Association between Interleukin-6 and Anemia of chronic Disease (ACD) in Patients with Systemic lupus Erythematosus

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Abstract

This study was performed to determine the concentrations of serum interleukin-6 (IL-6) and its correlation with iron status: serum iron, serum transferrin and serum ferritin in patients with systemic lupus erythematosus (SLE). We obtained serum samples from 50 patients with SLE who had visited Al-Diwanyia Teaching Hospital and 20 age- and sex-matched healthy controls, and we assessed the clinical parameters of disease, including seropositivity test for systemic lupus erythematosus (SLE), erythrocyte sedimentation rate (ESR). Serum levels of iron and total iron binding capacity (TIBC) were measured spectrophotometrically, and transferrin concentration were calculated mathematically. Serum concentrations of interleukin-6 (IL-6) and ferritin were measured using an enzyme-linked immunosorbent assay (ELISA).

Serum concentration of interleukin-6 (IL-6) was significantly elevated ($P< 0.0001$) in patients with SLE compared to those of healthy controls.

Serum concentrations of iron, total iron binding capacity (TIBC), and transferrin concentration were significantly decreased in patients with SLE compared to those of healthy controls. While serum level of ferritin was significantly elevated ($P< 0.0001$) in patients with SLE compared to those of healthy controls.

We concluded that the serum concentrations of iron and transferrin were significantly decreased in SLE patients. Most importantly, these changes correlated with the inflammatory state of the patients (significant elevation of IL-6, ferritin and ESR) and anemia of chronic disease. Taken together, altered iron handling, inflammation and anemia of chronic disease constitute an ominous triad in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can affect virtually any organ system, including the skin, joints, kidneys, serosal membranes, and heart. SLE is a major rheumatic disease, and more than 90% of persons with the disease have polyarthralgias. SLE is a fairly common disease with a prevalence of approximately 1 case

per 2000 persons in certain populations.[1] The peak incidence occurs between ages 15 to 40 years. There is a female-to-male ratio of 9 to 1, with the ratio becoming closer to 30 to 1 during the childbearing years. SLE is more common in African Americans, Hispanics, and Asians than whites, and the incidence in some families is higher than in others.[2]

The cause of SLE is unknown. The presence of a wide array of autoantibodies suggests a breakdown in the normal surveillance function of the immune system. Antibodies have been identified against a host of nuclear and cytoplasmic components of the cell that are neither tissue nor organ specific. Antinuclear antibodies (ANAs) are directed against several nuclear antigens, including DNA, histones, nonhistone proteins bound to RNA, and nucleolar antigens.[1] Another group of antibodies is directed against cell surface antigens of blood elements.

When IL-6 is activated, acute inflammatory responses such as fever or anemia are induced. IL-6 promotes the proliferation of B cells and thus is involved in the production of the autoantibodies[3]. Iron plays a potential role in oxidative stress-mediated injuries and pathologies of systemic lupus erythrmatosus (SLE). Four decades ago it was suggested that iron may have a crucial role in the progression of inflammation in SLE.[4] In vitro studies revealed that ferrous ions in trace amounts were very active in the depolymerization of purified hyaluronic acid and this is correlated with the low viscosity of synovial fluid (SF) of patients with SLE. Spectrographic studies showed that SF from patients with SLE contained elevated concentration of iron [5]. By emission spectrometric analysis, the mean concentration of iron was higher in the SF of SLE patients than normal subjects. [6] Muirden and Senator (1968) were one of the first to suggest the critical role that iron could play in the pathogenesis of SLE.[7]

In SLE, it is estimated that 30-60% of patients are anemic. One of the most frequent causes of anemia in SLE patients is iron deficiency anemia (IDA). Anemia of chronic disease (ACD) which does not usually respond to iron supplementation is another major cause of anemia in patients with SLE.[8] However, Bloxham and his coworkers found that the majority of anemic patients were ACD, with rather fewer patients demonstrating iron deficient.[9]

MATERIALS AND METHODS

Subjects and clinical assessment

This study was conducted in 50 patients who had visited Al-Diwanyia Teaching Hospital between February and May 2012, and who fulfilled the American College of Rheumatology (ACR) 2010 revised criteria for the diagnosis of SLE.[10] Twenty age-and sex-matched healthy adults without any evidence of chronic inflammatory disease served as the controls.

The patients underwent thorough clinical and laboratory evaluation, including complete medical history, seropositivity test for systemic lupus erythematosus (RF), C-reactive protein (CRP), and estimation of erythrocyte sedimentation rate (ESR).

Six ml of blood samples were collected intravenously from each patient, 1ml for evaluation of ESR, whereas serum samples were collected in glass tubes without anticoagulant, stored for one hour at room temperature, centrifuged (2.500 r.p.m. for 10 minutes at 4°C) and then aliquoted in plastic tubes before being stored at -20°C until analysis.

Study design

The patients were classified according to the duration of SLE disease [9] as follows:

1. Group I (GI): the group of patients with disease length of (less than one year) was considered as the group with very early disease duration.
2. Group II (GII): the group of patients with disease length of (1-5) years was considered as the group with early disease duration.
3. Group III (GIII): the group of patients with disease length of (6-15) years was considered as the group with median disease duration.
4. Group IV (GIV): the group of patients with disease length of (16-25) years was considered as the group with long disease duration.
5. Group V (GV): the group of patients with disease length of (more than 25) years was considered as the group with very long disease duration.

Methods

1. **Systemic lupus erythematosus (SLE) Latex serology test**

Procedure:

The AVITEX SLE is a rapid latex agglutination test kit for the presumptive detection of the Systemic Lupus Erythematosus (SLE) in a human serum by the detection of antibodies in serum to double stranded (ds) DNA. A drop of latex was mixed with 50µl of sample using disposable stirrer stick. The mixture was spreading homogenously over the entire area enclosed by the separate circle on the test card. Shake the card for 2 minute by a rotating motion at (100 r.p.m.). The eventual agglutination was observed using artificial light.

2. **Erythrocyte sedimentation Rate (ESR)**

The International Committee on Standardization in Hematology (ICSH) recommends the use of the Westergren method [10,11].

3. **C - reactive protein (CRP) serology test**

C - reactive protein was measured by agglutination test

4. **Iron status measurement**

4.1.   Serum iron and Total Iron Binding Capacity (TIBC)

Serum levels of iron and total iron binding capacity (TIBC) were measured spectrophotometrically in Biochemistry Laboratory Al-Diwanyia Teaching Hospital The Colorimetric test method was used via RANDOX reagents (RANDOX kit, U.K) according to (Ceriotti and Ceriotti, 1980).

4.2.   Transferrin saturation Percentage (%)

Transferrin saturation percentage (TS%) was calculated mathematically according to (Freeman and Arneson, 2007).

$$TS(\%) = \frac{\text{Serum iron}}{\text{TIBC}} \times 100$$

4.3.   Transferrin Concentration

Transferrin concentration was calculated mathematically according to (Morgan, 2002).

$$\text{Transferrin (g/L)} = \frac{\text{Serum iron (μmol/L)}}{3.98} \times (TS\%)$$

5. Measurement of IL-6and serum ferritin

The serum concentration of IL-6 was measured using an AssayMax enzyme-linked immunosorbent assay (ELISA) kit (Assaypro, USA) at Virology Laboratory of AL-Diwaniyia Teaching hospital. Fifty microliters each of serum sample and assay diluent were placed in each well of a 96-well plate coated with a monoclonal mouse IgG against IL-6. This mixture was incubated for two hours at room temperature, and each well was aspirated and washed five times with wash buffer. Subsequently, 50 μl of Biotinylated IL-6 Antibody was added to each well and incubated for two hours. Again, each well was washed five times with wash buffer. Following this, 50 μl of Streptavidin-Peroxidase Conjugate was added per well and incubated for 30 minutes and each well was aspirated and washed five times with wash buffer. Subsequently, 50 μl of substrate solution, which was prepared with equal amounts of stabilized hydrogen peroxide (H_2O_2) and tetramethylbenzidine, was added for a 20 minute reaction under dark conditions. The reaction was quenched by the addition of 50 μl stop solution (0.5 N of HCl). Within 30 minutes, the optical density was measured at a wavelength of 450 nm using the bioelisa reader ELx 800 (Molecular Device Co., biokit, CA, USA). The serum concentration of IL-6 was determined based on a standard concentration curve. The correlation coefficient (r) of the standard concentration curve was 0.990.

The serum ferritin was also purchased from Assaypro, USA, and determined using similar methods. The serum concentration of ferritin was determined based on a standard concentration curve. The correlation coefficients (r) was 0.993.

Statistical analysis

Data analyses were performed with SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).. All of the descriptive variables were expressed as the mean ± standard error (SE). The correlations between the concentrations of IL-6 and iron status were tested using Pearson's correlation test. The group analyses were performed using one-way ANOVA and Tukey's post-hoc analyses. For all tests, a p value less than 0.05 was considered statistically significant.

RESULTS

Table 1: Iron status in healthy control group and in the groups of patients suffering from SLE

Iron status	Healthy Control (n=20)	SLE patients (n= 50)				
		GI	GII	GIII	GIV	GV
S. Iron (µmol/L)	21.71±1.00	$8.44±2.07_a^{***}$	$7.81±1.49_a^{***}$	$7.88±1.49_a^{***}$	$10.12±1.79_a^{***}$	$10.66±1.07_a^{***}$
S.TIBC (µmol/L)	54.15±1.00	$32.58±0.90_a^{***}$	$34.29±1.70_a^{***}$	$34.5±1.05_a^{***}$	$35.98±1.93_a^{***}$	$32.28±1.48_a^{***}$
S. Tf (g/L)	2.33±0.22	$0.43±0.16_a^{***}$	$0.34±0.09_a^{***}$	$0.36±0.13_a^{***}$	$0.42±0.15_a^{***}$	$0.42±0.08_a^{***}$
TS (%)	39.57±2.30	$13.27±3.60_a^{***}$	$11.11±2.08_a^{***}$	$11.12±2.19_a^{***}$	$12.77±2.37_a^{***}$	$14.63±1.51_a^{***}$
Ferritin (ng/mL)	131.2±5.96	$220.4±23.73_a^{ns}$	$233.2±28.37_a^{**}$	$218.2±28.47_a^{*}$	$198.5±27.38_a^{ns}$	$217.5±21.26_a^{ns}$

- Data are expressed as means ± standard error (SE).
- The asterisks indicate significant difference based on Tukey's multiple comparison test.
- The same letters indicate non significant difference between groups based on Tukey's multiple comparison test.
- *** indicate extremely significant (P value <0.001).
- * indicate significant (P value 0.01 to 0.05).
- ns: not significant.

Serum concentration of IL-6

Serum level of IL-6 of SLE patients showed significant increase (P<0.0001) in the average values comparatively to the healthy group, in the very early duration of the disease was much higher (Figure 1).
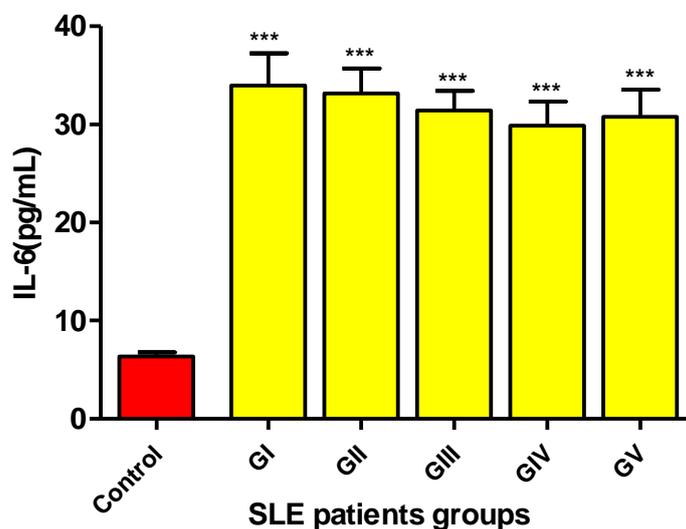

Fig 1: IL-6 in healthy control group and in the groups of patients suffering from SLE

- Data are expressed as means ± standard error (SE).
- The asterisks indicate significant difference based on Tukey's multiple comparison test.
- P value <0.0001
- *** indicate extremely significant (P value <0.001).

Erythrocyte Sedimentation Rate (ESR) (mm/hour)

ESR of SLE patients showed significant increase (P<0.0001) in the average values comparatively to the healthy group, in the long duration of the disease was much higher (Figure 2)
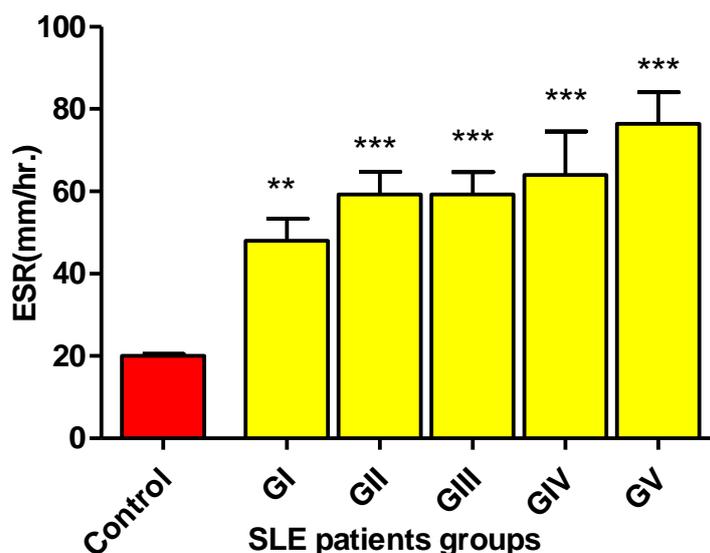
Fig 2: ESR in healthy control group and in the groups of patient suffering from SLE

- Data are expressed as means ± standard error (SE).
- The asterisks indicate significant difference based on Tukey's multiple comparison test.
- P value <0.0001
- *** indicate extremely significant (P value <0.001).
- ** indicate very significant (P value 0.001 to 0.05).

Relationship of IL-6 Levels to Iron Status

Serum concentration of IL-6 showed inverse correlations with serum iron, and serum TIBC, respectively (Table 2).

Table 2: Correlation between IL-6 and iron status in SLE patients.

Cytokine (pg/mL)	S. Iron (µmol/L)		S. TIBC(µmol/L)		S. Tf (g/L)	
	r	p	r	p	r	p
IL-6	-0.1358	0.2624	-0.01167	0.9236	0.1459	0.228

Relationship of IL-6 Levels to serum Ferritin and ESR

Serum concentration of IL-6 correlated positively and significantly with serum ferritin (r = 0.9480, *p*<0.0001) the highest coefficient correlation being with ESR (r = 0.9776, *p*<0.0001), respectively (Table 3).

Table 2: Correlation between IL-6 and serum ferritin and ESR in SLE patients.

Cytokine (pg/mL)	S. Ferritin (ng/mL)		ESR (mm/hr.)	
	r	p	r	p
IL-6	0.9480	<0.0001***	0.9776	<0.0001***

DISCUSSION

The current study showed that serum concentrations of IL-6, was significantly elevated in patients with SLE compared to those in healthy controls. As seen in previous reports, this finding supports the hypothesis that IL-6 is involved in the pathogenesis of SLE.[15-18]

IL-6 is a cytokine that causes an acute inflammatory response, and it is well-documented that IL-6 plays a crucial role in the pathogenesis of various inflammatory diseases including SLE.[19,20]

In addition to these previous observations, the significant increase in the IL-6 cytokine observed in the current study indicates that this cytokines might play a role in inducing inflammatory responses or mediating anti-inflammatory responses in the pathogenesis of SLE.[20]

The present study also indicates significant decrease in serum iron , serum transferrin in all groups of patients with SLE. As seen in previous reports, this finding supports the hypothesis that the anemia is the most frequent extra-articular manifestation of the disease [21,22].

In SLE, it is estimated that 30-60% of patients are anemic. One of the most frequent causes of anemia in SLE patients is iron deficiency anemia (IDA). Anemia of chronic disease (ACD) which does not usually respond to iron supplementation, is another major cause of anemia in patients with SLE [23].

The induction of iron sequestration in macrophages and the decrease in iron absorption in the small intestine were shown in infections and inflammatory diseases. This results in the development of anaemia, termed 'anaemia of inflammation' (AI), formerly known as 'anaemia of chronic disease' (ACD). The AI is a most common condition noticeable in patients suffering from inflammatory disorders (e.g. SLE). It is characterized by low to normal serum iron levels (i.e. hypoferremia), low serum iron binding capacity, and normal to elevated ferritin concentrations [27]. Several proinflammatory cytokines, such as IL-6, IL-1α and TNF-α, have been shown to contribute to the development of AI by the induction of hypoferremia [28].

Nemeth *et al*., (2004) showed that hypoferremia of inflammation is mediated by IL-6 which induces the synthesis of hepcidin, an iron-regulatory hormone. Hepcidin is recognized as a key factor in anaemia of chronic disease, in conjunction with the cytokine interleukin-6 (IL-6)[29]. Hepcidin is a hormone that lowers serum iron levels and regulates iron transport across membranes, preventing iron from exiting the enterocytes, macrophages, and hepatocytes. In addition, hepcidin inhibits intestinal iron absorption and iron release from macrophages and hepatocytes. [30]

Data from Masson, (2011) support a causal link between IL-6 production and the development of anaemia in patients with chronic disease. IL-6 diminishes the proportion of nucleated erythroid cells in the bone marrow and lowers the serum iron level, and these abnormalities can be corrected by administering an IL-6 antagonist. IL-6 stimulates hepcidin gene transcription, most notably in the hepatocytes.[31]

In summary, we found the serum concentrations of IL-6 was significantly increased in patients with SLE compared with those of normal controls. The cytokine levels were significantly correlated inflammatory state represented by serum ferritin and ESR. Taking together with handling of serum iron, IL-6 play major role in the pathogenesis of SLE including ACD.

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العلاقة بين الانترلوكين-6 وانيما المرض المزمن لدى مرضى داء الذئب الاحمراري

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الخلاصة

اجريت الدراسة الحالية لتحديد تركيز النترلوكين-6 وعلاقته بحالة الحديد المتمثلة بتركيز الحديد في مصل الدم وتركيز البروتين الناقل للحديد (الترانسفرين) والبروتين الخازن للحديد (الفرتين) لدى مرضى داء الذئب الاحمراري. اجريت الدراسة الحالية على (50) مريض بداء الذئب الاحمراري ارتادوا الى مستشفى الديوانية التعليمي في محافظة الديوانية و(20) شخص طبيعي مقارب لهم بالعمر والجنس اعتبروا كمجموعة سيطرة، تم تحديد المعايير السرسرية لكل مريض والتي تضمنت معدل ترسيب كريات الدم الحمراء (ESR) وعامل الترسيب لاختبار داء الذئب الاحمراري (SLE). تم قياس تركيز الحديد ومعامل ارتباط الحديد الكلي (TIBC) باستعمال المطياف الضوئي، اما تراكيز الانترلوكين-6 والفرتين فقد تم قياسهما باستعمال تقنية الـ (ELISA). بينما تم حساب تركيز الترانسفرين رياضيا.

اظهرت النتائج ارتفاعا معنويا في تراكيز الانترلوكين-6 مقارنه بالسيطرة . بينما انخفض معنويا تركيز كل من الحديد و معامل ارتبط الحديد الكلي و تركيز الترانسفرين مقارنه بالسيطرة. كذلك اظهرت النتائج ارتفاعا معنويا في تركيز كل الفرتين و معدل ترسيب كريات الدم الحمراء. بالرغم من عدم وجود علاقة معنويه بين الاترلوكين-6 وعدد كريات الدم الحمراء، خضاب الدم، حجم الكريات المرصوص ودلائل كريات الدم الحمراء، الا ان النتائج اظهرت علاقة موجبه معنوية مع خلايا الدم البيضاء والعدد التفريقي لها والصفيحات الدموية.

نستنتج من نتائج الدراسة الحالية على تركيز كل من الحديد و الترانسفرين قد انخفض معنويا لدى مرضى داء الذئب الاحمراري مقارنة بالأشخاص الاصحاء وهذا الارتفاع مرتبط ارتباطا قويا مع الحالة الالتهابي للمرضى (تركيز الاترلوكين-6 و ترسيب كريات الدم الحمراء) وانيما المرض المزمن . وهذا الاستنتاج يدل ان تغير تركيز الحديد والحالة الالتهابية وانيميا المرض المزمن تلعب دورا مهما في إمراضيه داء الذئب الاحمراري.