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Hemostatic Derangements in Egyptian Patients with ß-Thalassemia Major

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ABSTRACT

Background: Profound hemostatic changes have been observed in patients with ß-thalassemia major, which make it a high risk condition for development of thromboembolic events.

Aim: The present study aimed at evaluating coagulation parameters in Egyptian patients with ß-thalassemia major. Forty patients with ß-thalassemia major (Group I: 20 nonsplenectomised and Group II: 20 splenectomised) were included in the study. The study also included 16 age and sex matched healthy children as control. Hematological parameters as well as iron status and liver functions were analyzed. Coagulation was assessed by standard clotting tests; prothrombin time (PT) and activated partial thromboplastin time (APTT). The activity levels of anticoagulant proteins including protein C, protein S and antithrombin III (AT III) were also determined. In addition prothrombin fragment 1+2 (F1+2), a marker of thrombin generation was measured.

Results: Severe microcytic hypochromic anemia was present in all cases (mean Hb concentration was 6.66±1.36 and 7.19±0.19gm/dL and mean MCV was 66.42±6.26 and 68.54±6.71fL in group I and II respectively). They also suffered iron overload as both serum iron and serum ferritin were significantly higher in patients than control. PT and APTT were significantly prolonged ($p<0.001$). Protein C, protein S and antithrombin III were all significantly lower than the control ($p<0.001$) and they negatively correlated with serum ferritin in group I (r=-0.634, -0.616, -0.612 respectively and $p<0.01$) and in group II (r=-0.673, -0.666, -0.676 respectively and $p<0.001$). The level of F1+2 was significantly higher in splenectomised group than nonsplenectomised and the control ($p<0.001$).

In conclusion significant alterations in anticoagulant proteins and F1+2 exist in patients with ß-thalassemia major. Close monitoring and assessment of these parameters in thalassemic patients is recommended so that effective measures to control thromboembolic episodes can be implemented.

Key Words: *Hemostatic derangement – F1+2 – ß thalassemia major.*

INTRODUCTION

The homozygous ß-globin chain mutation in the adult hemoglobin (Hb A) gene gives rise to ß thalassemia major. In this genetic disorder, red blood cells have a short life span due to excess accumulation of the unpaired α-globin chain **[1]**. Possessing unstable molecular configuration, α-globin chains aggregate and precipitate in early hemoglobin producing cells in the bone marrow. This leads to apoptosis of these cells and ineffective erythropoiesis. The red cells that reach the peripheral blood also contain excess α-globin chains. This results in membrane damage and hemolysis of these cells preferentially with development of severe anemia **[2]**.

ß-thalassemia major is treated with regular blood transfusion and concomitant administration of chelating agents to reduce blood transfusion induced hemochromatosis **[3]**. Adequate chelation of such patients reduces iron accumulation and prevents organ damage, resulting in a consistent decrease of morbidity and mortality **[4]**. Survival of patients with ß-thalassemia major has improved by the development of comprehensive thalassemia care services. With continued improvement in survival, a number of late adverse effects has become increasingly apparent. The effects on heart, liver and endocrine system have been known for over four decades but focus on hemostatic derangement is relatively recent **[5]**.

Profound hemostatic changes have been observed in patients with ß-thalassemia major. The presence of higher than normal incidence of thrombotic anomalies in the majority of

patients, has led to the recognition of the existence of chronic hypercoagulable state in ß-thalassemia major **[6]**. The commonly seen clinical manifestations are transient ischemic attacks **[7]**, stroke **[8]**, deep vein thrombosis **[9]**, recurrent pulmonary hypertension **[10]** and myocardial infarction **[11]**. Similarly, autopsy findings in patients with ß-thalassemia major have clearly demonstrated hypercoagulability as a pathologic feature. Multiple microthrombi in the pulmonary arterioles, composed mainly of platelets were found in autopsies performed on thalassemic patients **[12]**.

Thrombotic events are associated with increased activity of the coagulation system and the generation of thrombin **[13]**. Thrombin itself is impossible to quantify because it lasts only seconds in the circulation. Thus, it is necessary to use a surrogate marker for thrombin generation. Prothrombin fragment 1+2 is an activation peptide which is generated during conversion of prothrombin to thrombin in blood coagulation **[14]**. Thus, it can be used as a biomarker for thrombin generation during blood coagulation and has the diagnostic potential for assessing thrombotic risk and monitoring anticoagulant therapy **[15]**. Studies of the coagulation proteins provide strong evidence for the existence of a chronic hypercoagulable state in ß-thalassemia major **[16]**. Profound changes in prothrombin time (PT), partial thromboplastin time (APTT), natural anticoagulants like protein C, protein S and Anti-thrombin III (AT III) have been described though the mechanisms involved in the thrombotic tendency seen in some patients have not been fully elucidated **[5]**.

ß-thalassemia major; the most common genetic disorder in Egypt, is a major health problem with an estimated carrier rate of 7% **[17]**. This study was therefore, planned with the objectives of evaluating the hemostatic derangements in Egyptian patients with ß-thalassemia major. So that, effective measures to control thromboembolic episodes can be implemented.

PATIENTS AND METHODS

This study was carried out on 40 patients with ß-thalassemia major. They were followed at the Hematology Department, Medical Research Institute, Alexandria. Their ages ranged from 8 to 14 years. The diagnosis of ß-thalassemia major was carried out by clinical signs, complete blood count and hemoglobin electrophoresis. The patients received regular blood transfusions and were under chelation therapy with Desferrioxamine (DFO).

Patients were divided into two groups; Group I: Which included 20 non splenectomised patients, 10 males and 10 females, their mean age was 10.80 years±1.10 and their median age was 10 years and Group II: Which included 20 splenectomised patients 8 males and 12 females, their mean age was 9.90 years±1.77 and their median age was 9 years. Our study also included 16 healthy normal children with matched age and sex as a control group. They were 9 males and 7 females, their ages ranged from 8 to 13 years with a mean of 10.13±1.96 and a median of 10 years.

This study was approved by the institutional review board and followed the Helsinki Declaration on human experimentation. Informed consent of the parents of all children was taken before starting the work.

All thalassemic children were subjected to the following:

• Careful history taking.

• Thorough clinical examination.

Blood samples were collected from thalassemic patients immediately before blood transfusion and from the healthy children.

• Two ml. of whole blood were collected on EDTA tube for complete blood picture and reticulocyte count **[18]**.

• Two ml. of whole blood without anticoagulant were collected for determination of AST (SGOT), ALT (SGPT), serum iron and serum ferritin.

• All assays for coagulation were carried out on blood collected in sodium citrate at a final concentration of 3.8% (w/v) and the ratio of anticoagulant to blood was 1:9 (v/v). Platelet poor plasma was prepared for all coagulation tests by centrifugation at 2000g for 15 minutes, it was kept at room temperature for all coagulation tests and kept at -70ºC until prothrombin fragment 1+2 was determined.

* Prothrombin time (PT) was estimated by Quick one stage method using calcium thromboplastin (thromborel-S) from Behringwerke, Marburg, Germany. Activated partial thromboplastin time (APTT) was estimated using C.K. prest kit supplied by Diagnostica Stago (France). PT and APTT were done using standard laboratory methods **[19]**.
* Protein C activity levels in plasma were determined by the synthetic chromogenic substrate method using Stachrom Protein C kit from Diagnostica Stago (France).
* Protein S and antithrombin III (AT III) quantitative determination was done by the immune-turbidimetric method using Liatest Protein S and Liatest AT III kits respectively, the kits were supplied by Diagnostica Stago (France).
* The levels of prothrombin fragment 1+2 (F1+2) were determined by ELISA **[20]** using Enzygnost F1+2 micro® from Dade Behring (Germany).
* Serum iron was quantitatively determined by colorimetry using Spinreact kit (Spain). Serum ferritin estimation was done by ELISA kit from Bioplus, San Fransisco, USA.

Protein C <70%, Protein S <70%, and AT III <80% were taken as low values.

Statistical analysis:

Statistical analysis was done using SPSS (version 10.0 for windows). Data were expressed as mean and standard deviation and were subjected to the Kolmogorov-Smirnov test to determine the distribution and method of analysis. As most of the data were normally distributed continuous variables, student's *t* test was used for numeric values. Pearson's correlation coefficient (*r*) was used to test the correlation between the continuous variables. *p* values <0.05 and <0.001 were considered significant and highly significant respectively.

RESULTS

The clinical data of nonsplenectomised and splenectomised groups of ß-thalassemia are summarized in Table (1). In group I, there was splenomegaly in 14 patients (70%), hepatomegaly in 12 (60%), pulmonary hypertension in 2 (10%). Right heart failure and leg ulcers were not detected. On the other hand, in group II, 15 patients (75%) had hepatomegaly, 3 (15%) had pulmonary hypertension, 2 (10%) had right heart failure and 2 (10%) had leg ulcers.

Table (2) summarizes the hematological parameters in both patient groups and control group. The red blood cell count (RBC), hemoglobin concentration (Hb), and cell indices including mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCH) were all markedly decreased in both patient groups when compared to the control indicating severe microcytic hypochromic anemia ($p<0.001$). However, the differences between group I and group II were not statistically significant. On the other hand, the two patient groups had higher reticulocyte percentage than the control group and the differences were statistically significant ($p<0.001$). Moreover, group II had higher normoblast percentage than group I and the difference was statistically significant ($p<0.001$). Regarding the platelet count, group II had statistically significant higher value than both; group I and the control ($p<0.001$).

Table (3) shows the iron status in the study groups and the controls. Both patient groups had statistically significant higher serum iron and serum ferritin levels than the control group ($p<0.001$), however the difference between the two patient groups was not statistically significant. Both levels of AST and ALT in the two patient groups were significantly higher than the control group ($p<0.001$) as shown in Table (4).

Table (5) illustrates the coagulation parameters in both groups of ß-thalassemia major and the control group and Table (6) shows the proportion of patients with abnormal results. PT and APTT were prolonged in both patient groups when compared to the control group and the differences were statistically significant ($p<0.001$). However, the difference between group I and group II was not statistically significant in PT while group II had more prolonged APTT than group I and the difference was statistically significant ($p<0.01$). The levels of naturally occurring anticoagulants namely; protein C, protein S and AT III were significantly lower in thalassemic patients whether splenectomised or not, than the control group ($p<0.001$), though both patient groups did not differ significantly from each other. There was significant

negative correlation between serum ferritin and the studied coagulation inhibitors; protein C, protein S and AT III in group I (r=-0.634, -0.616, -0.612 respectively and p<0.01) and in group II (r=-0.673, -0.666, -0.676 respectively and p<0.001). The level of F1+2 was significantly higher in group II than group I and the control group (p<0.001) (Fig. 1). However, group I had higher mean level of F1+2 than the control group but the difference did not reach statistical significance.

Table (1): Clinical findings in thalassemic patients.

Parameters	Group I n=20		Group II n=20	
	No.	%	No.	%
Splenomegaly	14	70	–	–
Hepatomegaly	12	60	15	75
Pulmonary hypertension	2	10	3	15
Right heart failure	0	0	2	10
Leg ulcers	0	0	2	10

Table (2): Hematological parameters in the patient groups and controls.

Parameters	Group I	Group II	Control
Hb g/dL	6.66±1.36 *p*a<0.001*	7.19±0.19 *p*a<0.001* *p*b>0.05	12.06±0.13
RBCsx10^{12}/L	2.88±0.64 *p*a<0.001*	3.15±0.4 *p*a<0.001* *p*b>0.05	4.43±0.29
MCV fL	66.42±6.26 *p*a<0.001*	68.54±6.71 *p*a<0.001* *p*b>0.05	86.12±4.05
MCH pg	23.38±2.29 *p*a<0.001*	22.94±3.28 *p*a<0.001* *p*b>0.05	27.41±1.41
Normoblasts/100 WBCs	9.25±3.35	19.05±4.35 *p*b<0.001*	
WBC x 10^9/L	8605±4090.36 *p*a>0.05	8500±2714.68 *p*a>0.05 *p*b>0.05	7625±1246.59
Platelet x 10^9/L	323.75±128.99 *p*a>0.05	419.30±100.30 *p*a<0.001* *p*b<0.01*	285±68.09
Reticulocyte%	4.23±1.07 *p*a<0.001*	4.8±0.89 *p*a<0.001* *p*b>0.05	0.93±0.23

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

Table (3): Iron status in thalassemic patients and controls.

Parameters	Group I	Group II	Control
Serum iron µg/dL	154.65±31.9 *p*a<0.001*	161.60±45.48 *p*a<0.001* *p*b>0.05	50.5±8.08
Serum ferritin ng/dL	1592.85±602.07 *p*a<0.001*	1546.65±565.83 *p*a<0.001* *p*b>0.05	34.25±8.81

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

Table (4): AST and ALT in thalassemic patients and controls.

Parameters	Group I	Group II	Control
AST U/L	52.10±29.36 *p*a<0.001*	59.20±23.62 *p*a<0.001* *p*b>0.05	16.20±9.08
ALT U/L	59.15±34.21 *p*a<0.001*	61.05±35.34 *p*a<0.001* *p*b>0.05	18.80±7.71

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

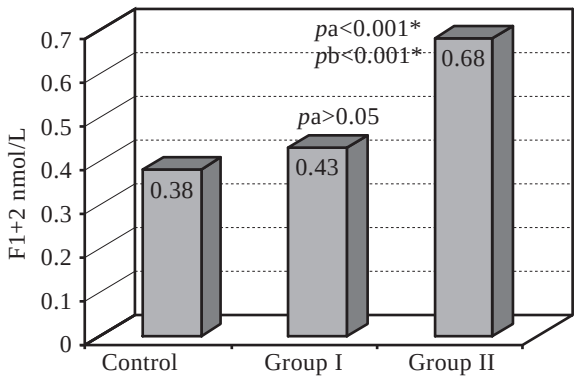
Table (5): Coagulation parameters in thalassemic patients and controls.

Parameters	Group I	Group II	Control
PT (seconds)	14.54±1.12 *p*a<0.001*	14.88±0.92 *p*a<0.001* *p*b>0.05	13.06±7.37
INR	1.23±0.18 *p*a<0.001*	1.29±0.15 *p*a<0.001* *p*b>0.05	1.0
APTT (seconds)	45.04±9.52 *p*a<0.001*	55.90±16.13 *p*a<0.001* *p*b<0.01*	32.37±2.58
F1+2 (nmol/L)	0.43±1.02 *p*a>0.05	0.68±0.22 *p*a<0.001* *p*b<0.001*	0.38±1.1
Protein C%	76.35±14.88 *p*a<0.001*	75.4±13.94 *p*a<0.001* *p*b>0.05	97.33±13.42
Protein S%	77.75±15.31 *p*a<0.001*	77.15±14.06 *p*a<0.001* *p*b>0.05	96.12±13.84
AT III%	79.35±13.83 *p*a<0.001*	80.75±14.22 *p*a<0.001* *p*b>0.05	101.44±7.06

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

Table (6): Proportion of patients with abnormal coagulation parameters in the two patient groups.

Parameters	Group I n=20		Group II n=20	
	No.	%	No.	%
APTT	9	45	15	75
Protein C	6	30	6	30
Protein S	5	25	7	35
AT III	6	30	5	25
F1+2	2	10	13	65



*p*a: Compared to control *p*b: Compared to group I
* Statistically significant

Fig. (1): Levels of F1+2 (nmol/L) in thalassemic patients and controls.

DISCUSSION

Patients with ß-thalassemia major are prone to develop thromboembolic complications. Zurlo et al. **[21]**, Michaeli et al. **[22]**, Borgna Pigantti et al. **[8]** and Eldor et al. **[9]** described thromboembolic complication in patients with ß-thalassemia major in previous reports. In the present study, 2 nonsplenectomised patients (10%) and 3 splenectomised (15%) complained of pulmonary hypertension, 2 splenectomised (10%) suffered from right heart failure and another 2 splenectomised (10%) had leg ulcers. Sonakul et al. **[10]** in an autopsy series found pulmonary artery obstruction in 44% of patients with ß-thalassemia major. On the contrary, Naithani et al. reported no thrombotic episodes in their patients **[5]**. Ibrahim has also studied 32 children with ß-thalassemia major and found no evidence of thrombotic manifestations. The plausible explanation for the lack of thromboembolic phenomenon in such studies could be the lower mean age of the studied groups when compared to patients evaluated by other investigators **[7]**. This might explain the lower incidence of thromboembolic events in our patients since their mean age was 10.80 years in nonsplenectomised and 9.90 years in splenectomised patients. Moreover, asymptomatic pulmonary vascular disease that could result from silent, recurrent thromboembolic events had been found in many patients with ß-thalassemia major. This was suggested by echocardiographic studies in 35 patients with ß-thalassemia major who had no clinical signs and symptoms of thromboembolic disease. Many of the patients showed pulmonary hypertension and right heart failure, which was more prevalent than left heart failure **[23]**. These findings suggest that the early right ventricular dysfunction, which precedes left heart failure in patients with ß-thalassemia, may be due to pulmonary hypertension secondary to microembolisation in the lungs and not from cardiomyopathy resulting from excessive iron deposition **[24]**.

In the present work, the mean PT and APTT were significantly prolonged than age-matched healthy children. This is in accordance with Naithani et al., who reported prolongation of the PT and APTT in their patients **[5]**. Parenchymatous liver damage or the circulating hemolysates can explain these effects. Ibrahim pointed that such prolongations could be due to a chronic activation of intrinsic coagulation and intravascular hemolysis **[7]**.

Andrew et al., investigated the reason for this contact activation and found a kallikrein like protease activity which could be released from tissues due to iron overload **[25]**. In keeping with iron overload, in the present study both levels of serum iron and ferittin were significantly higher in thalassemic patients than age-matched healthy children.

Low levels of the coagulation inhibitors, protein C, protein S, and AT III have been observed in patients with ß-thalassemia major from a variety of ethnic backgrounds **[9,16,26]**. Mussumeci et al., found low protein C activity in 94% and low AT III in 55% of cases but only 2 of their 74 patients had clinical thromboembolic manifestations **[27]**. Similarly, Ibrahim found low activity of protein C, protein S and AT III in 27,23 and 32% of patients, respectively but none had thromboembolic features **[23]**. In the present work low activity of protein C, protein S and AT III was found in 30,25, and 30% of cases respectively in group I and in 30,35 and 25% of patients respectively in group II. Furthermore, significant lower levels of the three parameters were observed in the patient groups when compared to age-matched healthy children. Inherited deficiencies of these factors are unlikely owing to such a high percentage of patients being deficient. Cappellini et al., explained that the deficiencies of these coagulation inhibitors may be due to a possible role of the liver dysfunction since protein C, protein S and AT III are very sensitive to mild degrees of impairment of the synthetic function of the liver **[26]**. This was evident in our study by the significant increase in liver enzymes. Moreover, severe iron overload can result in progressive organ failure **[6]**. In accordance with our results there was significant negative correlation between serum ferritin and the studied anticoagulant proteins; protein C. protein S and AT III in all patients whether splenectomised or not.

In the present study, F1+2; a marker for thrombin generation, was significantly higher in splenectomised patients than both non splenectomised patients and controls. Our results go in line with those of Opartkiattikul and his colleagues who found high level of F1+2 in splenectomised patients when compared to non-splenectomised patients and age-matched

healthy children **[28]**. On the contrary, Cappellini et al., reported normal levels of F1+2 in both splenectomised and non-splenectomised thalassemia major patients, however a much higher level of F1+2 was detected in his splenectomised patients with thalassemia intermedia when compared to non-splenectomised thalassemia intermedia patients and those with thalassemia major **[26]**. The reason for this difference might have been that thalassemia major in Italy undergo regular blood transfusion more than do our patients who suffered severe microcytic hypochromic anemia (mean Hb concentration in our study was 6.66 and 7.19gm/dL and mean MCV was 66.42 and 68.54 fL in group I and II respectively). This could be explained by the irregularity of blood transfusion in our patients due to lack of health education which makes the availability of matched blood for thalassemic patients a hindering problem. Atichartakran and his colleagues reported that smaller sized RBCs are more thrombogenic especially after splenectomy **[29]**. In addition Cappellini et al. postulated that thalassemic red cells and erythroid precursors present in the blood of splenectomised patients with ß-thalassemia would act as activated platelets and enhance the conversion of prothrombin to thrombin in the final stage of blood coagulation **[26]**. This is in agreement with our results as splenectomised patients had significantly higher percentage of normoblasts and F1+2 than non-splenectomised patients. Moreover, thalassemic red cells may provide a source of negatively charged phospholipids such as phosphatidyl serine which can increase thrombin generation **[30]**. Phosphatidyl serine exposed on the surface of thalassemic red cells functions as a signal for their recognition by phagocytes and their subsequent removal from the circulation and for the occurrence of apoptosis **[31]**. It is plausible that this phenomenon is more marked after removal of the spleen because splenectomy favors the persistence of these damaged red cells in the circulation **[26]**. These findings may explain that the incidence of thromboembolic events in our study was greater in splenectomised than non-splenectomised patients.

In conclusion, significant alterations in the hemostatic system exist in ß-thalassemia major particularly irregularly transfused patients, which make it a high risk condition for development of thromboembolic events. We suggest regular blood transfusion programs and close monitoring of thalassemic patients specially with increasing age so that effective measures to control thromboembolic episodes, can be implemented.

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