Mansoura Journal of Biology Vol. 38 (2) December, 2013.

ASSESSMENT OF ERYTHROCYTE PHOSPHATIDYLSERINE EXPOSURE IN B-THALASSEMIA

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Received: (9/ 2/ 2013)

ABSTRACT

`Phospholipid asymmetry is well maintained in erythrocyte (RBC) membranes with phosphatidylserine (PS) exclusively present in the inner leaflet. The appearance of PS on the surface of the cell can have major physiologic consequences, including increased cell-cell interactions. Eryptosis, the suicidal death of erythrocytes, is characterized by cell shrinkage, membrane blebbing and cell membrane phospholipids scrambling with PS exposure at the cell surface.

Erythrocytes exposing PS are recognized, bound, engulfed, and degraded by macrophages. Eryptosis thus fosters clearance of affected erythrocytes from circulating blood which may aggravate anemia in pathological conditions. Thalassemia patients are more sensitive to the eryptotic depletion and osmotic shock which may affect RBC membrane phospholipid asymmetry. We aimed in this work to determine the erythrocyte PS exposure in splenectomized and nonsplenectomized β-thalassemia (β-TM) patients and correlate it with the clinical presentation and laboratory data. Fresh whole blood was simultaneously stained for annexin V (AV) to detect phosphatidylserine (PS) exposure in 46 patients with β-TM (27 splenectomized and 19 nonsplenectomized) in addition to 17 healthy subjects as a control group. We reported significant increase in erythrocyte PS exposure in β-TM patients compared to control group. Erythrocyte PS exposure was significantly higher in splenectomized β-TM patients as compared with nonsplenectomized β-TM patients. No correlation was found between erythrocyte PS exposure and clinical or hematological data of β-TM patients but there was positive correlation between erythrocyte PS

exposure and ferritin level in β-TM patients. These findings suggest that β-TM patients have higher level of erythrocyte PS exposure and splenectomy was shown to aggravate erythrocyte PS exposure without aggravation of anemia.

INTRODUCTION

The thalassemias are a group of inherited hematologic disorders caused by defects in the synthesis of one or more of the hemoglobin chains. Alpha thalassemia is caused by reduced or absent synthesis of alpha globin chain, and beta thalassemia is caused by reduced or absent synthesis of beta globin chain. Imbalances of globin chains cause hemolysis and impaired erythropoiesis **(Muncie & Campbell, 2009).**

Historically, β-thalassaemia has been divided into three clinical syndromes; β-thalassaemia minor (heterozygous), a mild microcytic, hypochromic hemolytic anemia; β-thalassemia major (homozygous), a severe transfusion-dependent anemia; and β-thalassaemia intermedia, with symptoms of severity between the other two types. A fourth syndrome is now recognized, which has been designated as silent carrier status, for patients with genetic changes in one of the two β genes that results in no hematologic abnormalities **(Thein, 2004)**.

Individuals suffering from thalassemia major usually present at less than one year of age with servere anaemia. These are at risks of complications such as hepatosplenomegaly, bone deformities and growth delay **(Leung, et al., 2005)**. Current management of thalassaemia consists of blood transfusion, iron chelation, splenectomy and bone marrow transplantation **(Lukens, 1999)**. Splenectomy is performed if patients develop hypersplenism or an increased requirement for blood transfusion.

Thalassemia is characterized by morphological and functional RBC anomalies that lead to shortening of the RBC life span. Most patients suffer from chronic hemolytic anemia because of untimely RBC destruction in the bone marrow and spleen. Several studies have proposed mechanisms that lead to the premature removal of abnormal RBCs. These include extraordinary ineffective erythropoiesis in the marrow **(Finch, et al., 1970)** and altered deformability due to the rigidity of the RBC membrane, which impair passage of RBCs through sinusoidal walls of

reticuloendothelial organs, and which finally triggers the removal of these cells from circulation **(Schrier, et al., 1989; Shinar & Rachmilewitz, 1993; Schrier, 1994 and Schrier, 2002).**

The RBC membrane undergoes vesiculation under a variety of conditions, including increased cytoplasmic calcium concentration, reduced ATP content and disruption to the membrane lipid-protein organization **(Wagner, et al., 1986)**; all of which are features characteristic of thalassemic RBCs. Severly affected thalassemic RBCs are known to correlate with increased accumulation of unmatched globin chains in the cytoskeleton; skeleton associated globin results in altered membrane function by producing oxidative damage to adjacent cytoskeletal proteins **(Shinar & Rachmilewitz, 1993 and Schrier, 2002)**, which eventually induce loss of RBC membrane in the form of vesicles with phosphatidylserine (PS) exposure. It has been shown that vesicles and membrane-derived microparticles (MPs) from RBC strongly bind annexin V(AV), a protein known for its interaction with negatively charged phospholipids such as phosphatidylserine (PS) **(Shinar, et al., 1987; Borenstain-Ben Yashar, et al., 1993; Zwaal & Schroit, 1997; Kuypers, et al., 1998)**. Phosphatidylserine (PS) is one of the key membrane phospholipids that is normally located along the inner side of the membrane bilayer.

The aim of our research was to determine the erythrocyte PS exposure in splenectomized and nonsplenectomized β-thalassemia (β-TM) patients and their relationship to the degree of severity in β-TM patients.

SUBJECTS AND METHODS

Blood samples were collected from 46 β-TM patients (23 males and 23 females) with age ranged from (2 to 19 years) and 17 healthy volunteers with matched age (3.6-19 years) and sex (9 males and 8 females). The patients group included 27 splenectomized β-TM patients and 19 nonsplenectomized β-TM patients. They were selected in the period from January 2011 to December 2011 from Hematology Clinics of Children Hospital, Mansoura University. All patients were selected with no evidence of concurrent infection, and non had been hospitalized or

received blood transfusion for at least one month prior to the start of the study or during the sampling period.

Five ml of venous blood were obtained after attainment of informed consents. Three ml were preserved in K_2EDTA for both flow cytometric analyses of erythrocyte PS exposure using the EPICS XL flow cytometer (Coulter Electronic, Fl, USA) and hematologic parameters including complete blood count (CBC) using Cell- Dyn 3500 (Abott, USA), reticulocyte count and fetal hemoglobin percentage (HbF %) done by hemoglobin electrophoresis using Hydrasys (Sebia, France).

Serum was obtained after centrifugation of the remaining blood sample and used for the determination of serum ferritin level using Elisa reader (Adaltis, Italy).

RBCs binding assay:

A total of 200 µL of each whole blood sample was fixed in 200 µL of 1% paraformaldehyde in phosphate buffer saline (PBS) for at least 1h or, if necessary, stored in fixative at 4 ºC until staining and flow cytometric analysis was performed. A total of 5µL of fixed blood sample was then incubated with 10µL of phycoerythrin (PE) conjugated glycophorin A (GA), and 1µL of fluorescein isothiocyanate (FITC) conjugated annexin V (AV) for 30 min at room temperature in dark**.** All blood samples were collected at room temperature (23-25ºC) and processed within 2 h **(Pattanapanyasat, et al., 2004)**.

Flow Cytometric Analysis:

The Beckman coulter XL flow cytometry was used for analysis and data was collected in the list mode. The instrument was calibrated according to standard protocols to achieve day-to-day reproducibility. The red cell population was defined by PE-glycophorin-A positivity.

Fluorescence intensities were expressed in logarithmic mode. The control sample incubated without PE-glycophorin A and FITC-AV was used to set the region for positive fluorescence such that the fraction of cells with positive (auto-) fluorescence was lower than 0.2% of total. The population of cells labeled with FITC-AV and PE-glycophorin-A above background was determined from the fraction of cells in this region in excess of that obtained with the (unlabeled) control. This approach was of

particular importance because a number of thalassemic RBCs exhibited an increased autofluorescence as compared with normal control cells **(Kuypers, et al., 1998).** RBC PS exposure were distinguished by double positivity for both FITC-AV and PE-glycophorin-A which presented in the upper right quadrant, whereas intact RBCS were presented in the upper left quadrant of the biparametric histogram.

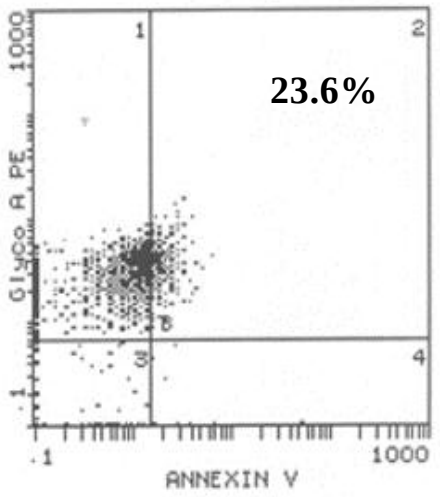

Fig 1. Representative flow cytometric dot plot of RBC phosphatidylserine (PS) exposure in a β-TM patient stained with FITC-conjugated annexin V(AV) and PE- conjugated anti-glycophorin A. percentages of positive events are indicated in the upper right quadrant.

Statistical Analysis:

Data analyses were performed using SPSS statistical package version 10. Quantitative data were presented as mean, standard deviation and range. Student t-test used to compare means of two groups. The one-way ANOVA procedure produces a one-way analysis of variance for a quantitative dependent variable by a single factor (independent) variable.

Mann Whitney-U test and Kruskal–Wallis H are non parametric tests.

Correlation between variables was done using Pearson correlation for parametric data and spearmen rank correlation for non parametric. For all above mentioned statistical tests, done, the threshold of significance was $P < 0.05$.

RESULTS

Hematologic data between β-TM patients and healthy control group

There were significant increase in RBCs count, Hb level, Hct %, MCV, MCH, Red blood cell distribution width (RDW) %, WBCs and platelets in β-TM patients compared to control group ($p< 0.05$). There was no significant difference in MCHC in β-TM patients compared to control group (table 1).

Significant decrease was found in RBCs count, Hb level, Hct %, MCV and MCH of splenectomized β-TM patients as compared to control group ($p<0.05$). Regarding RDW %, WBCs count and platelets, there was a significant increase in splenectomized β-TM patients group compared to control group. There was no significant difference in MCHC of splenectomized β-TM patients as compared to control group (table 1).

Significant decrease was found in RBCs count, Hb level, Hc t%, MCV and MCH in nonsplenectomized β-TM patients compared to control group ($p<0.05$). RDW % showed a significant increase ($p<0.05$) in nonsplenectomized β-TM patients group as compared to control group.

There was no significant difference in MCHC in nonsplenectomized β-TM patients compared to control group.

On the other hand, there was no significant difference in HbF % (P=0.094) between splenectomized β-TM patients group (median-27.4) %, range (12.3-91.9) % and nonsplenectomized β-TM patients group (median-54.6) %, range (10.7-96.2) % (table 2).

Table (2): Comparison between non-splenectomized and splenectomzed β-TM patients group regarding HbF%.

HbF%	**β-TM/S N=27**	**β -TM/NS N=19**	**P**
Median	27.4	54.6	0.094
Range	12.3-91.9	10.7-96.2	

Significant elevation was shown concerning ferritin level and RBC PS exposure % in β-TM compared to control group (table 3). On the other hand, there was no significant difference in ferritin level in splenectomized β-TM patients compared to nonsplenectomized β-TM patients but there was a significant elevation in RBC phosphatidylserine (PS) exposure in splenectomized β-TM patients compared to nonsplenectomized β-TM patients.

There was no significant correlation between RBC phosphatidylserine (PS) exposure % and hematological data in β-TM patients group (P>0.05) (table 4).

On the other hand, there was no significant correlation between RBC phosphatidylserine (PS) exposure % and HbF % in β-TM patients group but there is a positive correlation between RBC phosphatidylserine (PS) exposure and serum ferritin level (ng/ml) in β-TM patients group (table 5)

Table (5): Correlation between RBC phosphatidylserine (PS) exposure percentage, HbF %, and ferritin level in β-TM patients group.

		HbF %	**Ferritin (ng/ml)**
RBC phosphatidylserine (PS) exposure **%**	**r**	- 0.194	0.332
	p	0.197	0.024

r: Correlation between RBC PS exposure percentage, HbF %and ferritin level in β-TM patients group
p., significance difference between RBC PS exposure percentage, HbF %, and ferritin level in β-TM patients group.

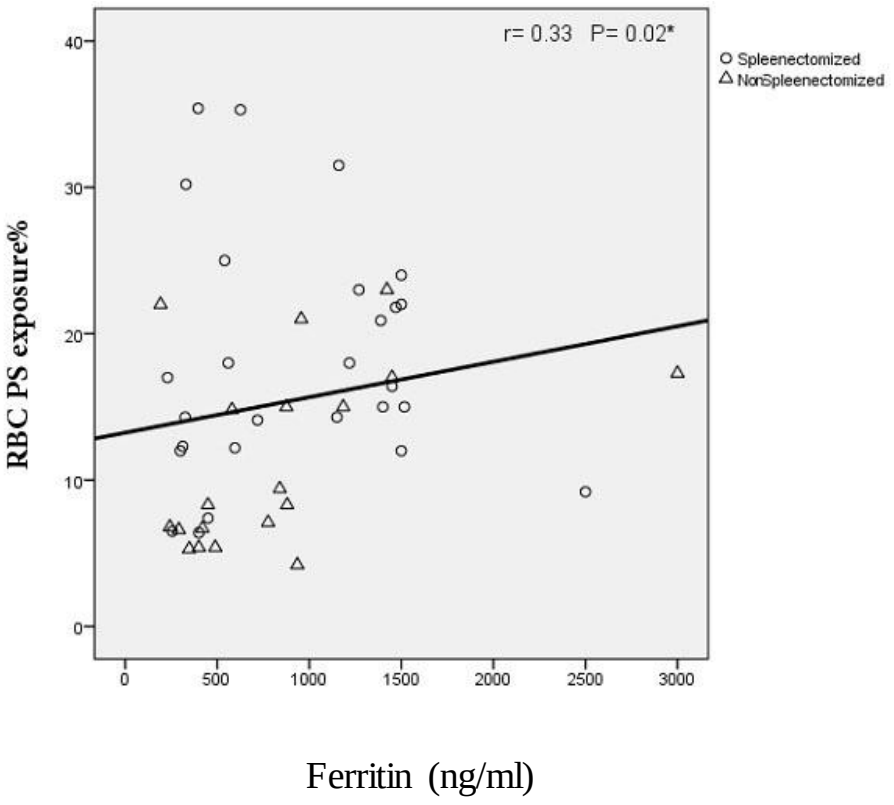

Fig.(2) Correlation between RBC phosphatidylserine (PS) exposure and Ferritin (ng/ml) in β-TM patients group.

DISCUSSION

Accumulation of unmatched globin chains at the cytoplasmic surface of the RBC membrane is an important feature of the pathophysiology of thalassemia. These extra globin chains, and the associated iron, heme, or hemochromes, induce oxidative damage to the membrane and, ultimately, contribute to premature cell destruction **(Schrier, 2002 and Shinar, et al., 1989).**

Normally, the erythrocyte membrane is asymmetric with respect to phosphatidylserine (PS). In β-thalassemia patients, a subpopulation of

erythrocytes were found in peripheral blood with higher level of PS exposed on the surface and is removed rapidly from the circulation **(Kuypers, et al., 1998 and Kuypers & de Jong, 2004)**. Changes in this asymmetry are one of the hallmarks of eryptosis **(Föller, et al., 2008).**

During in vivo ageing, the PS externalization triggers an "eat me" signal to the phagocytes **(Connor, et al., 1994 and Schlegal & Williamson, 2001).**

Annexins are a family of proteins that bind to acidic phospholipids, particularly phosphatidylserine (PS) **(Ernst, 1993 and Raynal & Pollard, 1994)**. Annexin V was used as a marker for PS positivity, while antiglycophorin – A (CD235) is a sialoglycoprotein expressed on the surface of erythrocytes **(Chasis & Mohandas, 1992 and Catimel, et al., 1993)** and was employed as marker for intact red cells and red cell derived microvesicles **(Setty, et al., 2000).**

The vesiculation process is probably more facilitated in β-thalassemic erythrocyte membrances, leading to faster shedding of glycophorin–containing microvesicles, leaving the highly PS-exposed erythrocytes accessible to the phagocytes or reticuloendothelial cells. This also explains the cause of survival of younger erythrocytes in normal individuals, where the cell surface glycophorins mask the exposed PS causing hindrance to phagocytic recognition **(Willekens, et al., 2008).**

In this study, we aimed to evaluate the PS externalization in 46 β-TM patients (27 splenectomized and 19 nonsplenectomized) and correlate it with the clinical presentation and laboratory data.

In the present study, there is a statistically increase in RDW %, WBC count and platelet count in splenectomized thalassemia cases as compared to control. Also, there is a statistically significant decrease in RBC count, Hb level, Hct %, MCV and MCH in splenectomized group compared to control group. Regarding MCHC, there is no statistically significant difference between splenectomized group and control group.

Also, there is statistically significant increase in RDW % in nonsplenectomized as compared to the controls. There is significant

decrease in RBC count, Hb level, Hct %, MCV and MCH in nonsplenectomized group as compared to the control group. Regarding MCHC, WBC count and platelet count, there is no statistically significant difference between nonsplenectomized group and control group. These results coincide with **(Hegazy, et al., 2008)** who found a statistically significant increase in WBCs count, RDW % and platelet count in splenectomized thalassemia cases as compared to controls. There was a statistically significant decrease in RBCs count and Hb level in splenectomized thalassemia group as compared to control group.

Regarding MCV, MCH and MCHC, there was no statistically significant difference between the 2 groups. Meanwhile, there was statistically significant increase in RDW % and platelet count in nonsplenectomized thalassemia cases as compared to controls. Also, there was significant decrease in RBCs count, Hb level, MCV and MCH in nonsplenectomized group as compared to the control group. Regarding WBCs count and MCHC, there was no statistically significant difference between splenectomized and nonsplenectomized thalassemia groups.

The statistical analysis of our results showed a highly significant elevation of serum ferritin levels in β-TM patients compared to healthy control (P= 0.000). This coincides with the finding of **(Ikram, et al., 2004)** who mentioned that the patients of β-TM have an increase in their serum ferritin levels. In β-TM repeated blood transfusion, ineffective erythropoiesis and increased gastrointestinal iron absorption lead to iron overload in the body. The management of the iron overload in these patients requires the administration of iron chelators continuously and evaluation of serum ferritin levels at regular intervals **(Giardina & Grady, 2001).**

In this study we found that percentage of erythrocyte PS exposure percentage were significantly elevated in β-TM patients group compared to control group. These results coincide with **(Willekens, et al., 2008)** who proved that 55 % of RBCs vesicles identified by glycophorin A were positive for annexin V. **(Lamchiagdhase, et al., 2004)** stated that vesicles are part of the red blood cells membrane which can be found in a small number in normal apoptotic process and increased in β-thalassemia.

In the present study there is elevation of erythrocyte PS exposure percentage in patients with β-TM/S as compared to β-TM/NS. This is in agreement with the finding of **(Pattanapanyasat, et al., 2004)**, who found higher percentage of RBCs vesicles in splenectomized β-thalassemia patients than nonsplenectomized β-thalassemia patients in which RBC vesicles identified by expression of both glycophorin A and annexin V.

These findings suggested that, although splenectomy improves Hb concentration and reduce the transfusion needs and total blood volume **(Aessppos, et al., 2005),** it also leads to increase in percentage of circulating RBCs vesicles and erythrocyte PS exposure.

In the present study there is no significant correlation between erythrocyte PS exposure percentage and hematological data in β-TM patients. These data are consistent with that of **(Hegazy, et al., 2008)** who didn't find any correlation between RBC vesicles percentage and hematological data in β-thalassemia patients.

In the present study we found no significant correlation between erythrocyte PS exposure percentage and HbF percentage in patients with β-TM which seems to contradict with the results obtained by **(Setty, et al., 2000)** who found an inverse relationship between the levels of both RBCs microvesicles and PS positive cells with HbF. This contradictory may be related to the difference in methodology used for evaluation of Hb F percentage.

On the other hand, we found a positive correlation between erythrocyte PS exposure percentage and ferritin level. This is in agreement with the finding of **(Lang, 2002)** who found that the accumulation of toxic quantities of iron cause tissue damage which induces oxidative stress in thalassemia major patients. This oxidative stress may contribute to shortened erythrocyte life span through accelerated eryptosis.

In conclusion, this study presented data that may be of important value to protect thalassemic patients from pathological complications by regular monitoring of erythrocyte PS exposure percentage and estimation of proper serum ferritin level.

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قياس تعرض الفسفاتيديل سيرين بكرات الدم الحمراء فى مرضى أنيميا البحر المتوسط من النوع بيتا

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يتم الاحتفاظ بشكل جيد بالفسفوليبيد فى أغشيه كرات الدم الحمراء مع وجود الفسفاتيديل سيرين حصرا فى داخل الخليه. يمكن ظهور الفسفاتيديل سيرين على سطح الخليه عواقب فسيولوجيه، تتضمن تفاعلات الخليه المتزايده. يتميز الموت المبرمج لكريات الدم الحمراء، من خلال انكماش الخليه وتعرج الخليه وبهرول غشاء خليه الفسفوليبيد مع تعرض الفسفاتيديل سيرين على سطح الخليه، يتم التعرض على الكريات الحمراء بتعريض الفسفاتيديل سيرين ، ملزمه، اجتاحت، تدهورت بسبب الميكروفاج، يعزز الموت المبرمج لكرات الدم الحمراء ازالة الكريات الحمراء المتضرره من الدم التى قد تؤدى الى تفاقم فقر الدم فى الحالات المرضيه. يعتبر أنيميا البحر المتوسط أكثر حساسيه لاستنزاف الموت المبرمج لكرات الدم الحمراء، و الصدمه الاسموزيه التى قد تؤثر على عدم تماثل الفسفوليبيد على غشاء كرات الدم الحمراء.

وهدف هذه الدراسه هو تعيين تعرض الفسفاتيديل سيرين بكرات الدم الحمراء فى مرضى أنيميا البحر المتوسط من النوع بيتا الذين أجروا عمليه استئصال الطحال والذين لم يجروا هذه العمليه وربط ذلك مع عرض البيانات السريريه والمعمليه.