Nitric Oxide, Peroxynitrite and Malondialdehyde Levels as Markers for Nitrosative/Oxidative Stress in Iraqi Patients with Systemic Lupus Erythematosus

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Abstract

Systemic lupus Erythematosus is an autoimmune disease of unknown aetiology affecting multiple organ system. Reactive nitrogen and oxygen species are claimed to play a role in this disease. However, the potential of Nitrosative/Oxidative Stress to elicit an autoimmune, response remain till now largely unexplored in humans. This study was done to investigate the status and contribution of nitrosative/oxidative stress in Iraqi patients for systemic lupus erythematosus. Blood samples from 19 patients with systemic lupus erythematosus and 19 age-and sex- matched apparently healthy controls were evaluated for serum levels of nitrosative/oxidative stress markers including nitric oxide, peroxynitrite and malondialdehyde. Nitric oxide levels were measured by spectrophetometric method depending on Griss method, while peroxynitrite levels were measured by spectrophetometric method based on peroxynitrite mediated nitration of phenol. Malondialdehyde levels were measured by the thiobarbitoric acid method. Serum nitric oxide levels were significantly elevated in SLE patients (mean $\pm$ SE 263.58 $\pm$ 35.42 µmol/L) as compared with healthy control (162.48 $\pm$10.42 µmol/L). Peroxynitrite levels were also significantly elevated in a disease group (mean $\pm$ SE 7.23 $\pm$0.92 µmol/L) as compared to healthy control (4.47 $\pm$ 0.38 µmol/L). On the other hand, malondialdehyde levels were slightly elevated in SLE patient (mean $\pm$ SE 4.53 $\pm$ 0.22 nmol/ml) as compared to control group (4.32 $\pm$ 0.58nmol/ml). The study findings support an association between nitrosative/oxidative stress and SLE through elevated level of NO, peroxynitrite and MDA in the serum of SLE patients.

Key words: Nitric oxide, Peroxynitrite, SLE.

قياس مستويات أوكسيد النتريك والبيروكسي نايترات والمالون داي الدهايد كعلامات لفرط النترجة والأكسدة عند المرضى العراقيين المصابين بداء الذئب الإحمراري ألجهازي

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الخلاصة

داء الذئب الإحمراري الجهازي هو مرض مناعي مسبباته غير معروفة ويصيب العديد من أجهزة الجسم. مسببات تفاعلات النترجة والأكسدة يُظن أن تلعب دوراً في هذا المرض، لكن طريقة استثارتها للجهاز المناعي الذاتي غير واضحة لحد الآن بالإنسان. هذه الدراسة أُجريت لمعرفة حالة مسببات النترجة والأكسدة في المرضى العراقيين المصابين بداء الذئب الإحمراري الجهازي. عينات الدم سُحبت من ١٩ مريضاً بداء الذئب الإحمراري الجهازي وكذلك من ١٩ من الأصحاء ظاهرياً المتماثلين بالعمر والجنس ليكونوا مجموعة السيطرة من الأصحاء، وذلك لتقييم تركيز علامات مسببات فرط النترجة والأكسدة والتي تشمل أوكسيد النتريك والبيروكسي نايترايت والمالون داي الدهايد. لقد تم قياس تركيز أوكسيد النتريك باستعمال طريق كرس باستخدام مقياس الضوء الطيفي وكذا تم قياس البيروكسي نايترايت بطريقة مقياس الضوء الطيفي المعتمدة على نترجة الفينول بالبيروكسي نايترايت، أما المالون داي الدهايد فقد تم قياسه بطريقة حامض الثايوباربجيورك. إن تركيز أوكسيد النتريك في مصل الدم لمرضى داء الذئب الاحمراري الجهازي قد كان مرتفعاً ارتفاعاً معنوياً (المعدل $\pm$ معيار الخطأ)(٢٦٣.٥٨ $\pm$ ٣٥.٤٢ مايكرومول/لتر) مقارنةً بمجموعة السيطرة من الأصحاء (١٦٢.٤٨ $\pm$ ١٠.٤٢ ميكرومول/لتر). تركيز البيروكسي نيترايت أيضاً كان مرتفعاً ارتفاعاً معنوياً عند مجموعة المرضى (٧.٢٣ $\pm$ ٠.٩٢ مايكرومول/لتر) مقارنةً بمجموعة السيطرة من الأصحاء (٤.٤٧ $\pm$ ٠.٣٨ مايكرومول/لتر). من ناحية أخرى، تركيز المالون داي الدهايد كان مرتفعاً ارتفاعاً طفيفاً عند مرضى داء الذئب الاحمراري الجهازي (٤.٥٣ $\pm$ ٠.٢٢ نانومول/مل) مقارنةً بمجموعة السيطرة (٤.٣٢ $\pm$ ٠.٥٨ نانومول/مول). نتائج الدراسة بينت أن هناك علاقة بين فرط النترجة والأكسدة ومرض داء الذئب الاحمراري الجهازي من خلال ارتفاع مستوى أوكسيد النتريك والبيروكسي نايترايت والمالون داي الدهايد في مصل الدم لدى المصابين بالمرض.

الكلمات المفتاحية : أوكسيد النتريك ، البيروكسي نايترات ، داء الذئب الإحمراري ألجهازي.

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Received : 8/1/2012
Accepted : 1/4/2012

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown aetiology affecting multiple organ system[1]. The most remarkable feature of SLE is autoantibody production, a function of the acquired immune response. However, an inappropriately active and sustained innate immune response is implicated in both the initiation and the pathogenic consequences of the autoantibody production in SLE[2]. An important part of the innate immune response is the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS)[3].Reactive nitrogen species include nitric oxide (NO) and peroxynitrite ($ONOO^-$), while reactive oxygen species include superoxide (SO) and hydrogene peroxide (H_2O_2). Nitric oxide is a biological messenger mediating many important physiological functions but also pathological process. It plays a vital role in host defense and immunity by modulating inflammatory processes[4].It's synthesized from L-arginine by both a constitutive NO synthase (cNOS) & inducible NO synthase (iNOS)[5].The effect of NO production on the cellular processes largely depends on its concentration and the local presence of other free radicals. Lower concentrations of NO have direct effects on processes e.g. proliferation and cell survival, while high concentrations have indirect effect through both nitrosative stress by modifying proteins and oxidative stress by influencing the cytoplasmic redox balance through generation of $ONOO^-$ following its reaction with SO[6]. Peroxynitrate can oxidize lipids such as those found in LDL or arachidonic acid[7]. Peroxynitrate can also act as peroxide substrate for peroxidoses such as those found in cyclooxygenase [8] and finally $ONOO^-$ can nitrate DNA [9]. Nitric oxide dependent tissue injury has been implicated in a variety of rheumatic diseases, including SLE and rheumatoid arthritis (RA), and recent evidence suggests that NO contributes to T cell dysfunction in these autoimmune disease[10] . In Murine model of SLE, NO production has shown to be increased with the progression of the disease and lead to glomerular, joint and dermal pathology[2]. On the other hand, pharmacological inhibition of iNOS in these models significantly reduced both NO and ROS production[11]. These findings suggest that iNOS activity and its products may contribute to the inflammatory lesions in SLE[3]. Like RNS, reactive oxygen species could play a significant role in a pathogenesis of SLE, in that, excessive generation of ROS (i.e.) super oxide anion (O_2^-) and/or hydroxyl radical (^-OH) have the potential to initiate damage to lipids, proteins and DNA[12,13]. Lipid peroxidation (LP), an oxidative degeneration of poly unsaturated fatty acids leads to the formation of highly reactive aldehydes such as malondialdehyde (MDA) which can bind covalently to proteins resulting in their structural modifications and affecting biological function[14]. It was reported that high level of MDA in SLE patients indicates that ROS damage might play a role in SLE[15].The potential for nitrosative/oxidative stress to elicite an autoimmune response or to contribute to SLE pathogenesis remains largely unexplored in humans. This study was undertaken to investigate the status of nitrosative/oxidative stress in patients with SLE.

Patients and Methods

Nineteen patients with SLE (17 females, 2 males) age range (19-45) years who were attending the rheumatology consultation clinic of Baghdad Teaching Hospital, and 19 apparently healthy controls (17 females, 2 males) age range (21-46) years were included in the study after obtaining their informed consent. SLE was diagnosed on the basis of the revised criteria of the American College of Rheumatology (ACR)[16]. Exclusion criteria were pregnancy, the presence of active infection and the presence of cancer, since all can affect serum NO level. Ten ml blood samples were collected from all patients by vein puncture; 2 ml of each sample were transferred to EDTA (ethylene diamine tetraacetate) tube for erythrocyte sedimentation rate (ESR) determination according to the Westergreen method. The rest 8 ml were transferred to 10 ml sterile plane tube, allowed to clot for 30 min at room temperature and centrifuged at 3000 g for 5 min to obtain serum. Serum aliquots were divided into three 1ml eppendroff's tubes for MDA, nitric oxide and peroxynitrite measurment.

Estimation of Biochemical Analysis

Determination of serum MDA level

Malondialdehyde level was estimated as described by Hunter et al.[17]. To 0.5 ml of serum was added 0.5 ml of 35% trichloroacetic acid (TCA). After vortex-mixing, 0.5ml Tris/HCl buffer (50m M; pH 7.4) was added followed by further mixing and incubation at room temperature for 10 min. One ml of 0.75% thiobarbituric acid (TBA) in 2M Na_2SO_4 was added and then the mixture was heated at 100^{o}C for 45 min. after cooling, 1ml of 70% TCA was added, the mixture was

vortexed and then centrifuged at 950 xg for 10 min. The absorbance of the supernatant was determined at 530 nm.Total TBA-reactive material were expressed as MDA, using a molar extinction coefficient for MDA of 1.5×10^5 cm^{-1} M^{-1}. Determination of NO level is done by 2 steps:

1. **Deprotinization step**: deprotinization of serum sample is done by addition of 6mg of zinc sulphate powder to 400µl of serum (15 gm/L) followed by vortex and centrifugation, clear supernatant is taken and kept frozen at -18°C until nitric oxide estimation.
2. **Serum NO measurement step:** measurement of serum NO was performed according to the method of Miranda et al. (2001). Deprotinized sample from step 1 was thawed at room temperature, and 70µl of supernatant was applied to a microtiter plate well, 70µl vanadium chloride (8mg/ml) was added to each well for reduction of nitrate to nitrite and this was followed by addition of the Griss reagents [35µl sulfanilamide (2%) and 35µl N-(l-Naphthyl) ethylendiamine dihydrochloride (NEDD) (0.1%)].

After 30 min, incubation at 37°C, absorbance was read at 540 nm using ELISA reader. Concentration of NO in serum samples were determined from linear standard curve established by 0-200 µmol/L sodium nitrite [18].

Determination of peroxynitrite level

Serum peroxynitrite level was determined according to the method described by Beckman et al. [19], cited by Van Uffelen et al. [20]. In which the peroxynitrite mediated nitration of phenol was measured spectrophotometrically at 412 nm. In brief 100µl of serum was placed in glass test tube, to which 5mM phenol in 5M sodium phosphate buffer pH 7.4 was added to a final volume of 2 ml, the resulting solution after mixing is then incubated for 2 hours and then 15 µl of 0.1 M NaOH was added and the absorbance is read at 412 nm.

Statistical Analysis

Data were translated into a computerized database structure. An expert statistical advice was sought for statistical analysis using SPSS version 12 computer software. Data in this study was presented as mean ± standard error (mean ± SE). student's t-test was used to compare the group means. A Pvalue <0.05 was considered to be statistically significant.

Results

Table (1) shows the demographic characteristics of the subjects. There was no significant difference between the control and SLE patients regarding gender, age, weight and body mass index (BMI). Serum analysis showed significantly elevated levels of NO in the 19 patients with SLE mean ± SE (263.58 ± 35.42 µmol/L) compared with controls (162.48±10.42µmol/L) Pvalue <0.05 serum level of peroxynitrite also were significantly elevated in the 19 SLE patients mean ±SE(7.23±0.92µmol/L) as compared with the controls (4.47±0.38µmol/L), Pvalue <0.05. Lipid peroxidation measured as serum MDA levels were higher in patients with SLE mean ± SE (4.53 ± 0.22 nmol/ml) as compared to healthy controls (4.35 ± 0.58 nmol/ml). Yet, it failed to reach a level of significance. Erythrocyte sedimentation rate levels were significantly higher in SLE patients (71.00 ± 7.05 mm/hr) as compared with the control group (14.21 ± 0.45mm/hr) P<0.05. Table (2) shows the level of NO, $ONOO^-$, MDA and ESR in serum of patients with SLE and healthy controls.

Table 1: Demographic data of the studied groups

Characteristic	SLE	Controls
Number	19	19
Gender F/M	17/2	17/2
Age	29 ± 2.42	31 ± 2.62
Weight	67.63 ± 2.82	70.37 ± 2.71
BMI	25.34 ± 0.99	24.82 ± 0.96

Values were expressed as mean ± SD.

Table 2 : Nitric oxide, peroxynitrite and malondialdehyde and erythrocyte sedimentation rates in serum of patients with systemic lupus erythematosus and healthy controls

	SLE patients	Healthy controls	P-value
NO (µmol/L)	263.58±35.42	162.48±10.42	0.017*
$ONOO^-$ (µmol/L)	7.23±0.92	4.47±0.38	0.025*
MDA (nmol/ml)	4.53±0.22	4.35±0.58	0.774
ESR (mm/hr)	71.00±7.05	14.21±1.57	0.000*

NO: nitric oxide, $ONOO^-$: peroxynitrite, MDA: malondialdehyde, ESR: erythrocyte sedimentation rates

Values were expressed as mean ± SD.

* P<0.05, students' t-test.

Discussion

Systemic lupus erythematosus is a puzzling disease due to its multifactorial etiology including genetic, hormonal and environmental triggers, the molecular mechanisms underlying this systemic autoimmune response remain largely unknown[15]. In recent years, free radical mediated reactions have implicated considerable attention as the potential mechanism in the pathogenesis of SLE[21]. Studies using animal models of SLE also suggested an association between nitrosative/oxidative stress and autoimmunity [2,22,23]. However, relevance of nitrosative / oxidative stress in the pathogenesis and progress of SLE in human is not fully understood. Our results present in this study show significantly elevated levels of NO as compared to healthy controls; this came in accordance with previous studies demonstrating higher level of NO in active SLE patients [3,24,25], also considerable evidence supports that NO production correlate with disease activity and damage in SLE[3]. On the other hand, this study also demonstrated significantly elevated level of $ONOO^-$ in SLE groups as compared to healthy controls. It is also came in accordance with previous studies which suggest that overproduction of iNOS and increased production of $ONOO^-$ may contribute to glomerular and vascular injury in SLE and other autoimmune diseases[24,26,27].Thus these RNS could play an important role in the pathogenesis of SLE, in that the potential of NO in disease pathogenesis could lie largely to the extent of its production and generation of superoxide radical (O_2^-), leading to the formation of peroxynitrite which is a potent nitrating and oxidizing agent[5]. Peroxynitrite can react with tyrosin residues forming nitrotyrosine forming a neoepitopes on nucleophilic domain of self antigens [21,28].In addition $ONOO^-$- mediated modifications of endogens proteins and DNA may enhance their immunogenicity leading to a break in the immune tolerance [21,29,30].Other important mechanism by which NO can play a central role in the pathogenesis of SLE is through its ability to regulate T-cell function[10]. Nitric oxide under physiological condition has been shown to regulate T-cell function, but overproduction of NO may contribute to T-cell dysfunction and result in NO-dependent tissue injury[31,32].On the other hand and concerning Lipid peroxidation, this study shows slightly elevated level of MDA in serum of SLE as compared to healthy controls which came in agreement with earlier reports and confirming the presence of increased oxidative stress in SLE [33-36].Malondialdehyde is the most abundant aldehyde resulting from lipid peroxidation and high level of it indicates that ROS damage might play a role in SLE [36,37].These ROS can cause cross linking of proteins or could cause oxidative inactivation of certain enzymes causing functional impairment of cells and libration of cytoplasmic proteases[38]. They can also induce damages in DNA which results in new antigenic determinants and stimulation of anti DNA antibody formation and autoimmunity[39,40].

Conclusion

The study findings support an association between nitrosative/oxidative stress and SLE through elevated level of NO, peroxynitrite and MDA in the serum of SLE patients which might have a role in the disease pathogenesis and progression, however, such suggestion need future studies to confirm it.

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