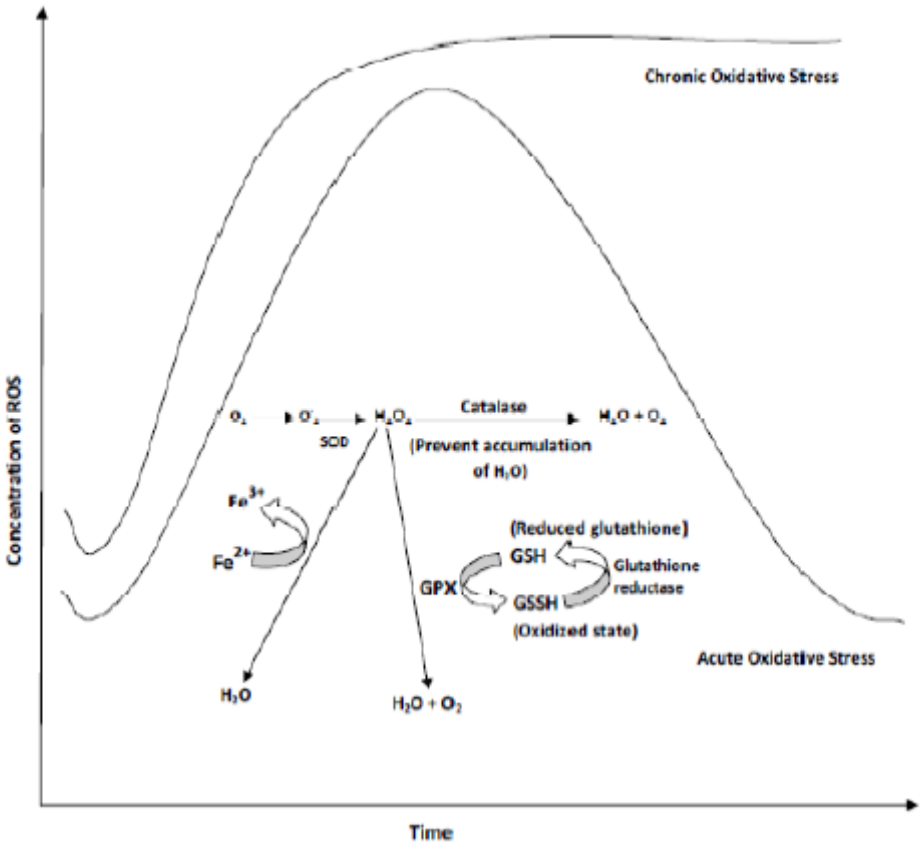

Figure 1. Acute and chronic oxidative stress in Rheumatoid arthritis

ROS = Reactive oxygen species; SOD = Superoxide dismutase; GPX = Glutathione peroxidase; GSH= Reduced glutathione; GSSH =glutathione disulphide, H_2O_2 = Hydrogen peroxide

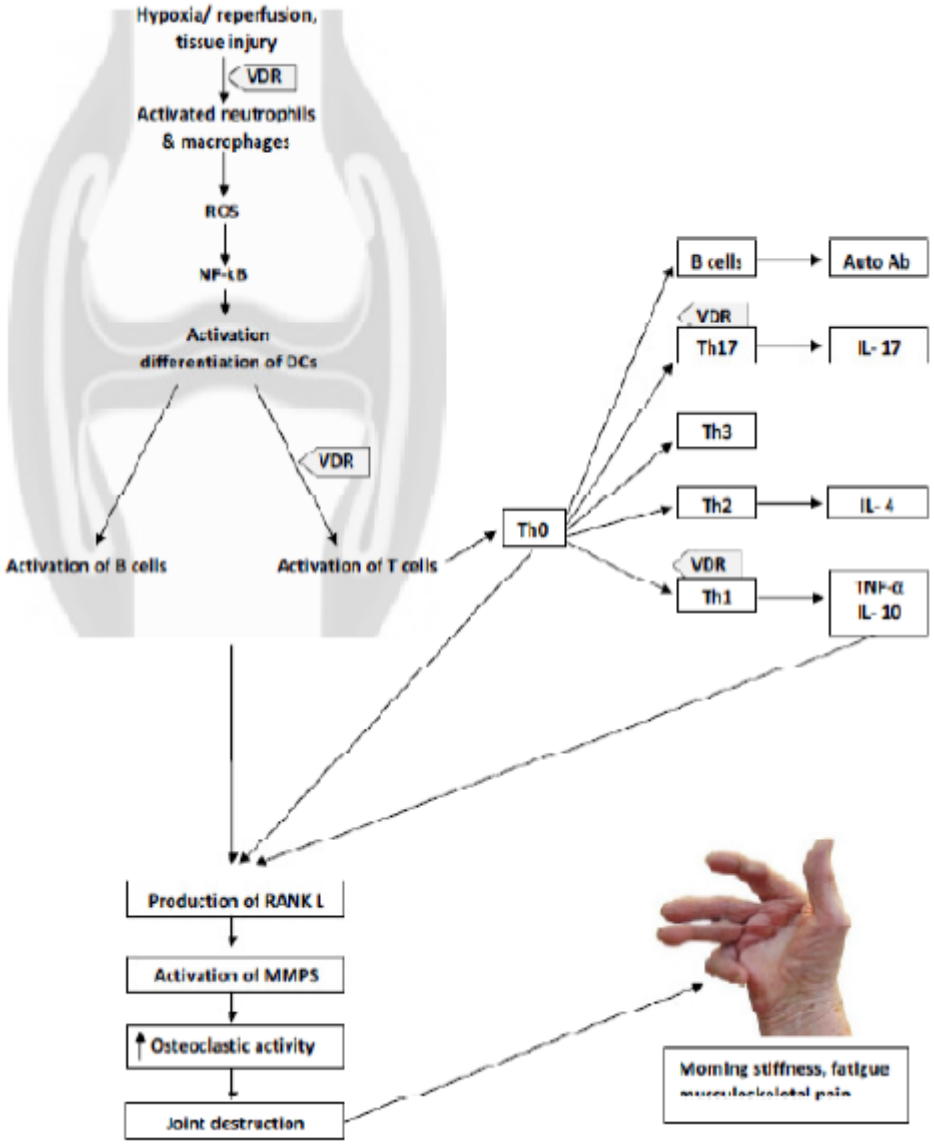

Figure 2. Impact of oxidative stress and vitamin D in Rheumatoid arthritis.

ROS = Reactive Oxygen species; NF-kB = Nuclear factor Kappa Beta; TNF-α=Tumor Necrosis Factor-α**;** IL = Interleukins; B cells = Beta cells; Auto Ab = Auto Antibody; VDR = Vitamin D Receptor; DC = Dendritic cell; Th = Helper T cells; RANKL = Receptor activator of nuclear factor kappa- B-ligand; MMP = Matrix Metalloproteinase.

Previous studies have highlighted oxidative stress in RA patients, but the results are controversial. To the best of the knowledge of the authors there is no study which compares oxidative stress status in patients who had not taken any pharmacological treatment with RA patients who are on methotrexate (MTX) monotherapy and healthy subjects. This study aims at estimating the oxidative stress markers and vitamin D status in RA and their relation to disease activity.

MATERIALS AND METHODS

Subjects and methods

The study was conducted in PSG Hospitals, Coimbatore, Tamilnadu, India. The inclusion criteria were RA diagnosis by rheumatologist according to 1987 American College of Rheumatology guidelines. Twenty four RA patients on MTX monotherapy for less than two years and fourteen RA patients who had not taken any pharmacological treatment were included. Twenty age-matched healthy subjects confirmed by rheumatologist, were considered as controls. The exclusion criteria were cardiovascular disease, diabetes, hyperthyroidism, Sjogren's syndrome, systemic lupus erythematosus, and osteoarthritis. Patients on antioxidant supplements and vitamin D_3 were omitted from the study.

The study was ethically approved by ethics committee of PSG Institute of Medical Sciences & Research (Ethical Committee Approval No: 13/111). All the participants of the study gave written informed consent. The physician measurements included tender joint count (TJC), and swollen joint count (SJC). The laboratory measurements included erythrocyte sedimentation rate (ESR). The disease activity score (DAS) was measured by online calculator [5].

Totally 5ml blood was drawn from antecubital vein from patients and controls. Samples were collected in ethylene diamine tetra acetic acid (EDTA) coated tubes.

Antioxidant assays were performed in the hemolysate prepared from the samples. Hemoglobin levels were measured spectrophotometrically [6]. The SOD was estimated using Kakkar *et al* method [7], CAT was estimated using Beers and Sizer method [8], reduced glutathione was estimated using Jollow *et al* method [9] and thiobarbituric acid reactive substances (TBARS) was estimated using Ohkawa method [10].

Vitamin D_3 was estimated in plasma by high performance liquid chromatographic (HPLC) liquid-liquid extraction technique, by using earlier reported method, with slight modification [11]. The C-18 column was the stationary phase and the mobile phase was methanol: isopropanol: water (88:10:2), with a flow rate of 1.6 ml/min. The injection volume was 20 µl and the absorbance of the eluent is monitored at 265 nm in ultraviolet-visible (UV) 2489 detector. The software used for vitamin D estimation was Empower 2.

Statistical analysis was done using Graph pad prism 4.03 version software. The data were expressed as Mean ± Standard Error of Mean (SEM). One-way analysis of variance (ANOVA) followed by post-hoc Bornferroni t test was applied. $P < 0.05$ was considered as statistical significance.

RESULTS

The age group of patients was 55.0 ± 6.30 (Mean ± SD) years, which was similar Between all the groups. The demographic characteristics of the participants were represented in Table 1.

Table-1 Demographic, Biochemical and clinical characteristics of Healthy, RA naïve and RA treated

Parameters	Healthy	RA Naïve	RA Treated	F value
Age	55.0 ± 6.30	51 ± 13.50	47.042 ± 12.067	2.941
Number of Participants	20	14	24	NA
Sex (F/M)	12/8	10/4	12/2	NA
Hb (gm / dl)	14.3 ± 2.20	10.982 ±1.39***	11.392 ± 1.580***	19.56
ESR (mm/hr)	6.75 ± 2.25	79 ± 35.81***	42.583 ± 21.538*** ###	43.61
SOD (U/gm Hb)	0.490 ±0.165	1.006 ±0.212***	0.450 ± 0.135###	50.75
CAT (µM of H_2O_2 utilised)	0.328±0.116	0.167±0.118***	0.284±0.06##	11.67
GSH (µM/gm Hb)	2.56 ± 0.954	0.236 ±0.086***	0.596 ± 0.098***	78
TBARS (nmoles/gm Hb)	110.83 ± 16.95	570.34 ± 135.06***	371.014 ± 86.996*** ###	119.3
TJC	-	5.625 ± 3.29	14.292 ± 8.951**	NA
SJC	-	5.875 ± 3.40	12.208 ± 9.921*	NA
DAS28-ESR	-	5.73 ± 0.99	5.438 ± 1.096	P = 0.417

F=Female; M=Male; Hb=Hemoglobin; RBC=Red Blood Cells; ESR=Erythrocyte Sedimentation Rate; SOD=Super Oxide Dismutase; CAT=Catalase; GSH=Reduced Glutathione; TBARS=Thio Barbituric Acid Reactive Substance; TJC=Tender Joint Count; SJC=Swollen Joint Count; DAS=Disease Activity Score; RA=Rheumatoid Arthritis; P<0.05* is considered as significant ** P<0.01*** ###P<0.001*Comparison with healthy subjects #Comparison with Rheumatoid arthritis treatment naïve patients. NA=Not applicable

The hemoglobin levels were found to be significantly lower in the RA patients group, when compared to RA patients on MTX and who were not on pharmacological treatment (P < 0.001).

The levels of SOD was found to be significantly elevated in the treatment naïve RA patients when compared to both RA patients on MTX and healthy controls (P < 0.001). The levels of CAT were found to be significantly reduced in the treatment naïve RA patients when compared to RA patients on MTX and healthy controls (p <0.001). The levels of reduced glutathione was found to be significantly lower in RA patients not on pharmacological treatment (P < 0.001).

The levels of TBARS were found to be significantly elevated in patients group, both naïve patients and those on treatment (P < 0.001). The TBARS and ESR levels were significantly elevated (P < 0.001) in the RA patients who had not taken any pharmacological treatment when compared to patients on MTX therapy and healthy controls.

The number of TJC (P<0.01) and SJC (0.05) significantly elevated in the RA patients on MTX (P < 0.01), when compared to the RA patients who were not on pharmacological therapy. The levels of disease activity represented by disease activity score (DAS) were similar in the RA patients group.

Table-2 Vitamin D3 levels in the plasma samples of rheumatoid arthritis patients on methotrexate monotherapy

Vitamin D (ng/ml)	N	Insufficient <30	Deficient 31-35	Healthy >50	Mean ± SD
	24	19 (79)	4 (17)	1 (4)	20 ± 11.60

N=Number of study subjects

Table-3 Correlation of Vitamin D3 levels and the disease activity parameters of rheumatoid arthritis patients on methotrexate therapy

	TJC	SJC	DAS
Vitamin D	$R^2 = 0.586$	$R^2 = 0.525$	$R^2 = 0.860$

R^2 = Regression coefficient, TJC=Tender Joint Count; SJC=Swollen Joint Count; DAS=Disease Activity Score

Vitamin D levels were found to be insufficient in most of the RA patients, which was as great as 79% (Table 2). A strong negative correlation was found with vitamin D levels and disease activity parameters such as TJC, SJC and DAS (Table 3).

DISCUSSION

Generation of ROS from hypoxia, reperfusion and tissue injury leads to leakage of neutrophils and macrophages. The oxidative burst in the synovium releases proinflammatory cytokines such as TNFα and interleukins which stimulate matrix metalloproteinases and cause erosion of the joint. Excessive ROS or insufficient antioxidant defense could contribute to chronic inflammatory pathogenesis of RA, which cause multiple clinical symptoms such as fatigue, morning stiffness and loss of functional ability. Another characteristic feature of RA is painful joints which are related to the deficiency of vitamin D. Thus it is important to assess the oxidative stress and levels of vitamin D to know the contributing features of pathogenesis and better clinical management of RA. Estimation of vitamin D is not done in routine clinical practice in developing countries like India, due to cost constraints. With this idea the current study investigated the levels of antioxidant enzymes in treatment naïve and RA patients on MTX monotherapy and compared with healthy subjects. The patients on MTX therapy were also taken up for assessment of vitamin D levels, because in routine clinical practice it is not measured, in spite of importance of vitamin D in RA.

In the present study the levels of oxidative stress was found to be high in both treatment naïve RA patients and those on MTX therapy. In the current study the levels of SOD were significantly elevated in treatment naïve RA patients. Similar to our reports the SOD levels were found to be increased in newly diagnosed RA patients [12]. SOD is the first line of defense and the elevation indicates a compensatory mechanism to scavenge the ROS and dismutates it to hydrogen peroxide. Elevated SOD also indicates excessive production of hydrogen peroxide. It has been postulated that levels of SOD determines the treatment progression and in the current study, patients on MTX therapy have reduced SOD when compared to naïve RA patients.

CAT is the second line of defense and the present study highlights the levels were greatly decreased in naïve RA patients when

compared to both healthy and RA patients on MTX therapy. Previous findings also support this and could be attributed to depletion of CAT as a compensatory response by the antioxidant defense mechanism to scavenge the excessive hydrogen peroxide liberated [13].

The excessive hydrogen peroxide could be handled in another pathway by iron released from hemoglobin of lysed erythrocytes. It is evident from the current study that both naïve and RA treated patients are anemic and thus free radical damage could occur in RA patients. This finding corroborates with previous results [14].

Reduced glutathione levels were found to be reduced in RA patients that indicate increased oxidative stress and our findings are similar to previous reports [15]. Glutathione is an important thiol antioxidant having anti-inflammatory role and regulates NF-κB and expression of several cytokines and chemokines, has a role in leukocyte trafficking, and prevents accumulation of free radicals. In the present study a significant negative correlation was observed between GSH and ESR in treatment naïve RA patients (data not shown).

Oxidative stress is found to be elevated in RA patients both naïve and treated subjects of the present study, indicated by increased levels of TBARS. The TBARs levels were very high in treatment naïve RA patients, in comparison with treated patients. Previous studies proved increase in TBARS levels could be attributed to increased production of free radical mediated adducts, and consequent leakage into the circulation which can trigger immunogenic response in the synovium [16]. The other postulated reasons for elevation of LPO are hypoxia mediated glucose-deficiency of synovium and accumulation of cyclooxygenase by-products [17].

In MTX treated patients the oxidative stress levels were less when compared to naïve patients. Since MTX possess multiple mechanisms which could modulate the immunological response, the immune mediated proinflammatory cytokine release and cytokine stress leading to oxidative stress could have been reduced in the MTX treated patients. But, the disease activity was similar between treatment naïve and MTX treated patients.

In the present study also a strong negative correlation was found with the disease activity parameters and vitamin D deficiency. Most of the RA patients on MTX therapy have insufficient levels of vitamin D_3. Vitamin D deficiency could have led to increased disease activity in MTX treated RA patients. Vitamin D deficiency is common and routine screening is essential to address this deficiency. Previous studies have reported that, for an increment of 10 ng/ml of vitamin D the DAS values reduce 0.3 and CRP levels reduce by 25% [18].

Recent studies indicate that antioxidant supplements could alleviate oxidative stress but does not decrease the TJC and SJC, which could be due to vitamin D deficiency [19]. Dietary intake of antioxidant vitamins such as vitamin C, vitamin E and β-carotene are the best nutritional supplements to alleviate the tissue injury created through oxidative stress [20].

In conclusion, the light of previous findings it is possible to conclude that increased oxidative stress have led to compensatory changes in antioxidant enzymes, but not sufficient to protect against formation of deleterious lipid endoperoxides in naïve RA patients and deficiency of vitamin D has led to worsening of functional status of MTX treated patients. Thus for effective management of RA nutrition could play an important role. Dietary intervention or supplementation with antioxidants could

Alleviate oxidative stress in RA patients not on pharmacological treatment and supplementation with vitamin D_3 in patients on monotherapy with MTX could be beneficial to improve the treatment outcomes.

CONFLICT OF INTEREST

The Author(s) declare(s) that they have no conflicts of interest to disclose.

ACKNOWLEDGEMENTS

The authors are grateful and thanks to Dr.V.N.Nagaprabu, Dr. Ramanujam, and Dr. B. Balaji for their valuable suggestions and support and technical assistance.

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