Leukocytes apoptosis and adipocytokines in children with beta thalassemia major

Khalid I. Elsayh1 · Wafaa S. Mohammed2 · Asmaa M. Zahran3 · Khaled Saad1

Abstract β-Thalassemia is a significant public health problem in Egypt. Infectious complications represent the second most common cause of mortality and the major cause of morbidity in β-thalassemia major (BTM). The increased susceptibility of these patients to infectious diseases has been attributed to the abnormalities of the immune system, which is evident by systemic inflammation and immune deficiency. In a case control study, 35 patients with BTM were compared with 30 sex- and age-matched children who served as controls. Serum ferritin, high-sensitive CRP (hsCRP), leptin and adiponectin levels were determined in all subjects. Apoptosis of neutrophils and lymphocytes was measured by the Annexin V-fluoroisothiocyanate binding assay. Serum leptin was significantly lower in patients when compared to controls. In contrast, adiponectin and hsCRP levels were significantly higher in the patients than the controls. Positive correlation was found between adiponectin and hsCRP. BTM patients had significantly higher total leukocytes, neutrophils and lymphocytes compared with controls. BTM children exhibited a significantly increased apoptosis in T-lymphocytes; however, there was no significant difference in the percentage of apoptosis of B-lymphocytes and neutrophils between the patients and the controls. There was a significant negative correlation between serum leptin and the percentage of apoptotic T-lymphocytes. Our BTM patients had a high percentage of apoptotic T-lymphocyte in comparison with controls. In addition, they had disturbed serum levels of adipocytokines and inflammatory markers. These derangements could have a role in the immunological disturbance observed in thalassemic patients.

Keywords Adiponectin · Apoptosis · Leptin · Inflammatory markers · Thalassemia

Introduction

β-Thalassemia is a significant public health problem in Egypt, where over one million newborns are expected to be affected with this disorder, and it is considered the most common genetically determined chronic hemolytic anemia (85.1 %) in our locality. A high frequency of carriers has been reported in Egypt, ranging from 4 to 10 %. This is due to high rate of consanguineous marriage, which helps to accumulate deleterious genes in Egyptian families [1, 2].

Infectious complications represent the second most common cause of mortality and the major cause of morbidity in β-thalassemia major (BTM), with a prevalence of 12–13 %. In addition to the high risk of blood-borne infections associated with multiple transfusions, the increased susceptibility of these patients to infectious diseases has been attributed to the abnormalities of the immune system, which is evident by systemic inflammation and immune deficiency [3–5].

Inflammation is known to play an important role in the development of complications in BTM patients. A chronic inflammatory state is present in BTM patients, with increased levels of pro-inflammatory cytokines and inflammatory markers such as C-reactive protein (CRP).
Adipocytokines are considered important players in the pathogenesis of numerous metabolic, immune, vascular and inflammatory disorders [6–8]. Among these cytokines, much awareness has been paid to leptin and adiponectin, both of which have substantial effects on the inflammatory process [6, 7].

Leptin is characterized by multiple and evident effects on metabolic and immune functions. Its immune effects include the stimulation of hematopoiesis and lymphopoiesis, the activation of monocytes, dendritic cells and macrophages, the activation of neutrophils and natural killer (NK) cells and the modulation of the adaptive immunity, by enhancing T cell survival and stimulating the production of pro-inflammatory cytokines and suppresses the production of anti-inflammatory Th2 cytokines such as IL-4 in CD4 T cell [7–10].

Adiponectin is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism, and also has anti-inflammatory, antiatherogenic and antidiabetic properties. In some chronic inflammatory/autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease, adiponectin may have pro-inflammatory effects and it reduces inflammation, oxidative stress and cytokine production [6, 7, 11].

Studies investigating leukocyte apoptosis in BTM children were few and had contradictory results; in addition, the data on the relationships between leukocyte apoptosis with adipocytokines and inflammatory markers are limited. The present study aimed to analyze the neutrophil, T- and B-lymphocyte apoptosis and to measure serum adiponectin and leptin levels in a cohort of Egyptian children with BTM.

**Patients and methods**

**Patients**

Thirty-five Egyptian BTM children (21 males) with an average age ± SD of 10.97 ± 3.81 year (5–13 years) were voluntarily recruited to this case-controlled study in the Pediatric Hematology Unit of the Assiut Children University Hospital, Assiut, Egypt. Thirty socio-economic, age- and sex-matched healthy children were included in this study as controls. The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki, and informed consent was obtained in every case from their legal guardians.

**Inclusion criteria**

Thalassemia patients on regular blood transfusion and chelation therapy, either oral chelation with defrasorix or deferiprone or subcutaneous administration of deferoxamine were included.

**Exclusion criteria**

Patients with known diabetes, cardiac, renal, infectious, inflammatory or pulmonary diseases and newly diagnosed BTM cases yet to receive a blood transfusion were excluded from the study. Splenectomized patients (to exclude hematological and immunological effects of splenectomy) were also excluded from the study.

**Methods**

All patients and controls were subjected to the following: thorough medical history and examination, including age, sex and duration of symptoms, blood transfusion and chelation therapy. Samples were obtained prior to a scheduled transfusion. All patients were abstained from medications (e.g., corticosteroids, antimicrobials), chelators and nutritional supplements for the previous 24 h. Fasting blood samples were collected from resting subjects through venipuncture in EDTA and plain tubes. The complete blood count was done on Celltac E automated hematology Analyzer (Nihon Kohden Corporation, Tokyo, Japan). Samples were centrifuged for 15 min at 3000 rpm at 4 °C, separated into aliquots, and immediately stored at −70 °C until tested. Assays were performed in duplicate according to the manufacturer’s instructions. Serum ferritin, high-sensitive CRP (hsCRP), leptin and adiponectin levels were determined in all participants using enzyme immunosorbent assay (ELISA). Serum ferritin was determined using ELISA kit manufactured by Diametra SRL, Italy, and hsCRP was determined using ELISA kit from Monobind Industry (Lake Forest, CA, USA). For adiponectin determination, we used the ELISA kit purchased from Assay PRO, USA. Serum leptin was determined using the ELISA kit manufactured by DBC Diagnostic Biochem Canada Inc.

**Flow cytometric detection of apoptosis of neutrophils and lymphocytes**

Apoptosis was measured by the Annexin V-fluoroisothiocyanate (FITC) binding assay according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Fifty μL of whole blood was stained with 5 μL of peridinin-chlorophyll-protein (Per-CP)-conjugated anti-CD3 (T-Lymphocyte marker), phycoerythrin (PE)-conjugated anti-CD13 (neutrophil marker) and allophycocyanin (APC)-conjugated anti-CD19 (B-Lymphocyte marker), washed twice with 2 mL of phosphate-buffered saline.
(PBS), and red blood cells were lysed. The cells were washed and resuspended in 100 μL of the Annexin V-conjugate binding buffer to which 5 μL of FITC-conjugated Annexin V was added. The mixture was incubated in dark at room temperature for 15 min, after which 400 μL of the binding buffer was added, and 10,000 cells were acquired and analyzed by FACSCalibur flow cytometry. Antihuman IgG was used as an isotype-matched negative control for each sample. Forward and side scatter histogram was used to define neutrophil and lymphocyte populations. Then, the percentages of CD19+ (B-lymphocytes) and CD3+ (T-lymphocytes) were assessed in the lymphocyte populations. Then the expression of Annexin V in T-lymphocytes, B-lymphocytes and neutrophils was detected (Fig. 1).

**Statistical analysis**

Data analysis was performed with the Statistical Package for Social Sciences (SPSS version 16). Data are expressed as mean ± standard deviation (SD) for all parameters. Due to the small sample size and a propensity for outliers in some of the variables, statistical differences between the groups were examined using the Mann–Whitney test. Spearman’s correlation was used to determine the correlation between studied parameters. A value of p ≤ 0.05 denoted a statistically significant difference.

**Results**

The clinical and demographic characteristics of the patients and the controls were shown in Table 1. Hemoglobin, height, weight and body mass index (BMI) of the patients were significantly lower than their respective values in the control group. Furthermore, as expected, serum ferritin levels of all patients were significantly higher than those of the healthy controls. Serum leptin was significantly lower in patients when compared to controls. In contrast, adiponectin and hsCRP levels were significantly higher in the patients than the controls (Table 2). Leptin was negatively correlated with ferritin levels (p < 0.001, r = −0.56, Fig. 2). Positive correlation of BMI leptin levels was statistically significant only in healthy controls (p < 0.01, r = 0.285). Positive correlation was found between adiponectin and hsCRP (p < 0.05, r = 0.25, Fig. 3).
When leukocyte subsets were investigated, BTM patients had significantly higher total leukocytes, neutrophils and lymphocytes compared with controls (Table 3). The mean number of B-lymphocytes was increased in the patients than controls, while T-lymphocytes and monocytes were comparable in the patients and the controls. Compared with the controls, BTM children exhibited an increased apoptosis in T-lymphocytes; however, there was no significant difference in the percentage of apoptosis of B-lymphocytes and neutrophils between the patients and the controls (Table 3). There was a significant negative correlation between serum leptin and the percentage of apoptotic T-lymphocytes ($R = -0.613, p = 0.0001$).

**Discussion**

The association of high adiponectin with elevated hsCRP levels found in the present study could potentially explain the pro-inflammatory effect of adiponectin that might be involved in the inflammatory vascular process and elucidate the association of inflammation/adipose tissue linkage in vivo [12]. Similar to our findings, previous studies reported elevated adiponectin levels in thalassemic children [6, 7]. Chaliasos et al. [7] reported that higher adiponectin levels were positively correlated with the increased levels of endothelin-1 (ET-1) in thalassemic patients. Adiponectin may take part in the equilibrium between the release of cytokines and adhesion molecules from the endothelium. Elevated adiponectin may be the expression of a counter-regulatory response aimed at modifying the endothelial damage and cardiovascular risk in BTM patients [7].

Lower leptin levels reported in our study and in previous studies [7, 13–16] and its negative correlation with ferritin possibly reflect the toxic effects of iron overload on cell

**Table 1** Demographic, clinical and some laboratory characteristics of thalassemia patients and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Thalassemia patients (35)</th>
<th>Control (30)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>10.97 ± 3.81</td>
<td>11.60 ± 1.91</td>
<td>N.S</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>24.77 ± 7.99</td>
<td>37.20 ± 5.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>123.91 ± 15.88</td>
<td>142.0 ± 9.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>15.70 ± 2.13</td>
<td>18.38 ± 1.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.31 ± 1.45</td>
<td>12.21 ± 2.34</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>2007.82 ± 646.63</td>
<td>81.0 ± 35.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of blood transfusion (year)</td>
<td>7.86 ± 3.721</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Chelation therapy: number (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Deferoxamine</td>
<td>18 (51.4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>*Deferiprone</td>
<td>9 (25.7)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>*Deferasirox</td>
<td>8 (22.8)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Mann–Whitney test

Data are presented as mean ± SD. $p < 0.05$ is significant

**BMI** body mass index

**Table 2** High-sensitive CRP, leptin and adiponectin levels in both thalassemia patients and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Thalassemia patients (35)</th>
<th>Controls (30)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP (mg/L)</td>
<td>4.87 ± 1.89</td>
<td>1.49 ± 0.37</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>2.92 ± 0.71</td>
<td>9.59 ± 5.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adiponectin (ug/mL)</td>
<td>13.39 ± 7.59</td>
<td>7.89 ± 3.25</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. $p < 0.05$ is significance difference

**hsCRP** high-sensitive C-reactive protein

**Fig. 2** Correlation between serum leptin and ferritin
membranes and proteins in BTM patients, since free iron causes peroxidative damage in lipid membrane and proteins with the generation of free radicals. Thus, iron overload (such as in BTM) results in the destructions of the adipocyte. Along with the destruction of the fat cell membrane and the dysfunction in adipose tissue, it leads to a decrease in leptin serum level. Furthermore, the replacement of red bone marrow with yellow bone marrow which contains adipocytes can be the cause of this decrease [7, 15, 16]. A positive correlation between leptin and BMI was detected only in healthy controls, a finding in agreement with previous reports [7, 12]. Since the BMI of the patients is lower than that of the matched control group, it could be concluded from our study and these reports [7, 12] that the adipocytes of thalassemic patients are unable to maintain adequate leptin production when a higher leptin secretion is required, possibly due to toxic effect of iron overload, suggesting that the adipose tissue dysfunction can be considered one of the endocrinopathies affecting thalassemic patients, and the consequent low leptin levels might play a role in the neuroendocrine and hematopoietic dysfunctions.

Few studies investigated the distribution of leukocyte subsets and leukocyte apoptosis in thalassemia patients [17–21]. A major cause of morbidity and mortality in thalassemic patients is infection, which assumed to be the result of immunological changes that could explain the higher total leukocytes, lymphocytes and neutrophil counts in our patients than the controls. The increased B-lymphocytes in our patients may be due to chronic stimulation of immune system in response to infection and related to increased production of immunoglobulin levels noted in thalassemia patients [22].

A previous study [17] reported that thalassemic patients had significantly higher total leukocytes and lymphocyte counts compared with the controls, and also B-lymphocyte percentage was significantly higher in patients with no significant difference in T-lymphocytes. Another study found that BTM patients showed a marked and persistent lymphocytosis with an increase in the number of both T and B cells with the chief increase in B cells [18]. Another study [19] found that the absolute number of total leukocytes, lymphocytes and neutrophils was increased in the thalassemic patients.

In our study, despite the fact that the percentage of apoptotic T-lymphocyte was more in the BTM patients than the controls, the T-lymphocyte count was not decreased. Thalassemia patients are chronically immunostimulated by transfusions, non-transferrin-bound iron, chelation and organ injury. This chronic stimulation of the immune system not only may lead to increased number of T-lymphocytes, but also lead to exhaustion of T-lymphocytes and increase their apoptosis. The negative correlation between the level of serum leptin and the percentage of apoptotic T-lymphocytes could indicate that the deficiency of leptin in our thalassemia patients may be responsible for the increased apoptosis of T-lymphocytes as leptin has direct antiapoptotic effect on T cells [23, 24]. Walter et al. [25] reported that thalassemic peripheral blood leukocytes

![Fig. 3 Correlation between adiponectin and hsCRP](image-url)

**Table 3** Leukocyte subsets and leukocyte apoptosis in thalassemia patients and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Thalassemia patients (35)</th>
<th>Control (30)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (10^9/L)</td>
<td>14.84 ± 3.70</td>
<td>9.32 ± 2.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils (10^9/L)</td>
<td>11.21 ± 2.70</td>
<td>6.31 ± 2.30</td>
<td>0.03</td>
</tr>
<tr>
<td>Monocytes (10^9/L)</td>
<td>0.68 ± 0.34</td>
<td>0.73 ± 0.27</td>
<td>0.562</td>
</tr>
<tr>
<td>Lymphocytes (10^9/L)</td>
<td>2.79 ± 0.44</td>
<td>2.36 ± 0.23</td>
<td>0.003</td>
</tr>
<tr>
<td>B-lymphocytes [CD19^+ (10^9/L)]</td>
<td>0.51 ± 0.10</td>
<td>0.33 ± 0.14</td>
<td>0.001</td>
</tr>
<tr>
<td>T-lymphocytes [CD3^+ (10^9/L)]</td>
<td>2.15 ± 0.09</td>
<td>2.12 ± 0.06</td>
<td>0.281</td>
</tr>
<tr>
<td>T-lymphocytes apoptosis %</td>
<td>37.24 ± 14.493</td>
<td>12.01 ± 2.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B-lymphocytes apoptosis %</td>
<td>10.98 ± 1.37</td>
<td>10.01 ± 2.45</td>
<td>0.669</td>
</tr>
<tr>
<td>Neutrophil apoptosis %</td>
<td>13.02 ± 5.71</td>
<td>11.60 ± 2.82</td>
<td>0.334</td>
</tr>
</tbody>
</table>

Mann–Whitney test

Data are presented as mean ± SD, p < 0.05 is significance difference

% Percentage
had increased levels of pro-apoptotic marker Bax (an inducer of mitochondrial dysfunction) and a high ratio of Bax/Bcl-2, indicating decreased stability of the mitochondrial outer membrane and increased potential for mitochondrial dysfunction and apoptosis. They demonstrated that markers of leukocyte apoptosis and mitochondrial dysfunction were high in thalassemia patients compared with controls [25]. Persistence or lack of immune cells at inflammatory sites or the development of chronic inflammation observed in β-thalassemia patients may result from dysregulation of the apoptotic cell death pathway [25, 26].

Conclusion

Our BTM patients had a high percentage of apoptotic T-lymphocyte in comparison with controls. In addition, they had disturbed serum levels of adipocytokines and inflammatory markers. These derangements could have a role in the immunological disturbance observed in thalassemic patients.

Conflict of interest None.

References