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 spectrophotometric method depending on Griss method, while peroxynitrite levels were measured by spectrophotometric method based on peroxynitrite mediated nitration of phenol. Malondialdehyde levels were measured by the thiobarbituric acid method. Serum nitric oxide levels were significantly elevated in SLE patients (mean + SE 263.58 ± 35.42 μmol/L) as compared with healthy control (162.48 ± 10.42 μmol/L). Peroxynitrite levels were also significantly elevated in a disease group (mean ± SE 7.23 ± 0.92 μmol/L) as compared to healthy control (4.47 ± 0.38 μmol/L). On the other hand, malondialdehyde levels were slightly elevated in SLE patient (mean ± SE 4.53 ± 0.22 nmol/ml) as compared to control group (4.32 ± 0.58 nmol/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.


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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown aetiology affecting multiple organ systems[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 hydrogen peroxide (H₂O₂). Nitric oxide is a biological messenger mediating many important physiological functions but also pathological processes. 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). 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[5]. Peroxynitrite can oxidize lipids such as those found in LDL or arachidonic acid[6]. Peroxynitrite can also act as peroxide substrate for peroxidases 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 been shown to increase 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 (O₂⁻) 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 elicit 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 eppendorf's tubes for MDA, nitric oxide and peroxynitrite measurement.

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 Na₂SO₄ was added and then the mixture was heated at 100°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 1.5×10³ 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-(1-Naphthyl) ethylenediamine 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 P-value <0.05 was considered to be statistically significant.

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**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) P-value <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**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SLE</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Gender F/M</td>
<td>17/2</td>
<td>17/2</td>
</tr>
<tr>
<td>Age</td>
<td>29 ± 2.42</td>
<td>31 ± 2.62</td>
</tr>
<tr>
<td>Weight</td>
<td>67.63 ± 2.82</td>
<td>70.37 ± 2.71</td>
</tr>
<tr>
<td>BMI</td>
<td>25.34 ± 0.99</td>
<td>24.82 ± 0.96</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SLE patients</th>
<th>Healthy controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO (μmol/L)</td>
<td>263.58±35.42</td>
<td>162.48±10.42</td>
<td>0.017*</td>
</tr>
<tr>
<td>ONOO⁻ (μmol/L)</td>
<td>7.23±0.92</td>
<td>4.47±0.38</td>
<td>0.025*</td>
</tr>
<tr>
<td>MDA (nmol/ml)</td>
<td>4.53±0.22</td>
<td>4.35±0.58</td>
<td>0.774</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>71.00±7.05</td>
<td>14.21±1.57</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

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 (21,22). Studies using animal models of SLE also suggested an association between nitrosative/oxidative stress and autoimmunity (23,24,25). 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 (24,26,27). Also considerable evidence supports that NO production correlate with disease activity and damage in SLE (25). 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 (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 (O₂⁻), leading to the formation of peroxynitrite which is a potent nitrating and oxidizing agent (25). Peroxynitrite can react with tyrosin residues forming nitrotyrosine forming a neoepitopes on nucleophile 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 functions (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 liberation 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.

References

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