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# Flow Cytometric Determination of Percentage of Red Blood Vesicles in β-Thalassemia

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The aim of this study was to determine the percentage of RBCs vesicles in the peripheral blood of β-thalassemic patients, using flow cytometry and study its relationship to the degree of severity of anemia. Thirty one β-thalassemic patients and 23, age and sex matched healthy controls were included in this study. All patients and controls were subjected to full history taking, complete clinical examination, complete blood picture, hemoglobin electrophoresis and estimation of RBCs vesicles percentage, by flow cytometry technique. We reported that RBCs vesicles were present in both normal and thalassemic blood samples, but their percentage was significantly higher in the β-thalassemia group as compared with the control group ($p = 0.002$). Also, the percentage of circulating RBCs vesicles was significantly higher in splenectomized thalassemic patients as compared with non-splenectomized cases ($p = 0.041$) and with controls ($p = 0.001$). No correlation was found between RBCs vesicles percentage and clinical or haematological data of β-thalassemia. From that study we concluded that flow cytometric determination of RBCs vesicles percentage is simple, reliable and may offer new insights in the assessment of possible thrombotic complications in patients with thalassemia.

**Key words:** Red cell vesicles, flow cytometric determination, β-thalassemia

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## INTRODUCTION

The thalassemias are one of the most common single gene disorders and form a heterogeneous group of inherited disorders of hemoglobin synthesis (Butwick *et al*., 2005). They are all caused by mutations in the globin gene cluster (Schrier, 2002).

The β-thalassemias are distributed widely across the Mediterranean region, the Middle East, the Indian subcontinent and throughout Southeast Asia and can also occur sporadically in any racial group (Butwick *et al*., 2005).

The clinical phenotypes of β-thalassemia include thalassemia minor, thalassemia intermedia and thalassemia major. Thalassemia major most often results from homozygosity or compound hetrozygosity for a mutant β globin allele (Chern *et al*., 2006).

Individuals suffering from thalassemia major usually present at less than one year of age with severe anaemia. The are at risks of complications such as hepatosplenomegaly, bone deformities and growth delay (Leung *et al*., 2005).

Increased frequency of thrombo-embolic events has been recently observed in patients with thalassemia major; causing hypoxemia and cor pulmonale. Studies to determine hypercoagulability showed impaired platelet aggregation, increased circulating platelet aggregates, shortened platelet survival (Amer and Fibach, 2004). Decreased plasma levels of protein C, protein S or antithrombin III. Erythrocytes from thalassemia major patients enhance thrombin formation in a prothrombinase assay (Eldor *et al*., 1993). The extent to which imbalance between coagulation inhibitors and clotting factors contributes to hypercoagulable states in thalassemia remains to be determined (Eldor and Rachmilewitz, 2002). Vascular endothelial cell injury is a possible pathogenic mechanism (Pignatti and Galanello, 2004).

In the β-thalassemia, there is an imbalance of α and β-globin chains with excess of α-globin. The α-globin tetramers are relatively unstable (Han *et al*., 2005), their accumulation at the cytoplasmic surface of RBCs membrane is an important feature of the pathophysiology of β-thalassemia (Pattanapanyasat *et al*., 2004). Oxidation of these α hemoglobin subunits leads to formation of hemichromes which bind to or modify various components of red cell membranes. After precipitation of hemichromes, heme disintegrates and toxic non-transferrin bound iron species are released. The resulting free iron catalyzes the formation of reactive oxygen species. Iron-dependent oxidation of membrane proteins cause thalassemia red cells to be rigid and deformed and to aggregate, resulting in premature cell removal (Rund and Rachmilewitz, 2005).

β-thalassemia is associated with RBCs anomalies, leading to impairment of their flow affecting properties, mainly red cells deformability, cells aggregability and adherence to endothelial cell (Ramot *et al*., 2008).

In the circulation, the presence of high levels of membrane-derived microparticles, stemming from RBCs, apoptotic cells, or activated platelets, have been shown to enhance procoagulant activity (Pattanapanyasat *et al*., 2004), they provide the catalytic surface necessary for the assembly of the procoagulant enzyme complexes, prothrombinase and tenase (Hugel *et al*., 1999).

Portal vein thrombosis is a recognized complication after splenectomy in β-thalassemia major due to chronic hypercoagulable state which has been recognized to exist in childhood thalassemia and contribute to thromboembolic events (Al-Hawsawi *et al*., 2004). So, splenectomized thalassemic patients should be considered at risk of thrombosis and should have prophylaxis when they are exposed to surgery, prolonged immobilization, pregnancy and oral contraceptive intake should be avoided (Cappellini *et al*., 2000).

The aim of research was to determine the percentage of RBCs vesicles in peripheral blood of β-thalassemia patients and their relationship to the degree of severity of anaemia, compared to normal blood samples.

## MATERIALS AND METHODS

The present study was conducted on 31 cases with β-thalassemia who were selected from pediatric haematology clinics of El Kasr el Aini Hospital, Cairo University for aperiod of one year starting at May 2006.

The group of patients included 14 splenectomized and 17 non-splenectomized β-thalassemia cases, their ages ranged from 5 to 20 years (mean +SD of 10.3+4.5 year). All cases were transfusion dependent β-thalassemia major, the blood samples were collected immediately prior to blood transfusion.

The diagnosis of the thalassemia for all subjects were made by standard haematological technique and hemoglobin electrophoresis.

All subjects had no history of any vaso-occlusive episodes prior to the start of the study or during the sampling period.

Twenty-three healthy volunteers, age and sex matched served as a control group.

All patients and controls were subjected to the following:

- Frequency of blood transfusion
- History of any vaso-occlusive episodes
- Date of last blood transfusion
- History of splenectomy

- Complete blood picture using cell Dyne 1600 automated counter (Abott Laboratories, Abott Park, Illinois 60064-USA
- Hemoglobin electrophoresis: was carried out on cellulose acetate membrane, in tris-EDTA borate buffer pH 8.4
- Estimation of RBCs vesicles percentage by flow cytometry technique (11), samples were processed within 2 h

This technique allows rapid measurement of particles or cells as they flow in a fluid stream one by one through a sensing point, measurements are made separately on each particle within the suspension in turn and not just as average values for whole population. The flow cytometer basically consists of one or more lasers for supplying excitation energy and a series of filters and detectors for measuring the resultant fluorescent emissions.

There are 2 reagents are used:

- Mouse antihuman CD41a FITC monoclonal antibody: Serotec (UK). This monoclonal antibody reacts with glycophorin A on the surface of red blood cells.
- Mouse antihuman glycophorin A PE monoclonal antibody. R and D systems (USA). This monoclonal antibody reacts with glycophorin A on the surface of red blood cells.

RBCs vesicles are smaller than intact RBCs population and overlapped in size with the platelet population (based on forward and side-light scattering measurements).

A flow cytometric gate was set for acquiring cell events in the platelet size range that would include both platelets and RBCs vesicles. Whole blood was doubly stained for RBCs and platelet markers:monoclonal antibodies to glycophorin A (PE) and CD41a (FITC) were used to discriminate RBCs vesicles from platelets and platelet microparticles.

**Statistical methods:** Computer software package SPSS13 was used in the analysis for quantitative variables, mean (as a measure of central tendency), standard deviation and range (as measures of variability) were presented.

To compare quantitative variables between two groups, student's t-test was used in the analysis.

To estimate association between RBCs vesicles and other variables, correlation (Pearson) was done, correlation coefficient and p-value were presented.

For RBCs vesicles percentages and all other values among the groups of subjects, the threshold for statistical significance for all comparisons was chosen as $p = 0.05$.

## RESULTS

Statistical comparison of haematological data between splenectomized β-thalassemia patients and healthy controls as shown in Table 1 showed significant increase in WBCs count, RDW and platelet count in splenectomized thalassemia cases as compared to controls ($p<0.05$). Also, there was a statistically significant decrease in RBCs count, HB level and packed cell volume in splenectomized group as compared to control group. Regarding MCV, MCH and MCHC, there was no statistically significant difference between the 2 groups.

Also, in Table 1 there was statistically significant increase in RDW and platelet count in non-splenectomized thalassemia cases as compared to controls ($p<0.05$). Also, there was significant decrease in RBCs count, HB level, packed cell volume, MCV, MCH in non-splenectomized group as compared to the control group. Regarding WBCs count and MCHC, there was no statistically significant difference between the 2 groups.

Also, there was a statistically significant increase in percentage in the percentage of circulating RBCs vesicles in splenectomized β-thalassemia patients as compared to the healthy controls p-value = 0.001 as shown in Table 2. There was no statistically significant difference in

Table 1: Comparison of haematological data between splenectomized, non splenectomized β-thalassemia cases VS healthy controls

| Hematological data | Controls No. 23 | Splenectomized No. 14 | p-value | Controls No. 23 | Non splenectomized No. 17 | p-value |
|---|---|---|---|---|---|---|
| WBCs (1000 $\mu L^{-1}$) | 7.8±2.5 | 49.9±28.3 | 0.001 | 7.8±2.5 | 24.9±41.1 | 0.056 |
| RBCs (1000000 $\mu L^{-1}$) | 4.5±0.4 | 2.7±0.7 | 0.001 | 4.5±0.4 | 2.6±0.7 | 0.001* |
| HB (9μ%) | 11.9±1.0 | 7.4±2.1 | 0.001 | 11.9±1.0 | 6.2±1.1 | 0.001* |
| HCt (%) | 34.6±3.1 | 22.2±5.4 | 0.001 | 34.6±3.1 | 18.2±2.8 | 0.001* |
| Mcv (fl) | 77.5±8.3 | 82.1±5.9 | 0.109 | 77.5±8.3 | 71.6±10 | 0.032* |
| MCH (pg) | 26.6±2.6 | 27.3±2.5 | 0.441 | 26.6±2.6 | 24.1±2.8 | 0.006* |
| MCHC (g%) | 34.3±0.8 | 33.2±2.7 | 0.079 | 34.3±0.8 | 33.8±2.7 | 0.043* |
| RDW (%) | 16.1±1.8 | 24.3±6.3 | 0.001* | 16.1±1.8 | 24.5±3.2 | 0.001* |
| Platelet count (1000 $\mu L^{-1}$) | 303.3±70.4 | 583.9±198.4 | 0.001* | 303.3±70.4 | 493.1±267.6 | 0.002* |

NB: *Means significant difference at p-value <0.5

Table 2: Comparison of RBCs vesicles percentage between splenectomized, non splenectomized β-thalassemia cases VS healthy controls

| | Controls No. 23 | Splenectomized No. 14 | p-value | Controls No. 23 | Non splenectomized No. 17 | p-value |
|---|---|---|---|---|---|---|
| RBCs vesicle percentage | 7.5±2.4 | 15.0±7.6 | 0.001* | 7.5±2.4 | 10.8±6.7 | 0.073 |

NB: *Means signification difference at p-value <0.05

Table 3: Comparison of RBCs vesicles percentage between patients with β-thalassemia and healthy controls

| | Cases and controls | Mean±SD | Min-Max | p-value |
|---|---|---|---|---|
| RBCs vesicles percentage | Controls (23) | 7.5±2.4 | 3.2-13.4 | 0.002* |
| | Cases (31) | 12.7±7.3 | 3.4-33.5 | |

NB: *Means signification difference at p-value <0.05

Table 4: Comparison of haematological data and RBCs vesicle percentage between splenectomized and non splenectomized β-thalassemia cases

| | Splenectomized No. 14 | | Non splenectomized No.17 | | |
|---|---|---|---|---|---|
| Hematological data | Mean±SD | Min.-Max. | Mean±SD | Min.-Max. | p-value |
| Date of last blood transfusion (since …weeks) | 4.5±1.7 | 3.0-8 | 4.9±5.1 | 2.0-24 | 0.791 |
| WBCs (*1000 $\mu L^{-1}$) | 49.9±28.3 | 8.7-90.0 | 24.9±41.1 | 4.7-163.8 | 0.140* |
| RBCS (*1000000 $\mu L^{-1}$) | 2.7±0.7 | 1.3-4.2 | 2.6±0.7 | 1.8-4.0 | 0.663 |
| HB (g $dL^{-1}$) | 7.4±2.1 | 3.7-13.1 | 6.2±1.1 | 4.5-7.8 | 0.018* |
| Hct (%) | 22.2±5.4 | 10.1-34.4 | 18.2±2.8 | 13.2-22.8 | 0.005* |
| MCV (fl) | 82.1±5.9 | 71.0-91.6 | 71.6±10.0 | 56.8-87.7 | 0.001* |
| MCH (pg) | 27.3±2.5 | 23.5-31.3 | 24.1±2.8 | 19.6-28.1 | 0.002* |
| MCHC (g $dL^{-1}$) | 33.2±2.7 | 28.7-38 | 33.8±2.7 | 27.4-38.6 | 0.530 |
| RDW (%) | 24.3±6.3 | 14.2-34.6 | 24.5±3.2 | 15.9-29.6 | 0.847 |
| Platelet count (*1000 $\mu L^{-1}$) | 583.9±198.4 | 140.0-990 | 493.1±267.6 | 182.0-990 | 0.183 |
| RBCs vesicles percentage | 15.0±7.6 | 5.5-33.5 | 10.8±6.7 | 3.4-25.3 | 0.041* |

NB: *Means signification difference at p-value <0.05

Table 5: Correlation between RBCs vesicles percentage, clinical and haemetological data of β-thalassemia cases

| Clinical and hematological data | RBCs vesicles (%) r | p-value |
|---|---|---|
| Age | -0.165 | 0.380 |
| Last blood transfusion | 0.050 | 0.790 |
| RBCs | 0.135 | 0.470 |
| HB | 0.231 | 0.212 |
| Hct | 0.200 | 0.281 |
| MCV | 0.015 | 0.934 |
| MCH | 0.152 | 0.416 |
| MCHC | 0.181 | 0.329 |
| RDW | -0.094 | 0.617 |
| Platelets | 0.248 | 0.178 |
| WBCs | 0.035 | 0.850 |

NB: *Means correlation is significant at the 0.05 level (2-tailed)

percentage of circulating RBCs vesicles between non-splenectomized β-thalassemia patients and healthy controls p-value = 0.073 as shown in Table 2.

There was a statistically significant increase in the percentage of circulating RBCs vesicles in the β-thalassemia group as compared to the control group ($p<0.05$) as shown in Table 3. Comparing the haematological data and RBCs vesicles percentage between splenectomized and non splenectomized β-thalassemia cases as shown in Table 4 it was found that there was significant increase in WBCs count, HB level, packed cell volume, MCV, MCH, in splenectomized group as compared to non-splenectomized group ($p<0.05$), but no statistically significant between 2 groups regarding RBCs count, MCHC, RDW and platelet count. Also, there was a statistically significant increase in percentage of circulating red cell vesicles in splenectomized group as compared to the non-splenectomized group ($p<0.05$) as shows in Table 4.

Correlation between RBCs vesicles percentage, clinical and haematological data of β-thalassemia cases as shown in Table 5, there was no correlation ($p>0.05$).

## DISCUSSION

Increased frequency of thromboembolic events has been recently observed in patients with thalassemia major, causing hypoxemia and cor pulmonale. Studies to determine hypercoagulability showed: impaired platelet aggregation, increased circulating platelet aggregates, shortened platelet survival and decreased plasma levels of protein C, protein S or antithrombin III. Erythrocytes from thalassemia major patients enhanced thrombin formation in a prothrombinase assay (Eldor *et al*., 1993).

Alteration of normal phospholipids asymmetry in the membrane bilayer, allowing phosphatidylserine to be exposed on the outer surface, affording an explanation for enhanced erythrophagocytosis and for hypercoagulative reactions frequently found in β-thalassemia patients (Pattanapanyasat *et al*., 2004).

Although microcirculatory obstruction can be explained by RBCs membrane rigidity and reduced sialic acid content and hence, a reduced surface charge, the mechanisms responsible for increased incidence of

thrombotic events in thalassemia remain unclear (Pattanapanyasat *et al*., 2004).

In the circulation, the presence of high levels of membrane-derived microparticles, stemming from RBCs, apoptotic cells or activated platelets, have been shown to enhance procoagulant activity (Pattanapanyasat *et al*., 2004).

In this study, we provided evidence of presence of circulating RBCs vesicles in β-thalassemia major as well as normal blood samples, but their percentage in the patients group (12.7±7.3%) showed a broad range when compared to the control group (7.5±2.4%). This may be explained by the fact that, in thalassemia patients with different degrees of severity, there may be different levels of RBCs membrane damage evidenced by vesiculation. Different body iron status, degree of hepatosplenomegaly, degree of anaemia, different HBF levels, concomitant inheritance of other non β globin genes and genetic factors that reduce the overall globin chain synthesis imbalance may contribute to variable numbers of RBCs vesicles seen in β-thalassemia patients. However, mechanism responsible for this variability and its relationship to hypercoagulability remain to be defined (Pattanapanyasat *et al*., 2004).

In this study, there was a statistically significant increase in percentage of circulating RBCs vesicles in β-thalassemia patients compared to healthy controls (Table 3), these results coincide with Lamchiagdhase *et al*. (2004). They proved that 10-50% of RBCs vesicles identified by glycophorin. A were positive for annexin V, a protein that interacts with negatively charged phospholipids such as phosphatidyl serine. Moreover, the percentage of annexin V positive events in RBCs vesicles was significantly higher than for intact RBCs, which proved that RBCs vesicles for both normal and thalassemia patients express higher percentage of phosphatidyl serine than their associated intact RBCs.

Present study showed that percentage of circulating RBCs vesicles in splenectomized β-thalassemia patients was significantly higher than non-splenectomized group and healthy controls as well (Table 2), this is consistent with a study done by Pattanapanyasat *et al*. (2004), which proved that β-thalassemia/HBE patients who had been splenectomized showed higher of RBCs vesicles than non-splenectomized patients. These findings suggest that, although splenectomy improves HB concentration and reduces the transfusion needs and total blood volume (Aessopos *et al*., 2005), it leads to increase in percentage of circulating RBCs vesicles and subsequently, enhancing their procoagulant effect.

Although our study showed that the percentage of circulating RBCs vesicles is higher in non-splenectomized β-thalassemia patients than healthy controls, this difference was not statistically significant (Table 2), this is not in agreement with Pattanapanyasat *et al*. (2004) who found a significantly higher percentage of circulating RBCs vesicles in non splenectomized β-thalassemia/HBE patients as compared to healthy controls.

When clinical and haematological indices were compared with RBCs vesicles percentage in order to research for evidence of changes in vesicles percentage and their relationship to the degree of severity of anaemia in β-thalassemic patients, we didn't find any correlation between them and so this relationship could not be proved (Table 5). However, these results contradict with those of Pattanapanyasat *et al*. (2004) who reported an inverse relationship between levels of RBCs vesicles and HB concentration. Negative correlations were also noted between levels of vesicles and various other haematological parameters, including RBCs count and HCT. These findings proved the presence of an inverse relationship between the degree of severity in thalassemic patients and the number of RBCs vesicles.

In conclusion, the presence of high levels of phosphatidyl serine positive RBCs vesicles, or other circulating microparticles originating from other blood cells, might act as one of the confounding factors responsible for dissemination of prothrombotic manifestations in thalassemia, moreover they can be considered a valuable biological parameter for the assessment of possible thrombotic complications in patients with thalassemia. Also, flow cytometric determination of RBCs vesicles percentage is simple, reliable and may offer new insights in assessment of possible thrombotic complications in thalassemic patients.

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