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EVALUATION OF RED BLOOD CELL MICROPARTICLES IN THALASSEMIA

By

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ABSTRACT

Background: Thalassemia is a group of heterogeneous disorders with variable severity characterized by defect in globin chain synthesis due to genetic defect. Hypercoagulable state in thalassemia is well known complication affecting 1.1 % of patients. It is a multifactorial event with variable degree of severity and clinical presentation. Cell derived vesicles are very small vesicles < 1?m released from cells in different conditions and for different purposes. They play an important role in cell signaling, adhesion and coagulation.

Objective: to evaluate erythrocyte microparticles in patients with thalassemia.

Patients and methods: Blood samples were collected from 50 thalassemic patients and 50 normal individuals. Platelet poor plasma was separated and used for analysis of erythrocyte microparticles by flow cytometry using CD235a.

Results: A highly significant difference was observed between all groups in basic hematological parameters. The level of microparticles was significantly higher in patients with thalassemia and was higher in adult than in children. This high level is positively correlated with HbF levels and negatively correlated with Hb levels and Hb A levels.

Conclusion: The levels of erythrocyte microparticles were higher in patients with thalassemia which may contribute to the hypercoagulable state of the disease. Further studies on medications that inhibit microparticles formation may be of value in prevention and treatment of thrombosis caused by these vesicles.

Keywords: Thalassemia, Microparticles, Hypercoagulability.

INTRODUCTION

Beta-Thalassemia is a heterogeneous family of inherited disorders affecting hemoglobin synthesis. It is characterized by the complete absence or reduced synthesis of the β chain of hemoglobin, resulting in increased, but ineffective, erythropoiesis. The β-thalassemia phenotypes are variable, ranging from severe conditions requiring blood transfusions (thalassemia major [TM]) to milder forms (thalassemia intermedia [TI]) (Borgna-Pignatti et al., 2004).

Patients with thalassemia frequently develop leg ulcers and have an increased predisposition to thrombosis, especially if splenectomised. Such events include deep vein thrombosis, portal vein thrombosis, stroke and pulmonary embolism (Taher et al., 2008).

Microparticles (MPs) are shed submicrometric plasma membrane fragments (~0.1-1 ?m) harboring phosphatidylserine

(PS) in their extracellular membrane leaflet. They mainly derive from apoptotic or activated cells, and generally present a procoagulant potential. Increased levels of circulating MPs were described in many disorders with major vascular and thrombotic symptoms (Piccin et al., 2007).

It was shown that microparticles of red blood cell are elevated in patients with TI versus controls; these have a potential to aggravate thrombotic events (Habib et al., 2008).

This study was performed to evaluate the level of RBCs MPs in patients with thalassemia with different severity as a factor predisposing to the hypercoagulability in hemoglobinopathies and compare the results with that of controls.

SUBJECTS AND METHODS

This study was done at Al-Azhar University Hospitals in the period between November 2013 and April 2016 on a total of 100 individuals, 50 patients with thalassemia (20 males and 30 females, aged ranged from 4-41 years with average 16.6 ± 8.2) and 50 age and sex matched normal individuals (23 males and 27 females, aged ranged from 4-40 years with average 25.2 ± 9.5) as a control group.

Patients with thalassemia were selected by history and laboratory evidence of the disease (microcytic hypochromic anemia with electrophoretic pattern consistent with thalassemia).

Individuals in control group were apparently healthy with no history of hematological disease and no laboratory evidence of thalassemia.

According to the severity, patients with thalassemia were subdivided into 3 subgroups:

a) Thalassemia minor: Ten patients with no history of transfusion, no splenomegaly by examination, Hb levels >9 g/dl, high HbA2 with no or very mild elevation of Hb F.

b) Thalassemia intermedia: Eleven patients diagnosed after age of 2 years and no or occasionally transfused, Hb levels > 7, with splenomegaly on examination, or history of splenectomy and with Hb F > 10 %.

c) Thalassemia major: Twenty nine patients who were regularly attending to the hospital for transfusion with splenomegaly on examination or history of splenectomy.

All participating individuals gave a written informed consent for applying in the study.

Blood samples were collected. In patients with thalassemia, blood was withdrawn before transfusion. The blood sample was then divided into 4 tubes with the following order: 2 ml on 3.2% trisodium citrate vacutainer tube for Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT); 2 ml on 3.2% trisodium citrate vacutainer tube for microparticles separation; 2 ml on plain tube for serum ferritin level, and 2 ml on K-EDTA vacutainer tube for complete blood count (CBC), reticulocytic count and Hb electrophoresis.

C.B.C. was done using Sysmex Kx-21 N automated cell counter, PT and aPTT were done using Sysmex® CA 1500 automated coagulation analyzer, and

ferritin was analyzed using Cobas C311 automated chemistry analyzer.

Reticulocytic count was done manually by adding one volume of Brilliant cresyl blue stain to one, two or three volumes of blood (according to the degree of anemia), then incubated for 20 minutes. Smear is then prepared and examined.

Hb electrophoresis was assayed using Helena SAS electrophoresis with the following procedure:

- Blood samples were washed and packed RBCs were then lysed using hemolysate regent; 35 µL of Hb samples were then loaded into the wells; Samples were transferred onto the gel plate by sample comb; Machine settings was adjusted at 200 volts for 30 minutes.
- Gel plate was then removed from the electrophoretic unit and loaded in to stain and destain unit; Clear gel plate was then scanned for identification of bands.
- Control sample was run with each gel plate.

Microparticles were assayed using BD FACSCalibur™ Cell Analyzer with the following procedure:

- Platelets free plasma (PFP) was prepared by serial centrifugations, 10 minutes at 1,800×g, 15 minutes at 3,000×g, and 5 minutes at 3,000×g at room temperature and within one hour of blood collection (Nielsen et al., 2014).
- Samples were incubated at dark for 30 minutes with FITC-conjugated antibody against CD235a.
- Control tubes were prepared by incubation of samples with FITC-conjugated antibody against mouse IgG.
- After incubation, phosphate buffered saline (PBS) were added to the samples and analyzed within 30 minutes.
- Data was acquired using logarithmic amplification, low flow rate; with acquisition of at least 10.000 events; the area of interest was identified through back gating.

Statistical analysis:

- All results were analyzed using Statistical package for social science (SPSS V.15, IBM Corp. U.S.A).
- Descriptive statistics used for quantitative data were; Mean ± SD, while categorized data were represented as numbers and percentages.
- Chi square test was used to compare sex.
- T-student test was used to compare age.
- Friedman One way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) were used to compare means of parametric data of different groups.
- Pearson correlation coefficient was used to check for correlation between two quantitative parametric data.
- For all analysis, a two-tailed test was used and $p < 0.05$ was considered statistically significant.

RESULTS

Our study included a total of 100 individuals. 50 thalassemic patients recruited to Al-Azhar university hospitals and 50 apparently healthy individuals. No significant difference was observed between patients and controls as regard to age and sex.

There were highly significant differences ($p<0.01$) between groups in all parameters of CBC except for MCV, between thalassemia major and thalassemia intermedia (60.0 ± 5.93 versus 62.73 ± 6.97, p=0.120), MCHC, between normal individuals and thalassemia major (32.31 ± 0.78 versus 32.3 ± 1.59, p =0.938), WBCs, between normal individuals and thalassemia minor (7.90 ± 2.82, versus 7.95 ± 2.70, p=0.979) and between thalassemia major and thalassemia intermedia (16.5 ± 9.21 versus 13.59 ± 6.75, p=0.170), and Platelets, between normal individuals and both thalassemia minor (310 ± 106.65 versus 357 ± 169.78, p=0.408) and thalassemia intermedia (310 ± 106.65 versus 381 ± 160.94, p=0.19) and between thalassemia minor and thalassemia intermedia (357 ± 169.78 versus 381 ± 160.94, p =0.74).

Regarding to reticulocytic count, highly significant differences existed between groups ($p<0.01$). Serum levels of ferritin showed statistically significant differences between groups ($p<0.01$) except between normal individuals and thalassemia minor (109.1 ± 45.8 versus 158.6 ± 61.6, p=0.789). Similarly, highly significant differences were found between groups in Hb variants (Hb A, HbA2 and HbF). These differences were not observed between normal individuals and thalassemia minor as regard to HbA (97.57 ± 0.27 versus 94.41 ± 0.92 p=0.243), between thalassemia minor and thalassemia intermedia as regard to Hb A2 (5.04 ± 0.61 versus 4.49 ± 0.82, p=0.114) and between normal individuals and thalassemia minor as regard to HbF (0 ± 0 versus 0.54 ± 0.71, p=0.841).

Regarding to PT, high significant differences existed between normal individuals and both thalassemia major (12.80 ± 0.87 versus 12.37 ± 0.68, $p<0.01$) and thalassemia intermedia (12.80 ± 0.87 versus 12.26 ± 0.65, p=0.01). Comparison between the study groups as regard to aPTT showed highly significant difference between normal individuals and both thalassemia minor (31.44 ± 5.8 versus 37.42 ± 5.5, $p<0.01$) and thalassemia major (31.44 ± 5.8 versus 37.54 ± 4.4) and between thalassemia intermedia and thalassemia major (33.74 ± 4.6 versus 37.54 ± 4.4, $p<0.01$). Interestingly, all results of PT and aPTT were within normal range. No history of thrombosis or bleeding was recorded during history taking.

Erythrocyte microparticles (CD235a labeled) were significantly higher in thalassemic individuals than normal controls ($p<0.01$) even in thalassemia minor. It was significantly higher in thalassemia major than in thalassemia intermedia and thalassemia minor. The range of percentage of microparticles were 0-2.3 % in normal individuals, 1.2-5.3 % in individuals with thalassemia minor, 2.3-10.1 % in patients with thalassemia intermedia and 3-25.9 % in patients with thalassemia major. Between patients with thalassemia, it was observed that mean levels of MPs was higher in adult than in children (9.11 ± 5.86 versus 5.20 ± 2.48, p=0.05).

Levels of CD235a labeled MPs had significant negative correlation with the levels of hemoglobin and HbA (r=-0.418 and -0.467 respectively) while showing significant positive correlation with the levels of HbF. No other significant correlations could be elicited between the levels of CD235a labeled MPs and other parameters.

Table (1): Demographic and laboratory data of all studied groups.

Groups / Parameters	Controls (n=50)	Thalassemia			p-value
		Thalassemia minor (n=10)	Thalassemia intermedia (n=11)	Thalassemia major (n=29)	
Age (years)	25.2 ± 9.5	26.6 ± 8.2			<0.01
Sex (M/F)	23/27	20/30			<0.01
Hb (g/dl)	13.78 ± 1.39	9.84 ± 0.80	8.30 ± 0.69	6.3 ± 1.06	<0.01
MCV (fl)	83.86 ± 3.27	71.89 ± 5.47	62.73 ± 6.97	60.0 ± 5.93	<0.01
MCH (pg)	28.11 ± 0.72	22.57 ± 2.01	19.74 ± 2.93	16.9 ± 2.08	<0.01
MCHC (g/dl)	32.31 ± 0.78	33.40 ± 1.93	31.06 ± 1.06	32.3 ± 1.59	<0.01
TLC (thousand/µL)	7.90 ± 2.82	7.95 ± 2.70	13.59 ± 6.75	16.5 ± 9.21	<0.01
Platelets (thousand/µL)	310 ± 106.65	357 ± 169.78	381 ± 160.94	509.7 ± 230.1	<0.01
Reticulocytes (%)	1.19 ± 0.53	3.37 ± 1.05	6.25 ± 2.83	9.13 ± 4.94	<0.01
Ferritin (ng/mL)	109.1 ± 45.8	158.6 ± 61.6	976.7 ± 441.8	1897.5 ± 947.6	<0.01
HbA (%)	97.57 ± 0.27	94.41 ± 0.92	75.22 ± 6.61	56.48 ± 13.8	<0.01
HbA2 (%)	2.43 ± 0.27	5.04 ± 0.61	4.49 ± 0.82	3.58 ± 1.31	<0.01
Hb F (%)	0 ± 0	0.54 ± 0.71	20.26 ± 6.77	40.28 ± 13.76	<0.01
PT (sec)	12.80 ± 0.87	12.39 ± 0.73	12.26 ± 0.65	12.37 ± 0.68	<0.01
aPTT (sec)	31.44 ± 5.8	37.42 ± 5.5	33.74 ± 4.6	37.54 ± 4.4	<0.01
CD235a labeled MPs (%)	0.62 ± 0.49	2.96 ± 1.38	6.47 ± 2.5	9.27 ± 5.54	<0.01

Table (2): Comparison between levels of microparticles in children and adult in thalassemic patients.

MPs / Parameters	**Number of patients**	**MPs Mean ± SD**	**p-value**
Adult (age ≥ 18 years)	28	9.11 ± 5.86	0.05
Children (age < 18 years)	22	5.20 ± 2.48	

Table (3): Correlation between CD235a labeled MPs and other studied parameters in thalassemic group.

Parameters / CD235a	**Hb**	**Reticulocytes**	**PT**	**aPTT**	**Hb A**	**Hb A2**	**HbF**	**Ferritin**
r-value	-.418	.105	.009	-.106	-.467	.012	.452	.084
p-value	0.003	0.468	0.948	0.466	0.001	0.932	0.001	0.561

DISCUSSION

Thromboembolic complications of beta thalassemia are well identified (Eldor et al., 1999), and occur in about 1.1 % of patients (Borgna-Pignatti et al., 2004). Many factors play intractable role in the hypercoagulable state observed in patients with thalassemia. These factors include endothelial dysfunction with expression of adhesion molecules and MPs release, vasoconstriction due to depletion of NO, enhanced platelet activity with thrombocytosis, red cell defects with exposure of negatively charged PL and enhanced aggregability and splenectomy which further aggravates the condition (Cappellini et al., 2012).

In our study, we evaluated the erythrocyte microparticles in patients with thalassemia using flow cytometry.

Comparing 50 thalassemic patients with age and sex matched, 50 normal individuals as regard to CD235a labeled microparticles, we found that the levels of erythrocyte microparticles were significantly higher in thalassemic group, and being higher in adult patients than in children. There was also a significant difference between each group of thalassemic patients, where the highest percentage was found in patients with thalassemia major. Our study agreed with all studies that showed elevation in erythrocyte microparticles in patients with thalassemia intermedia and thalassemia major (Pattanapanyasat et al., 2004; Habib et al., 2008; Westerman et al., 2008; Tantawy et al, 2013, and Agouti et al., 2015).

This finding could be explained by the fact that the release of RBCs microparticles occurred in many different conditions that were found collectively in thalassemia. These included, increased cytoplasmic calcium concentration, reduced ATP content, and disruption to the membrane lipid-protein organization (Wagner et al., 1986).

Disruption of phospholipid membrane asymmetry in RBCs of β-thalassemia patients, allowing exposure of PS on the surface of subpopulations of thalassemic red cells and release of MPs expressing PS. Both could provide a surface on which to assemble various complexes to activate clotting factors, and play a role in the hypercoagulable state (Kuypers et al., 1998, and Ataga and Key, 2007).

None of our patients experienced venous or arterial TEE by history. Habib et al. (2008) and Tantawy et al. (2013) reported thrombosis in patients with thalassemia major and thalassemia intermedia with incidence of 15 % and 8.3 % respectively. However, the number of patients in both studies was higher and the mean age of patients was also high.

While all our results of PT and aPTT were in the normal reference value, there was a significant difference between groups.

Regarding to RBCs microparticles, the only relationships found were between CD 235a and Hb level (negative correlation), CD 235a and Hb A level (negative correlation) and between CD235a and HB F (positive correlation).

In agreement with our results, Pattanapanyasat et al. (2004) and Tantawy et al. (2013) also observed inverse relationship between the levels of RBC microparticles and the Hb concentration.

In addition, the positive correlation with Hb F level was also reported.

On the other hand, Agouti et al. (2015) found no significant correlation between total erythrocyte MPs and hemolysis criteria including Hb value and reticulocytic count. Also, no correlation with Hb F was observed. Furthermore, that study was done on 37 TM patients who are treated with regular blood transfusions since infancy with mean hemoglobin level of 9.1 g/dl and mean level of Hb F was 6.4 % (far less than our's).

Our study results agreed with Agouti et al. (2015) who fail to find any correlation between levels of MPs and serum ferritin concentration.

Contrary, Tantawy et al. (2013) found that MPs levels were significantly increased in patients with TM with serum ferritin >2500 ng/mL, suggesting a relationship between MPs and iron overload, a relation that we failed to prove. They chose this cutoff because it is best for prediction of iron overload related complications in thalassemia (Vermylen, 2008).

On the other hand, an Italian study elicited this correlation only in splenectomized patients which is possibly caused by the iron overload of non-splenic macrophages (Ferru et al., 2014).

CONCLUSION

Erythrocytic microparticles increased in the patients with beta thalassemia, and this increase was well correlated with the degree of thalassemic erythropoiesis (Hb F levels). It is recommended to evaluate the levels of microparticles in patients with high risk of thrombosis in order to find a way to prevent its share in thromboembolic events.

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تقييم الجسيمات الدقيقة لخلايا الدم الحمراء في أنيميا البحر المتوسط

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قسمي الباثولوجيا الأكلينيكية وطب الأطفال – كلية طب الأزهر

خلفية البحث: أنيميا البحر المتوسط هي مجموعة من الأمراض مختلفة الشدة تنتج عن اعتلال في بناء سلسلة بروتين الجلوبين بسبب خلل وراثي في الجينات الخاصة به. فرط تخثر الدم هو من العواقب المعروفة والذي يؤثر على 1,1 % من المرضى وله أسباب متعددة ويظهر بشدة مختلفة و بصور إكلينيكية مختلفة. الحويصلات المنبثقة من الخلايا هي حويصلات متناهية الصغر أقل من 1 ميكرومتر وهي تنبثق من الخلايا تحت ظروف مختلفة وتؤدي وظائف مختلفة و تلعب هذه الحويصلات دورا هاما في حمل الاشارات بين الخلايا و التصاق الخلايا وتجلط الدم.

الهدف من البحث: تقييم الجسيمات الدقيقة الخاصة بخلايا الدم الحمراء في مرضى أنيميا البحر المتوسط.

المرضى وطرق البحث: تم أخذ عينات الدم من خمسين مريض بأنيميا البحر المتوسط وخمسين شخص طبيعيا.وتم فصل البلازما الخالية من الصفائح وإستخدامها لتحليل الجسيمات الدقيقة بواسطة التدفق الخلوي بإستخدام .CD235a

النتائج: لوحظ وجود إختلاف ذو قيمة إحصائية بين المجموعات فيما يخص فحوصات الدم الأساسية. وقد كانت الحويصلات الدقيقة أعلى في مرضى أنيميا البحر المتوسط وقد كانت أعلى في البالغين عن الأطفال، وهذا الإرتفاع يرتبط بشكل طردي مع نسبة هيموجلوبين الجنين وبشكل عكسي مع النسبة الكلية للهيموجلوبين ونسبة هيموجلوبين البالغين.

الاستنتاج: مستوى الحويصلات الدقيقة الخاصة بخلايا الدم الحمراء كان أعلى في مرضى انيميا البحر المتوسط والذي قد يساهم في حالة زيادة تخثر الدم المصاحبة للمرض.