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Elevated plasma asymmetric dimethylarginine levels in children with beta-thalassemia major may be an early marker for endothelial dysfunction

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ABSTRACT

Objectives: Beta-thalassemia major is associated with the increased risk of cardiovascular morbidity and mortality. Asymmetric dimethylarginine (ADMA) has been implicated in the pathogenesis of endothelial dysfunction and atherosclerosis. In this study, we aimed to investigate circulating ADMA concentrations in children with beta-thalassemia major.

Methods: Thirty-one beta-thalassemia major children aged between 4 and 16 year old and age, gender-matched 36 healthy controls were enrolled in the study. Plasma ADMA was measured along with the soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), P-selectin, and Pentraxin-3.

Results: Age, gender and body mass index were similar in two groups. Plasma ADMA, sVCAM-1, and sICAM-1 measurements were significantly higher in beta-thalassemia major patients than the control group ($p < 0.004$ for ICAM-1, $p < 0.001$ for other parameters). There were positive significant correlations between ADMA, sVCAM-1 and sICAM-1 ($r = 0.437$, $p < 0.001$; $r = 0.544$, $p < 0.001$; $r = 0.405$, $p < 0.001$, respectively) in the whole group.

Discussion: The findings of the current study show us that increased plasma ADMA levels in children with beta-thalassemia major may be an early marker for endothelial dysfunction and may play a role in the development of premature atherosclerosis in beta-thalassemia major patients.

KEYWORDS

Beta-thalassemia major; asymmetric dimethylarginine; adhesion molecules; endothelial dysfunction

Introduction

Beta-thalassemia major requires regular blood transfusions to maintain an adequate supply of hemoglobin and sustain life, and patients suffer from the long-term consequences of iron overload which may lead to various complications, including damage of parenchymal organs and cardiovascular system [1–3]. *In vitro* studies have revealed that iron-loading has been linked with endothelial dysfunction due to peroxidative tissue injury resulting from blood transfusions, circulating cholesterol oxidation products, and possibly coronary artery disease [4,5]. Endothelial cell activation and impaired flow-mediated dilation in the brachial arteries of patients with beta-thalassemia major implicated endothelial dysfunction in the pathogenesis of vascular complications [6].

According to a new disease paradigm involving hemolysis-associated endothelial dysfunction, the process of hemolysis affects the arginine–nitric oxide pathway [7]. Nitric oxide (NO) is a paracrine factor that controls vascular tone (as a potential vasodilator), inhibits platelet function, prevents adhesion of

leukocytes, and reduces proliferation of the intima [8]. It originates in the endothelium via the conversion of L-arginine to L-citrulline in the presence of endothelial NO synthase (eNOS), which is nicotinamide adenine dinucleotide phosphate dependent [9]. The addition of two methyl radicals to arginine, through the action of methyltransferase nuclear proteins produces asymmetric dimethylarginine (ADMA), which competes with L-arginine and promotes a reduction in nitric oxide formation in the vascular wall [10]. Elevated levels of ADMA inhibit NO synthesis and therefore impair endothelial function and thus promote atherosclerosis. Large body of evidence has demonstrated that ADMA is an early marker for endothelial dysfunction and also regarded as an independent predictor of future cardiovascular events [11,12]. In addition, endothelial adhesion molecules [soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1) and P-selectin] are also considered as early endothelial dysfunction markers, and there is scarce data that highlights the significance and role of these molecules in the

endothelial dysfunction development in pediatric beta-thalassemia major patients [13].

Based on all these informations, in the present study we aimed to assess circulating levels of ADMA between patients with beta-thalassemia major and control group and determine its correlation with markers of endothelial adhesion molecules (sICAM-1, sVCAM-1 and P-selectin), and Pentraxin-3 as prognostic factors for vascular risk stratification and subclinical atherosclerosis.

Patients and methods

This study was approved by the local ethics committee of Gulhane School of Medicine, which was conducted according to the Helsinki II Declaration. A total of 31 children with beta-thalassemia major aged between 4 and 16 year old (study group) and 36 age and gender-matched healthy controls were enrolled in the study. Exclusion criteria were: presence of chronic diseases including diabetes mellitus, hypertension, renal failure, hepatic disease and hypo-/hyperthyroidism; familial history of hypercholesterolemia, coronary artery disease and premature atherosclerosis; previously diagnosed diseases like cholestatic hepatitis, nephrotic syndrome, cardiac and vascular disease; and children with body mass index (BMI) $\geq$ 95th percentile.

The subjects underwent routine medical history, physical examination, anthropometry and laboratory assessment. Body mass index (BMI, kg/m²) was calculated as weight (in kilograms) divided by height (in meters) squared and used as an index of body fat. Fasting blood samples were obtained from the antecubital vein. The samples for the measurement of sICAM-1, sVCAM-1, P-selectin, Pentraxin-3 and ADMA were centrifuged for 15 min at 2000g, aliquoted, and immediately frozen at -80°C until analysis. Circulating levels of sICAM-1, sVCAM-1, P-selectin, Pentraxin-3 and ADMA were determined with commercially available ELISA (enzyme-linked immunosorbent assay) kits (ADMA were by Immunodiagnostic Systems, Boldon, U.K.; Pentraxin-3 was by R&D Systems, U.S.A.; sICAM-1, sVCAM-1

and P-selectin were by eBioscience, San Diego, CA, U.S.A.). According to kit specifications, minimal detection limit of ADMA was 0.05 $\mu\text{mol/l}$. The calculated overall intra- and inter-assay coefficient of variation (CV) for Pentraxin-3 were 4.4 and 6.1%, respectively with a minimum detectable concentration; 0.025 ng/ml. The intra- and inter-assay CV values for sICAM-1 were 9.5 and 12.9%; for sVCAM-1 were 3.1 and 5.2% and for P-selectin were 7.8 and 5.4%, respectively. ELISA measurements were carried out using the Bio-Tek Synergy HT plate reader (Biotek Instruments Inc., Winooski, VT, U.S.A.).

Statistical analysis

All statistical analyses were performed using the SPSS for Windows v. 15 (SPSS Inc., Chicago, IL, U.S.A.). Demographical, biochemical and histological features were classified as continuous or categorical variables. Kolmogorov-Smirnov analysis was used to test for Gaussian distribution. For Gaussiandistributed variables, the data were expressed as arithmetic mean $\pm$ standard deviation. For those variables that were not Gaussian distributed, the data were expressed as median (25th–75th interquartile range). Comparisons between two groups were performed by using the Student's *t* test or Mann-Whitney U test as appropriate. The relationship between the variables was evaluated with Spearman correlation analysis. All of the reported *p* values were twotailed and those less than 0.05 were considered to be statistically significant.

Results

The comparison of demographic, laboratory and metabolic features of patients with beta-thalassemia major and control group are shown in Table 1. According to these results, no statistical significance was found between groups in terms of age, sex, BMI, white blood cell and platelet counts, serum P-selectin and pentraxin-3 levels ($p > 0.05$). Comparing the two groups, the beta-thalassemia major patients had

Table 1. Comparison of demographic, laboratory and metabolic features of patients with beta-thalassemia major and control group.

Variables	Study group ($n = 31$)	Control group ($n = 36$)	<i>p</i> -value
Female/male	13/19	16/20	0.809
Age (years)	12 (4–16)	11 (4–16)	0.491
BMI (kg/m ²)	16.7 (15.4–19)	16.8 (16.3–19.8)	0.148
WBC ($10^3/\text{mm}^3$)	8.1 (6.8–9.8)	7.6 (5.9–8.8)	0.220
Hemoglobin (g/dl)	10.3 $\pm$ 0.6	13.3 $\pm$ 0.8	<0.001
Platelet count ($10^3/\text{mm}^3$)	342 $\pm$ 128	313 $\pm$ 84	0.269
Ferritin (ng/ml)	2235 (390–5119)	32 (18–90)	<0.001
ADMA ($\mu\text{mol/l}$)	0.90 (0.7–1.09)	0.49 (0.46–0.56)	<0.001
VCAM-1 (ng/ml)	1828 (1478.74–2423.25)	1207.54 (1078.65–1386.73)	<0.001
ICAM-1 (ng/ml)	419.29 $\pm$ 133.41	345.08 $\pm$ 53.54	0.004
P-selectin (ng/ml)	192.51 $\pm$ 52.97	173.42 $\pm$ 41.57	0.115
Pentraxin-3 (ng/ml)	2.90 (1.45–3.48)	1.90 (1.43–2.96)	0.347

BMI: body mass index; WBC: white blood cell; ADMA: asymmetric dimethylarginine; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1.

Bold values denote significance at $p < 0.05$.

Table 2. ADMA in correlation with clinical characteristics and laboratory parameters.

Variable	Spearman's correlation coefficient	p-value
Age (years)	−0.029	0.816
BMI (kg/m ²)	−0.265	0.031
WBC (10 ³ /mm ³)	−0.045	0.722
Hemoglobin (g/dl)	−0.735	<0.001
Platelet count (10 ³ /mm ³)	−0.119	0.341
Ferritin (ng/ml)	0.585	<0.001
VCAM-1 (ng/ml)	0.544	<0.001
ICAM-1 (ng/ml)	0.405	0.001
P-selectin (ng/ml)	−0.034	0.792
Pentraxin-3 (ng/ml)	0.155	0.214

ADMA: asymmetric dimethylarginine; BMI: body mass index; WBC: white blood cell; VCAM-1: vascular cell adhesion molecule-1; ICAM-1: intercellular adhesion molecule-1.

Bold values denote significance at $p < 0.05$.

significantly higher serum ferritin, sICAM-1, sVCAM-1 and ADMA levels than controls ($p < 0.001$ for ferritin, sVCAM-1, ADMA; and $p = 0.003$ for sICAM-1, respectively).

The correlation between ADMA and clinical characteristics/laboratory parameters in subjects is shown in Table 2. According to these results, there was a significant positive correlation between ADMA and ferritin, VCAM-1 and ICAM-1 levels while a negative correlation was noted between ADMA and BMI, and Hb concentrations ($p < 0.001$ for Hb, ferritin, VCAM-1; $p = 0.031$ for BMI; and $p = 0.001$ for ICAM-1). On the other hand, there was no significant correlation between ADMA and age, WBC count, platelet count, serum P-selectin and Pentraxin-3 measurements ($p > 0.05$).

Discussion

With the increase in life span of patients with beta-thalassemia major, premature coronary artery disease may emerge as one of the important late cardiovascular complications. As is known, endothelial dysfunction is a well-established response to cardiovascular risk factors and precedes the development of atherosclerosis. The present study provides evidence of impaired endothelial function in children with beta-thalassemia major, with increased plasma ADMA, sVCAM-1, and sICAM-1 levels.

Accumulating evidence suggests that endothelial dysfunction is an early stage of atherosclerosis [14]. Proinflammatory cytokines and inflammation process play a key role in atherogenesis, since they promote leukocyte adhesion to endothelium and subendothelial migration, directly or indirectly by stimulating the expression of adhesion molecules such as sVCAM-1 and sICAM-1 on endothelial cells and by reducing endothelial-derived NO and its bioavailability [15,16]. Thus, reduced release of NO and increased levels of ADMA are thought to be an early hallmark of endothelial dysfunction. ADMA is known as an endogenous inhibitor of eNOS and competes with L-arginine for the same binding site on eNOS, thus resulting in eNOS

uncoupling, increased superoxide production and hence decreased NO production [16]. However, there are very few studies investigating association between hemolytic disorders, particularly children with beta-thalassemia major, and elevations in free plasma/serum ADMA levels in the literature. In a study by Mohamed et al. [17], plasma ADMA levels were determined significantly higher in children with beta-thalassemia major, and there was a positive association with the hemolytic rate. In another study investigating plasma ADMA levels in 52 HbSS/HbSbeta (0)-thalassemia and 24 HbSC/HbSbeta (+)-thalassemia patients, ADMA levels were determined significantly higher in HbSS/HbSbeta (0)-thalassemia patients with pulmonary hypertension [18]. Consistent with these studies and as expected, serum ADMA level was significantly higher in patients with beta-thalassemia major than in the control group in our study. It may be a result of iron overload which may reduce endothelium-derived NO bioactivity and increase ADMA levels [19]. Oxidative stress triggered by iron overload interferes with the metabolism of ADMA following inhibition of the dimethylarginine dimethyl aminohydrolase which hydrolyzes ADMA to dimethylamine and citrulline [20,21]. In addition, according to another aspect, intact erythrocytes play an important role in storage of ADMA, whereas upon erythrocyte lysis large amounts of free ADMA are generated by proteolysis of methylated proteins, which may affect plasma levels in hemolysis-associated diseases [22]. As the result, increased ADMA levels with beta-thalassemia major may contribute to the development of premature atherosclerosis due to endothelial dysfunction and rearrangement of vascular wall as a result of chronic hemolytic process.

Also, as the endothelium progresses to a dysfunctional state, vascular homeostasis becomes impaired, leading to reduced anti-oxidant, anti-inflammatory and anti-thrombotic properties (due to reduced NO bioavailability), enhanced endothelial permeability (barrier dysfunction), upregulated proinflammatory cytokine levels, and expression of adhesion molecules such as VCAM-1 and ICAM-1, which play a key role in the early formation of atherosclerotic plaque by facilitating leukocyte rolling, adhesion and transmigration into the endothelial space [23]. Thus, elevated plasma levels of adhesion molecules are considered as early markers of endothelial dysfunction and can be used as an indirect measure of endothelial dysfunction. Recently, an increasing number of studies have been performed on evaluating noninvasive procedures and markers to assess endothelial function in subjects with beta-thalassemia major [1,3,4,6,24]. In a study by Kyriakou et al. [24], endothelial activation markers (sICAM-1, sVCAM-1, E-selectin, tumor necrosis factor- α , and interleukin-1 β) which may be related to the vascular complications were found to be significantly

higher in both transfusion-dependent and non-dependent thalassemia patients and these markers have been suggested for the follow-up of the vascular disease in these patient group. In another study by Aggeli et al. [1], increased levels of Interleukin-6, sVCAM-1 and sICAM-1 were determined in transfusion-dependent patients with beta-thalassemia major compared to controls and potential role of inflammation and endothelial dysfunction in the complications of the disease were attributed to these increase. On the other hand, in a study including 35 β -thalassemia intermedia patients aged 8–63 years, levels of sVCAM-1, sICAM-1, P-selectin, E-selectin and C-reactive protein levels were significantly increased in patients and not influenced by treatment. However, there was no correlation between endothelial adhesion molecules and inflammation markers [25]. Considering these widely used adhesion molecules, we found that serum sICAM-1 and sVCAM-1 levels were increased in our patient group compared to controls while there was no statistically significant difference in terms of serum P-selectin levels. We believe this increased peripheral release/activity of the soluble forms of adhesion molecules was caused by iron overload which leads to increase in oxidative stress and activation of macrophages.

The main limitation of our study is the lack of data on proinflammatory cytokines and/or inflammation markers (like C-reactive protein) which possibly play a key role in inflammation process in endothelial dysfunction. Another limitation is the relatively small sample size. The present data should therefore be interpreted with caution and need reconfirmation in a larger cohort.

In conclusion, as far as we know, this is the first study evaluating ADMA and endothelial adhesion molecules together in children with beta-thalassemia major and this makes the *results of our study more meaningful compared to previous studies*. These findings support the hypothesis that a serious degree of endothelial activation and damage underlie the pathophysiology of beta-thalassemia major and endothelial dysfunction may participate in the progression of common complications encountered in these subjects.

Disclosure statement

No potential conflict of interest was reported by the authors.

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