Original article

Serum nitric oxide and malondialdehyde as potential activity and severity markers of oxidative stress in juvenile idiopathic arthritis

Background: *The imbalance between the oxidant and antioxidant defense systems may play a role in the etiopathogenesis of juvenile idiopathic arthritis (JIA).Nitric oxide (NO) is a free radical and malondialdehyde (MDA) is a product of polyunsaturated fatty acid peroxidation and both are possibly implicated in the pathogenesis of chronic synovitis.* ***Objective:*** *We sought to investigate the role of serum NO and MDA as markers for oxidative stress in JIA and their relation to activity and therapeutic modalities used.* ***Methods:*** *This comparative prospective study included 20 children with pediatric JIA enrolled consecutively from the Pediatric Allergy, Immunology and Rheumatology Unit, Children's Hospital, Ain Shams University. They were compared to 20 matched healthy control subjects. They underwent clinical evaluation and measurement of serum NO and MDA by enzymatic immunoassay was performed in both groups.* ***Results:*** *A significant positive correlation was found between serum NO and MDA concentrations and JIA activity (p=0.0150; p=0.037 respectively).Patients who had arthralgia, arthritis and/or morning stiffness had significantly higher Serum NO and MDA concentrations. According to ROC analysis, serum NO level above 158.9 µ mol/L and serum MDA level above 6.75 m mol/L had 100% sensitivity and specificity in prediction of disease activity (AUC= 0.99-1.0).Patients treated with methotrexate (MTX) had significantly higher MDA concentrations and a significant positive correlation between serum NO and MTX dosage.* ***Conclusion:*** *Serum NO and MDA levels were significantly elevated among patients during disease activity which may suggest a significant role in the etiopathogenesis of JIA. Further studies are needed to validate their usefulness as predictors of JIA outcome.*

Keywords: Juvenile Idiopathic Arthritis, Oxidative Stress, Nitric Oxide, Malondialdehyde.

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INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common connective tissue disease of childhood with onset before the age of 16 years. It is characterized by leukocyte infiltration in the synovium leading to a chronic inflammation in the joints, which persists for more than 6 weeks, with consequent destruction of articular tissue.[1] In the oligoarticular and polyarticular forms of JIA, dysregulated adaptive immunity is a likely factor in disease pathogenesis. In the systemic form of JIA, however, dysregulation of innate immune pathways appears more central to disease pathogenesis, resulting in augmented levels of interleukins IL-1β, IL-6, and IL-18. A final common pathological pathway in JIA is the activation of monocytes and neutrophils which are the principal mediators of joint inflammation and damage.[2]

Apart from the immunological reaction, Oxidative stress is involved in the pathogenesis.[3,4] Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS), Reactive Nitrogen Species (RNS) and a cell's antioxidant networks to scavenge these excessively produced reactive intermediates or to repair the resulting damage of all components of the cell, including proteins, lipids, and DNA.[5, 6,7]

Neutrophils are known to contribute to rheumatoid arthritis pathogenesis by the release of oxygen radicals and tissue-degrading enzymes, which can lead to the degradation of the articular cartilage.[8,9] Overproduction of tumor necrosis factor alpha (TNF-α) is thought to be the main contributor to increased ROS release in RA patients[10] that amplify the synovial inflammatory-proliferative response.[11]

RNS are derived from the oxidation of the guanidonitrogen of L-arginine, with the production of a nitrogen-centered radical, nitric oxide (NO), by the enzyme nitric oxide synthase (NOS) in various cells as chondrocytes, synoviocytes and leukocytes.[12] NO is a short lived gaseous free radical that plays an important role at the site of synovial inflammation, including cytokine production, signal transduction, mitochondrial functions and apoptosis.[13] The effect of NO production on cellular processes is largely dependent on its concentration and the local presence of other free radicals.[14,15]

Raised levels of reactive nitrogen species in serum and synovial fluid have been reported in patients with rheumatoid arthritis.[16] NO Effects such as vasodilation with increased permeability, stimulation of tumor necrosis factor- (TNF-α) and interleukin 1 (IL-1) release and the angiogenic activity leading to chronic synovitis with cartilage damage suggest that NO metabolites (nitrite and nitrate) inside the synovium may be acting as pathogenic mediators in adult RA.[17] T cells from Rheumatoid arthritis patients produce more than 2.5 times more NO than healthy donor T cells in addition genetic factors including endothelial nitric oxide synthase (eNOS) were implicated in pathogenesis of rheumatoid arthritis.[13,18]

Lipids are susceptible targets of oxidation and lipid peroxidation products are potential biomarkers for oxidative stress.[19] Lipid peroxidation generates a variety of relatively stable decomposition end products, mainly unsaturated reactive aldehydes, such as Malondialdehyde (MDA) which is the principal and most studied product of polyunsaturated fatty acid peroxidation that can be shown to increase following oxidative stress.[20] MDA is reactive and potentially mutagenic and can be measured in various biological samples (serum/plasma and urine) as an indirect index of oxidative stress.[21,22] Matrix degradation arising from cytokine-stimulated chondrocytes was shown to be primarily due to lipid peroxidation.[23]

JIA patients presented concentrations of plasma MDA significantly higher than those of controls.[24] Vasanthi et al. described an increase in the MDA concentration in adult patients with rheumatoid arthritis contributing to connective tissue destruction with subsequent articular and periarticular deformities.[25] An understanding of the complex interactions of ROS might allow the development of novel therapeutic strategies for RA.[11]

We sought to evaluate serum nitric oxide and malondialdehyde levels in JIA patients as markers for oxidative stress in relation to different JIA categories, disease activity, degree of joint destruction and therapeutic modalities received. Our ultimate objective is to highlight the contribution of routine antioxidants or future modalities to the disease course and severity.

METHODS

This controlled prospective study was conducted on patients diagnosed as JIA, following up at the Pediatric Allergy, Immunology and Rheumatology Unit, Children's Hospital, Ain Shams University during the period from July 2014 to December 2014. The study sample comprised 20 patients who were enrolled consecutively and 20 age and sex matched healthy children as a control group enrolled from the Outpatient Clinic Children's Hospital, Ain Shams University after exclusion of any history of rheumatological illness or family history of it.

All patients enrolled met the American College of Rheumatology (ACR) criteria and International League of Association for Rheumatology (ILAR) classification criteria of JIA.[26] Patients with allergic, parasitic, active infectious disease, psoriatic arthritis, or enthesitis-related arthritis were excluded as well as those receiving more than 20 mg/day of prednisone or equivalent to avoid any interference of corticosteroids on nitric oxide production.[27]

Ethical consideration: A consent was obtained from each patient legal guardian before enrollment in the study. The study protocol was accepted by the local ethical committee of the Pediatric Department, Ain Shams University.

Study methods:

Clinical evaluation

Detailed medical history was recorded concerning demographic data including; age, gender, parental consanguinity, family history of similar conditions or other rheumatologic illnesses, age of disease onset, disease duration, disease activity record; relapses form, frequency, detailed medication history; corticosteroids, non-steroidal anti-inflammatory (NSAIDS), disease modifying anti-rheumatological drugs (DMARDS) and biological therapy such as {anti-TNF- monoclonal antibody, IL-1 receptor antagonist}and response to treatment, co-morbid illnesses; diabetes, hypertension and obesity.

Each patient was evaluated for: Physician's global assessment of overall disease activity, which was measured on a 10 cm visual analog scale (VAS, 0 = no activity and 10 = maximum activity);

Parent's global assessment of the child's overall well-being, which was measured on a 10 cm VAS (0 = very good and 10 = very poor), number of joints with pain upon movement/tenderness; number of swollen joints; number of joints with limited range of motion; number of joints with active arthritis (defined as the number of joints with swelling or with limitation of movement and pain upon movement/tenderness); duration of morning stiffness; laboratory variables: erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level. The above criteria were gathered to score disease activity level according to American College of Rheumatology recommendations.[28] Patients were followed up clinically and laboratory every 2 months to detect any disease flare.

Laboratory investigations:

Complete blood count (CBC) using coulter counters (Hematology Analyzer, Coulter Corporation, Miami, FI 33116.9015) as well as Leishman-stained peripheral blood film examination for differential white blood cell counting. C-reactive protein (CRP) was assayed using nephelometric method (Dade Behring BN II, USA). Erythrocyte sedimentation rate (ESR) was measured by the Westergren method. Serum lipid profile (triglycerides, cholesterol, LDL and HDL) was assayed by synchron CX7 autoanalyzer, Bechman instrument, Brea, California, USA. Rheumatoid factor (RF) was determined by the Latex agglutination technique and antinuclear antibodies (ANA) were measured by ELISA (ORGENTEC Diagnostika GmbH, Germany).

Serum Nitric oxide (NO) assay for patients and healthy controls**:** Serum Nitric oxide (NO) were measured by enzyme linked immunosorbent assay (ELISA Kit: Biotechne, USA) with a kit Detection range of 3.12 - 200 umol/L.

Serum Malondialdehyde (MDA) for patients and healthy controls**:** Serum Malondialdehyde (MDA) will be measured by enzyme linked immunosorbent assay (ELISA Kit: Kamiya Biomedical Company, Seattle, WA, USA) with a kit range of 0.3mmol/L - 7mmol/L.

Statistical analysis

The collected data were revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Data are presented as mean, standard deviation (± SD) and range for parametric numerical data as well as median and interquartile range (IQR) for non-parametric numerical data. The Student T Test was used to compare means and ANOVA test was used to assess the difference between more than two group means. Post Hoc Test was used for comparison of pairs of group means. Correlation analysis was used to assess the strength of association between two quantitative variables. The ROC Curve (receiver operating characteristic) was employed to evaluate the sensitivity and specificity of the quantitative test measures. Probability (p) values <0.05 were considered significant.

RESULTS

The patients were enrolled consecutively and comprised one patient with extended Oligo-articular onset, four patients with poly-articular onset and 15 patients with systemic onset juvenile idiopathic arthritis (SoJIA). The demographic, clinical and Laboratory are displayed in table 1. Ages of the enrolled patients ranged from 3 to 15 with mean ± SD of 7.58 ± 3.15 years. They comprised 11 male (55%) and 9 females (45%). Obesity was detected in 25% of cases and two patients had associated co-morbidity (IDDM in one and hypertension in another). The mean disease duration was 2.47±2 months. Arthralgia, arthritis, and morning stiffness of affected joints was detected among 65%, 30% and 45% of patients respectively. Disease activity was low in 50% of cases, moderate in 40% and severe in 10%. Among our series, 85% were receiving corticosteroids, 60% methotrexate while biological agents were used in 10% of cases.

The laboratory evaluation revealed anemia, leukocytosis, and thrombocytosis in 45% of cases, elevated C-reactive protein (CRP) in 65%, and elevated ESR in 60%. Rheumatoid factor (RF) positivity was detected in 15%, while the antinuclear antibody (ANA) was positive in 10%, and dyslipidemia was observed in 50% of cases. Serum NO and MDA levels were significantly higher among cases compared to controls (P= 0.0001). with cases showing higher mean NO (260.2 Vs 104.4) and higher mean MDA levels (14.2 Vs 2.3) (table 2).

Among the JIA patients, although serum NO and MDA concentrations were comparable among the JIA subtypes, a significant positive correlation was found between serum NO and MDA concentrations and JIA activity (p=0.0150; p=0.037 respectively). Patients who had arthralgia, arthritis and/or morning stiffness had significantly higher Serum NO and MDA concentrations. According to ROC analysis, serum NO level above 158.9 µ mol/L and serum MDA level above 6.75 m mol/L had 100% sensitivity and specificity in prediction of disease activity (AUC= 0.99-1.0).

Analysis of our data revealed no significant correlation between serum NO and MDA concentrations and gender or diseases duration. The relation between serum NO and MDA and different laboratory parameters among cases are shown in table 3. Patients who were treated with methotrexate (MTX) had significantly higher MDA concentrations and there was a significant positive correlation between serum NO and dosage of MTX (10, 13 and 15mg/m²); figure 1. Serum NO and MDA concentrations did not vary significantly with other treatment modalities.

Table 1. Descriptive data of the JIA group

Age (mean ± SD)		7.58±3.15	
Sex	Male (n %)	11	55.0%
	Female (n %)	9	45.0%
Consanguinity	Yes (n %)	2	10.0%
	No (n %)	18	90.0%
Co-morbidity	Positive (n %)	2	10.0%
	Negative (n %)	18	90.0%
Family History	Positive (n %)	0	0.0%
	Negative (n %)	20	100.0%
Activity	Low (n %)	10	50.0%
	Moderate (n %)	8	40.0%
	Severe (n %)	2	10.0%

Table 2. Variation of serum NO and MDA levels in the studied sample

Parameter	**Group**				**P**
	JIA		**Control**		
	Mean	±SD	Mean	±SD	
Serum NO	260.29	32.60	104.45	12.86	0.0001*
Serum MDA	14.20	3.78	2.38	0.84	0.0001*

*Highly significant

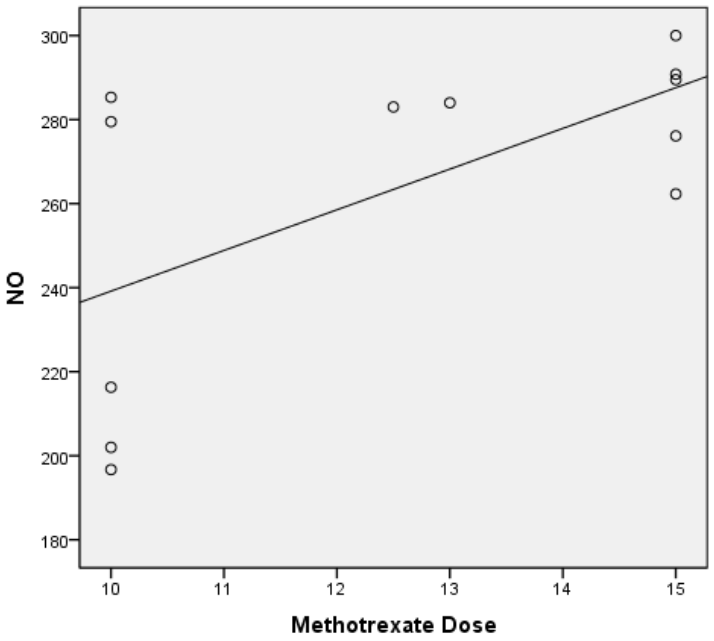

Figure 1. Positive correlation between serum NO and methotrexate dose.

Table 3. Relation of serum NO and MDA results to other laboratory data in the JIA patients

Studied Parameters			Mean	±SD	p
Serum NO	CBC	Abnormal	264.69	37.53	0.599
		Normal	256.70	29.34	
	CRP	Normal	227.91	27.64	0.0001*
		High	277.73	18.96	
	ESR	Normal	229.84	26.16	0.0001*
		High	280.60	16.60	
	RF	Positive	235.87	30.83	0.165
		Negative	264.60	31.80	
	ANA	Positive	222.65	29.20	0.085
		Negative	264.48	30.86	
	Dyslipidemia	Positive	272.87	25.66	0.084
		Negative	247.72	35.12	
Serum MDA	CBC	Abnormal	15.07	3.84	0.365
		Normal	13.48	3.76	
	CRP	Normal	11.13	0.87	0.004*
		High	15.85	3.71	
	ESR	Normal	11.31	0.96	0.002*
		High	16.12	3.74	
	RF	Positive	12.17	0.45	0.326
		Negative	14.55	4.00	
	ANA	Positive	12.15	0.64	0.435
		Negative	14.42	3.92	
	Dyslipidemia	Positive	16.06	3.04	0.023*
		Negative	12.33	3.63	

* Significant

DISCUSSION

The studied JIA patients had significantly higher levels of serum NO and serum MDA as compared to healthy controls. That results agreed with several studies in JIA and adult RA patients.[16, 29-33] We did not observe a significant relation between serum NO and serum MDA levels and the subtype of JIA onset whether systemic onset, poly- or oligo-arthritis. However, our sample was not evenly distributed among the various subtypes (one patient with Oligo-articular, four patients with polyarticular and 15 patients with systemic onset JIA) and this hinders the value of comparison. Similar results were reported in some other studies.[16,30] On the contrary, Bica et al.[34] noted that the highest serum NO levels were detected in patients with seropositive polyarticular onset (positive RF) while the highest MDA concentrations were observed among patients with systemic onset JIA.[35]

On the other hand, parameters of oxidative stress were higher in extensive oligoarticular sub-groups compared to Juvenile enthesitis-related arthritis and polyarticular JIA[36]**.**

Among our sample of patients, serum NO and MDA were significantly related to and going along with the level of disease activity with the highest concentrations observed in JIA cases suffering from utmost activity and this agrees with relevant studies.[29,34,37] The results of these studies along with our study confirmed the evident role of oxidative stress in the pathogenesis of JIA with a strong correlation between the serum and synovial fluid levels of NO and the severity of the disease, variability and the degree of joint damage. In addition, serum NO and MDA levels above 158.9 µ mol/L and 6.75 m mol/L respectively had excellent sensitivity and specificity in the prediction of disease relapse (AUC = 0.99-1.0). Serum NO and MDA had the highest significant correlation to the clinical stigmata, namely arthritis and morning stiffness as well as to the laboratory acute phase reactants CRP and ESR. A significant positive correlation was previously reported in RA between JADAS-27 (a score for disease activity estimation) and the ESR and serum NO levels [30] and this was agreed upon in several other publications.[14,16,34]

A significantly elevated levels of lipid peroxidation in RA in correlation with CRP and ESR levels was previously reported [38] and this augments our observation and may point to the

assumption that these parameters can serve as surrogate markers for disease activity.[39] The positive correlation between serum NO and disease activity in RA was significantly confirmed from some other publications.[29,40, 41]

We observed a significant positive relation between serum MDA level and dyslipidemia among cases of JIA. In agreement, a highly significant positive correlation between MDA and LDL-cholesterol was previously reported.[42] This may be explained by a relation between JIA activity and hepatic lipid handling owing to the effect of the hepatic cytokine signaling alteration as for instance the reduction of lipoprotein lipase activity by TNF-α [43] or may reflect the effect of corticosteroids on the lipid profile [44]. In addition, patients with active RA have a dysfunctional proinflammatory HDL, lacking the antioxidant capacity of conventionally cardioprotective HDL [45]. The finding is limited by the sample size.

In the current study, patients who received methotrexate had significantly higher serum MDA level reflecting disease activity and/or severity that necessitated the addition of methotrexate as a disease modifying anti-rheumatic drug (DMARD) to the therapeutic regimen. This may augment a previous observation that many DMARDs have an influence on oxidative stress.[46] We also observed a significant positive correlation between the serum NO expression and serum methotrexate dosage in an escalating manner which may suggest a similar effect for NO on the therapeutic decision making in JIA.

Supporting this perspective, lipid peroxidation products significantly decreased post methotrexate therapy in RA patients indicating a possible antioxidant role of methotrexate in these patients [47].

The serum NO and/or MDA expression did not vary according to corticosteroid or anti-TNF-α biologic administration (infliximab or etanercept) in the current study. On the contrary, studies on adult rheumatoid arthritis reported significant relations between these therapeutic modalities especially etanercept and the reduction of oxidative stress markers.[48-50] The age difference and overexpression of the SoJIA subtype in our sample may count for the difference.

TNF-α inhibitors was found to reduce levels of inflammatory markers (CRP and pro-inflammatory cytokines) and induce some potential beneficial changes in lipid levels in JIA.[51] In another perspective, adult patients undergoing anti-TNF-α treatment for severe RA, still had elevated serum NO levels indicating that NO may be acting at a step that precedes the production of TNF-α.[52]

Our study has several limitations. First, the results are limited by the sample size. Second, the consecutive manner of case enrollment led to unequal distribution of the JIA subtypes among the studied series, and this hindered the interpretation of some correlations. A stratified non-random sampling might be suitable in face of the small sample size. A long-term prospective design would be more informative in terms of analyzing the relation to therapeutic modalities used.

In conclusion, the significantly higher serum NO and MDA levels among JIA patients than the healthy controls with the highest levels in those with active disease supports the validity and the role of those oxidative stress markers in the disease etiopathogenesis. Further studies on a wider scale are needed to strengthen the finding of this study and for better understanding of the role of these biomarkers as predictor of outcome of JIA patients.

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