Assessment of Some Biochemical Parameters to Evaluate the Endothelial Function in Patients with Sickle Cell Disease

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Abstract

Sickle cell disease (SCD) is hematological disease that affect the endothelial function, the hemoglobinopathy of this disease triggers erythrocyte polymerization. The sickling process leads to vascular occlusion, tissue hypoxia and subsequent reperfusion injury, thus inducing inflammation and endothelial injury (EI).

Thirty sickle disease patients (15 females and 15 males) with a mean age of (27.0 ± 8.9) and 30 healthy controls (18 females and 12 males) with a mean age of (29.7 ± 9.1) participated in the study Assessment of endothelial function done by studying biochemical parameters which includes; serum nitric oxide, serum endothelin-1, vascular cell adhesion molecule-1, and serum interlokin-6

The results showed that all the biochemical parameters that measured in this study were not significantly different between the patients with sickle cell disease and control subjects, except the serum level of nitric oxide level in patients with sickle cell disease (2.90 ± 2.57) was significantly lower ($P = 0.02$) than that of controls (8.49 ± 9.36).

From all these findings, we can conclude that SCD patients have some degree of impairment of endothelial function.

Key wards: Sickle cell disease, nitric oxide, endothelin-1, vascular cell adhesion molecule-1, and interleukin-6.

Introduction

Sickle cell disease (SCD), also known as sickle cell anemia (SCA) and drepanocytosis, is a hereditary blood disorder, characterized by an abnormality in the oxygen-carrying hemoglobin molecule in the red blood cells. This lead to a propensity for the cell to assume an abnormal, rigid, sickle-like shape under certain circumstances (1).

The endothelium is the largest organ in the body, it is the inner most layer of the blood vessels. It senses mechanical stimuli, such as pressure and shear stress, and hormonal stimuli, such as vasoactive substances. In response, it releases agents that regulate vasomotor function, trigger inflammatory processes, and affect hemostasis (3). The vascular endothelium is an active paracrine, endocrine, and autocrine organ that is indispensable for the regulation of vascular tone and the maintenance of vascular homeostasis (4,5).

In normal conditions, endothelial stimulation induces the production and release of nitric oxide, which diffuses to surrounding tissues and cells, exerts its cardiovascular protective role by relaxing media-smooth muscle cells, preventing leukocyte adhesion and migration into the arterial wall, muscle

cell proliferation, platelet adhesion and aggregation, and adhesion molecule expression (6).

The vasoconstrictor endothelin-1 (ET-1), a 21-amino acid peptide, is the most abundant member of the family of endothelins including ET-1, ET-2, and ET-3. ET-1 is derived mainly from vascular ECs and acts in an autocrine and/or paracrine manner, mediating vasoconstriction predominately by binding to its endothelin receptors type A (ETA) receptors on the underlying SMCs (7).

Vascular Cell Adhesion Molecules-1 (VCAM-1) are membrane proteins necessary for anchoring leukocytes to the vessel wall and are well established markers of endothelial dysfunction in inflammatory conditions such as atherosclerosis (8). Although VCAM-1 was first characterized on vascular endothelial cells, it is clear that a number of other cell types can also express these molecules. These include follicular dendritic cells, interdigitating reticulum cells, kupffer cells, type B synovial lining cells and renal proximal tubule epithelial cells (9).

Interleukin-6 (IL-6) is a protein of 185 amino acid glycosylated at position 73 and 172. It is synthesized as a precursor protein of 212 amino acid. IL-6 belongs to a family of 10 cytokines (10). The inflammatory cytokine IL-6 is an important mediator of increased endothelial permeability via alterations in the ultrastructural distribution of tight junctions and morphologic changes in cell shape (11).

Subjects and Methods

A randomized controlled trial had been applied to evaluate the role of some biochemical parameters for their value in early detection of endothelial dysfunction in patients with SCD.

A total of 60 subjects were included in this study; thirty of them (15 males and 15 females) were patients with homozygous sickle cell disease proved by the result of hemoglobin electrophoresis, and 30 (18 males and 12 females) were apparently healthy were considered as control. Both the patients and the controls were of a comparable age group. The study had been conducted for 19 months throughout the period from the first of April 2013 to the first of November 2014.

Patients were been collected from hematology unit of Al-Imamain Al-Kadhimiyan Medical City, Department of Inherited Blood Diseases in Al-Karama Teaching Hospital, and the National Center for hematology diseases and researches (NCH). All patients were previewed and examined by specialist or consultant hematologist to define eligibility to be enrolled in this study at the aforementioned centers. Patients were confirmed to have SCD clinically and by laboratory measures. The examination, assessment and the investigations needed for the research done in the central laboratory of Al-Imamain Al-Kadhimiyan Medical City and in Al-Nahrain College of Medicine.

The inclusion criteria for this study were:

1. Male or female 15-50 years of age.
2. A homozygous Hb SS disease on clinical & laboratory basis
3. Hb > 6 g/dL

The exclusion criteria for this study includes:

1. SCD patients in any crises form within the last week.
2. SCD patients with severe anemia < 5 g/dl.
3. Recent blood transfusion (last 4 weeks).

4. Pregnancy or lactation.
5. Serum creatinine greater than 1.5 mg/dL.
6. Serum alanine aminotransferase (ALT) > 3 times the upper limit of normal.
7. Subjects taking tonic supplement as L-arginine, fibrates (clofibrate or finofibrate) within the last week.
8. Subjects taking any statin drug within the last four weeks.
9. Subjects taking drugs with known clinically significant metabolic interaction with statin like flucanazole, erythromycin, clarithromycin, cyclosporine, niacin).
10. Subjects with significant cardiac disease and/or ECG abnormalities.
11. Condition that may independently affect endothelial function, i.e., DM or fasting blood sugar > 120mg/dL, cigarette smoking within one month, and hypertension (diastolic blood pressure > 90 mmHg).

Detailed history and general physical examinations were carried out for each individual included in this study, an assessment of vital parameters, and systemic examination performed to assess any possible complications. Anthropometrics measurements were performed and these includes; height was measured without shoes to the nearest 0.1 cm, body weight was obtained by a balance scale in the morning after a 12-hr fasting at the day of investigation, and the body mass index (BMI) which was calculated by dividing body weight in kilograms by the square of height in meters (12).

For each individual included in this study, the biochemical assessments were done after an over-night fasting. Three milliliters (mls) of fasting venous blood sample (13) was aspirated from the antecubital vein using 5 ml disposable syringe; then the blood transferred immediately into a plain tube and left to clot for 30 minutes at room temperature, then was centrifuged for 10 minutes at 3000 rpm for the separation of serum. The serum was transferred to a new plain tube and was stored at - 8 OC until the time of analysis. The biochemical parameters that are measured in this study were:

1. Determination of nitric oxide (NO):

This is done using NO assay kit, BioAssay™ (US Biological Life Sciences, United State). It is common practice to quantitate total NO2-/NO3- as a measure for NO level. The kit is designed to accurately measure NO production following reduction of nitrate to nitrite using improved Griess method (14). The procedure is simple and the time required for sample pretreatment and assay is reduced to only 40 min.

2. Determination of endothelin-1(EN-1):

Endothelin-1 was determined using E-1 Human BioAssay ELISA Kit (US Biological Life Sciences; USA). This assay employs the quantitative sandwich enzyme immunoassay technique (15). A monoclonal antibody specific for ET-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ET-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for ET-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of ET-1 bound in the initial step. The color development was stopped and the intensity of the color was measured by a spectrophotometer.

3. Determination of vascular cell adhesion molecule-1 (VCAM-1):

Vascular cell adhesion molecule-1 was determined by using RayBio Human VCAM-1 ELISA Kit (Ray

Biotech Co. USA). The RayBio Human VCAM-1 ELISA Kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human VCAM-1. This assay employs an antibody specific for human VCAM-1 coated on a 96-well plate. Standards and samples are pipetted into the wells and VCAM-1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human VCAM-1 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of VCAM-1 bound. Then the stop solution was added, which change the color from blue to yellow, and the intensity of the color is measured at 450 nm (16).

4. Determination of interleukin-6 (IL-6):

Interleukin-6 was determined by using RayBio Human IL-6 ELISA Kit (RayBiotech Co.USA). The RayBio Human IL-6 ELISA Kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human IL-6 in serum, plasma, cell culture supernatant and urine. This assay employs an antibody specific for human IL-6 coated on a 96- well plate. Standards and samples are pipetted into the wells and IL-6 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IL-6 antibody is added. After washing away unbound biotinylated antibody, HRP- conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IL-6 bound. Then the stop solution was added, which changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. (17,18).

Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means. The significance of difference of different percentages (qualitative data) were tested using Pearson Chi-square test (χ2-test) with application of Yate's correction or Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value for the test of significance was equal or less than 0.05.

Results

Table 1: Summarizes the distribution of BMI of both studied groups; it equals to 22.9 ± 4.0 kg/m2 for the SCD patients versus 26.1 ± 4.7 kg/m2 of the control group. Categorization of BMI was done according to the WHO criteria into 4 groups. In general, SCD patients appear to have lower BMI when compared to the control subjects (P = 0.006), and even when they were categorized (P = 0.022).

Other than the serum nitric oxide metabolites (NOx) level, all the biochemical markers were not significantly different between sickle cell disease patients and control subjects. Serum NO level in SCD

patients significantly lower (P = 0.001) than that of controls (table 2).

Discussion

Sickle cell disease (SCD) is the most common inherited hematological disease, the outlook for patients with SCD continues to be poor. Today there is no doubt that general survival is improving with more medical care and improvement of environmental and social factor (19).

This study had revealed that SCD patients have lower body mass index as compared to controls. This finding goes in agreement with many previous studies (20, 21), this could be a consequence of the increased resting energy expenditure caused by the increased erythropoietic and cardiac activities (22), or it could reflect the greater increase in height than weight usually seen in adolescents with SCA (19 - 23).

In this study, nitric oxide metabolites (NOx) had been found to be significantly lower in SCD patient at baseline than that for the controls. Although reports have varied, some investigators have found low NOx (nitrate & nitrite) levels in SCD patients and these reduced levels are consistent with impaired endothelial generation of NO. Decreased NO bioavailability has been observed at 'steady state', and in association with vaso-occlusive pain and acute chest syndrome (ACS) (24, 25), however, in a study by Sullivan KJ, et al. 2001 (26) on SCD patients aged 6 - 18 years although NOx was less in SCD patients but was not significant.

There was no difference in ET-1 level among the studied groups, this in agreement with other studies (27 - 28) demonstrating that circulating ET-1 levels were not different in SCD children in steady state than in an age and race-matched group of healthy controls and raised with simultaneous increase in the intensity of pain. However, it is in disagreement with other studies that had shown that ET-1 levels are increased in SCD patients compared to healthy controls, mentioning that these increased levels are more pronounced during an acute VOE (29) and that the time course of ET-1 plasma elevations parallels VOE-associated pain symptoms with a peak in plasma levels and pain at the height of the VOE with slow return to baseline that requires several weeks (29 - 32), or the study of Rybicki and Benjamin, who related the increase in ET-1 level to the ongoing process of inflammation which is important in initiation and maintenance of pain crisis (33).

The cause for insignificant difference in ET-1 levels between patient and control is that all SCD patients enrolled in the study were in steady state with no history of VOE or blood transfusion.

This study had shown no significant difference in VCAM-1 level in the studied groups, this may be related to the steady state of SCD patients enrolled in this study or could be due to the effect of HU on VCAM-1 although small number of patients were on HU which may have no statistical significance on the VCAM-1 level. This is in disagreement with the study of Dworkis, et al. 2011 (34), who had found increased level of VCAM-1 in SCD patients, however, it was associated with the disease severity, and also in disagreement with study of Kato, et al. 2005 (35). who relating the increased VCAM-1 level to multi-organ dysfunction and disease severity.

No significant difference was found in IL-6 between SCD patients and control, this is in agreement with the study of Graido-Gonzalez, et al.,

1998 (30). IL-6 is produced by a number of different cell types, such as monocytes, macrophages, fibroblasts, endothelial cells, epithelial cells, T- and B-cells and keratinocytes (36). IL-6 is also expressed in a variety of situations involving host immune responses and inflammatory reactions (37).

Conclusion

The studied biochemical parameters is not fully assess the endothelial functions in vivo. Because the endothelium regulates several vascular functions, the assessment of each of them is a potential way to evaluate its integrity. Vascular ECs are not only a primary cellular target for the action of pro-inflammatory cytokines but also a source of cytokines and chemokines.

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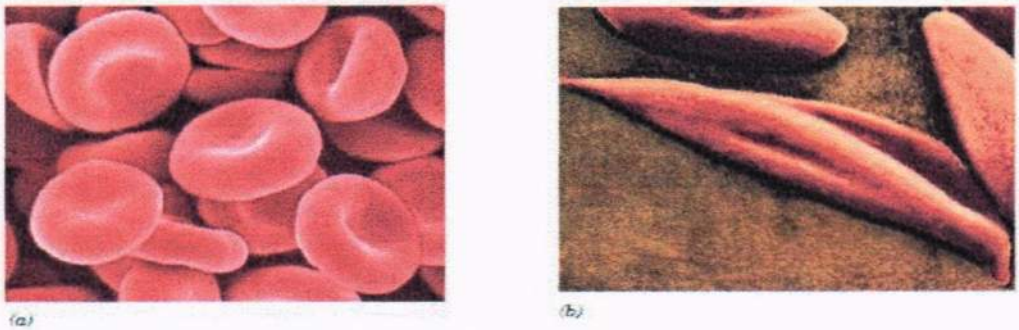
Table (1): Body mass index of the sickle cell disease patients and control subjects.

BMI (kg/m^2)	Groups				P value
	SCD patients		Control subjects		
	No.	%	No.	%	
Underweight (<18.5)	4	13.3	1	3.3	0.022*
Normal (18.5 - 24.9)	20	66.7	12	40.0	
Overweight (25 - 29.9)	4	13.3	13	43.3	
Obese (≥ 30)	2	6.7	4	13.3	
Mean BMI ± SD	22.9 ± 4.0		26.1 ± 4.7		0.006

Table (2): Biochemical markers of the sickle cell disease patients and control subjects.

Biochemical Parameters	SCD Patients	Control	P Value
NOx (µM)	2.90 ± 2.57	8.49 ± 9.36	0.02*
ET-1 (pg/ml)	3.91 ± 3.63	4.07 ± 2.96	0.937
VCAM-1 (ng/ml)	18.23 ± 18.78	18.94 ± 12.06	0.210
IL-6 (pg/ml)	28.24 ± 26.67	25.63 ± 13.41	0.378



(a) (b)

Figure (1): Scanning electron micrographs of human erythrocytes. (a) Normal erythrocytes are flexible, biconcave disks that can tolerate slight distortions as they pass through the capillaries. (b) Sickled erythrocytes are elongated and rigid and cannot easily pass through capillaries [2].