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The hypercoagulation state among major β-thalassemia patients at H. Adam Malik Hospital, Medan, Indonesia

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ABSTRACT

Background: Thalassemia is the most common hereditary blood disorder in the world and Indonesia. Major β-thalassemia is classified as Transfusion-Dependent Thalassemia (TDT). Specific changes in the composition of red cell membrane lipids and hemosiderosis may lead to hypercoagulation. This study aimed to determine the difference in Protein C, Protein S, Antithrombin III (AT-III), and D-dimer between major β-thalassemia patients and normal people.

Methods: This was an observational analytical study with a cross-sectional design, including 18 major β-thalassemia patients in H. Adam Malik Hospital Medan who met the study criteria and 18 normal controls. Subjects were examined for Protein C, Protein S, AT-III and D-dimer. The assessment was also carried out among patients based on their transfusion frequency and serum ferritin.

Results: There were significant differences in Protein C, Protein S, and D-dimer levels between major β-thalassemia patients and normal controls ($p < 0.05$). AT-III level in major β-thalassemia patients was lower than healthy controls, however the difference was not significant. The level of Protein C in patients who infrequently and frequently underwent blood transfusions were 34.88 ± 8.6 and 45.19 ± 7.8 respectively, which was significantly different ($p < 0.05$). A significant differences in Protein C, Protein S and D-dimer were also found between patients with serum ferritin level < 2,500 ng/ml and ≥ 2500 ng/ml.

Conclusion: Significant changes were found in anticoagulation proteins of major β-thalassemia patients. Frequent blood transfusions and chelation therapy should be emphasized for major thalassemia patients because they affect anticoagulation protein levels.

Keywords: antithrombin, D-dimer, major β-thalassemia, protein C, protein S

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INTRODUCTION

Thalassemia originates from the Greek word Thalassa (sea) and Haema (blood), which refers to a synthesis disruption of the globin-α chain or globin-β chain, which is a subunit of hemoglobin Hb A (α2; β2) so that the erythrocytes lysed faster. As a result, sufferers must undergo blood transfusions throughout their lifetime.[1]

According to the Indonesian Ministry of Health in 2007, the prevalence of thalassemia in Indonesia reached 1.5 per 1,000 inhabitants.[2] Data obtained from medical records at H. Adam Malik General Hospital Medan showed that in 2009-2010, there were 160 people with thalassemia and in 2011-2014, there were 113 people with thalassemia. The latest data on the number of one-day care of thalassemia patients showed 370 patients with thalassemia from August 2016 to May 2017. This fact supports thalassemia as one of the most common hereditary diseases.

Major β-thalassemia is a clinical condition characterized by severe anemia due to ineffective erythropoiesis.[1] The severity of the anemia symptoms in patients with major β-thalassemia results a condition of transfusion dependency as a major part of its treatment in improving quality of life and prolonging survival, so it is classified as Transfusion-Dependent Thalassemia (TDT).[3]

It is widely recognized that major β-thalassemia patients have an increased risk of thrombosis. A study conducted by Michaeli J et al. reported 4% prevalence of thrombosis in thalassemia patients. Various cases have been reported, including neurological disorders that occur due to cerebral thrombosis.[4] Some etiologic factors may also contribute to the occurrence of a hypercoagulable state in thalassemia patients. The presence of specific changes in the lipid composition of red blood cell membranes and hemosiderosis can contribute to the activation of the coagulation system.[5]

Based on these data, this study aimed to determine the difference between Protein C, Protein S, Antithrombin III, and D-dimer in major β-thalassemia patients and healthy people.

METHODS

This was a cross-sectional analytical study, which aimed to determine the difference in the mean level of Protein C, Protein S, Antithrombin III and D-dimer between thalassemia patients and

healthy people. This study included 18 children with major β-thalassemia and 18 healthy children. This study was conducted in the Clinical Pathology Department of the Faculty of Medicine, University of North Sumatra/Haji Adam Malik Hospital Medan from April 2018 to July 2018. The inclusion criteria included subjects under or equal to 18 years old who have been diagnosed as with major β-thalassemia and have received transfusion for at least one year. Patients with liver disorders and those who were using drugs that could affect hemostasis were excluded.

Written informed consent was requested in advance from the parents of the research subjects. History taking and physical examination were then performed. The data and results of the examination were recorded in the special status for this study.

The independent variables measured in this study were age, sex, transfusion frequency, splenectomy history, erythrocyte index, thrombocyte, and ferritin. Transfusion frequency was grouped into frequent and infrequent. Frequent transfusion was defined as more than 12 times per year, while infrequent was defined as ≤ 12 times per year. Ferritin level was grouped into < 2,500 ng/ml and ≥ 2,500 ng/ml.

Pretransfusion blood samples were taken from the median cubital vein and put in a 2 ml vacutainer citrate tube (with a ratio of 9:1). The examination was carried out using TEChrom Protein C reagent, TeClot Protein S, TEChrom AT (anti-Xa), and Blue D-dimer from TECO (Neufahrn, Germany) in the Coatron A4.

The independent t-test was performed when data were normally distributed, and Mann-Whitney test was performed when data were not normally distributed. The analysis was carried out at a 95% confidence interval with a p-value < 0.05 considered as significant.[7]

RESULTS

In this study, examinations were carried out in 18 people with major β- thalassemia and 18 normal controls with adjusted sexes and ages. The characteristics of patients and controls in this study are presented in Table 1.

As shown in Table 2, there were significant differences in Protein C, Protein S, and D-dimer between major β-thalassemia patients and healthy controls ($p < 0.05$). AT-III level in major β-thalassemia patients was lower than healthy controls, however the difference was not significant.

In Table 3, we can see a significant difference in the level of Protein C between major β-thalassemia patients with frequent transfusions and those with infrequent transfusions.

In addition to the effects of frequency of transfusion, we also evaluated the effects of iron accumulation on thalassemia patients who routinely underwent transfusion to anticoagulation proteins, by comparing Protein C, Protein S, Antithrombin III and D-dimer based on their ferritin levels.

As shown in Table 4, there were significant changes in Protein C, Protein S and D-dimer levels in patients with ferritin levels < 2,500 ng / ml and > 2,500 ng / ml. Ferritin level > 2500 ng / ml was associated with the prognosis of poor long-term survival.

DISCUSSION

The number of male patients in this study was greater than female patients, although there is still inadequate evidence linking sex as a risk factor for decreasing the thalassemia gene. In addition, there was no significant difference in the level of natural anticoagulants between males and females, meaning that both sexes had the same risk of thromboembolic events.[6]

Hemoglobin levels, MCV and MCH were significantly lower in the patient group, which was considered as hypochromic microcyter anemia. The increase in RDW in the patient group was also in accordance with the morphology of erythrocytes in peripheral blood smears, which in this study found anisocytosis and poichylocytosis in the form of Nucleated Red Blood Cell (NRBC), target cells, polycromate cells and basophilic stippling. Pretransfusion Hb was supposedly maintained in the range of 9 – 10.5 g / dL based on TIF recommendations in 2014.[7] Low pretransfusion Hb values in patients (median 7.8 g/dl) of this study, may be due to patient noncompliance with regular transfusions on schedule.

In the group of patients, there was a significant decrease in platelet count when compared with controls. This could be due to the occurrence of recurrent and chronic platelet consumption in patients with major β-thalassemia.[5] The shortening of the platelet lifespan also occurs in both major and intermedia thalassemia patients as well as splenectomized and nonsplenectomized thalassemia patients, which is due to the increase in platelet counts that are used, destroyed and removed from the circulation.[8] However, splenectomy is a risk factor that increases the likelihood of thromboembolism.[9] In this study, two patients had undergone splenectomy. The erythrocyte index in both patients was not different when

Table 1 Baseline characteristics of respondents

Characteristics	Unit	Patient (n = 18)		Control (n = 18)		p value
		n	%	n	%	
Sex						
Male	Person	11	61.11	11	61.11	
Female	Person	7	38.89	7	38.89	
Age (Mean ± SD)	Year	11.39 ± 5.07		11.39 ± 5.07		
Transfusion frequency						
Infrequent (< 12 x/ year)	Person	8	44.44	-		
Frequent (> 12 x/ year)	Person	10	55.56	-		
Splenectomy						
Nonsplenectomy	Person	16	88.89	-		
Post splenectomy	Person	2	11.11	-		
Erythrocyte Index						
Hb (Median (Min - Max))**	g/dl	7.8 (6 – 10.7)		12.4 (11.2 – 14.3)		0.001^
MCV (Mean ± SD)*	fl	73.89 ± 5.34		81.5 ± 4.34		0.001^
MCH (Mean ± SD)*	pg	23.98 ± 2.5		27.2 ± 2.1		0.001^
RDW (Mean ± SD)*	%	23.9 ± 5.37		14.7 ± 1.99		0.001^
Thrombocyte (Median (Min-Max))**	10^3/µL	204.5 (118 – 411)		340 (239 – 444)		0.001^
Ferritin (Median (Min-Max))	ng/ml	2766.5 (338.9 – 15,275)		-		

^ Significant difference if p < 0.05, difference test used:
* = Independent t-test, ** = Mann-Whitney test

Table 2 The difference of Protein C, Protein S, Antithrombin III and D-Dimer between patient and control.

Characteristics	Unit	Patient (n = 18)	Control (n = 18)	P value
Protein C (Mean ± SD)*	%	40.61 ± 9.56	80.19 ± 24.77	0.001^
Protein S (Mean ± SD)*	%	32.16 ± 18.39	58.3 ± 37.71	0.012^
Antithrombin III (Median (min-max))**	%	79.75 (30.20 – 104.30)	88.7 (64.5 – 103.8)	0.327
D-dimer (Median (min-max))**	ng/ml	170.50 (31– 7500)	87 (17– 466)	0.034^

^ Significant difference if p <0.05, difference test uses:
* = Independent T-test, ** = Mann-Whitney test

Table 3 The difference of Protein C, Protein S, Antithrombin III, and D-dimer between frequent and infrequent transfusion

Characteristics	Unit	Frequency of transfusion		P Value
		Frequent (n = 8)	Infrequent (n = 10)	
Protein C (Mean ± SD)*	%	34.88 ± 8.6	45.19 ± 7.8	0.018^
Protein S (Median (min-maks)) **	%	35.15 (16.3 – 57.4)	18.4 (7 – 60.30)	0.477
Antithrombin III (Median (min-maks)) **	%	60.9 (34.70 – 97.60)	95.35 (30.20 – 104.30)	0.076
D-dimer (Median (min-maks))**	ng/mL	146.5 (42- 7500)	179 (31 – 7500)	0.657

^ Significant difference if p <0.05, difference test uses:
* = Independent T-test, ** = Mann-Whitney test

compared with other patients who had not undergone splenectomy. Platelet counts in these two patients were 274 x 103 / µL and 411 x 103 / µL, where both of these values were higher when compared with the average platelet count in the patient group. Hasan et al. found increased platelet in patients who had undergone splenectomy, which is caused by the reduction in the destruction and removal of platelets from the circulation by the spleen.[10]

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Table 4 **The difference in Protein C, Protein S, Antithrombin III, and D-dimer between low and high ferritin level.**

Characteristics	Unit	Ferritin level		P value
		< 2,500 ng/ml (n = 8)	≥ 2,500 ng/ml (n = 10)	
Protein C (Mean ± SD)*	%	47.08 ± 749	35.43 ± 7.87	0.006^
Protein S (Mean ± SD)*	%	20.45 ± 12.26	41.5 ± 17.4	0.008^
Antithrombin III (Median (min-maks)) **	%	95.8 (37.80-104.30)	68.85 (30.20-103. 50)	0.091
D-dimer (Median (min-maks))**	ng/mL	115 (31- 193)	337 (117 – 7500)	0.006^

^ Significant difference if p <0.05, difference test uses:
* = Independent T-test, ** = Mann-Whitney test

In this study, significant changes in Protein C, S Protein, and D-dimer levels were found in major β-thalassemia patients compared to normal controls. Based on the data obtained, it appears that the process of changes in natural coagulation inhibition and fibrinolysis, which is a risk of thromboembolism, occurs in patients with major β thalassemia although clinical signs and symptoms have not appeared and it happens since an early age.

In Table 3, a significant difference can be seen in the levels of Protein C between major β-thalassemia patients with frequent and infrequent transfusion. A study by Hassan et al. found a significant difference in the amount of Protein C between patients who rarely underwent transfusion and those who frequently underwent transfusion. They found the amount of protein C was lower in patients who often underwent transfusion.[10] Similarly, Trinchero et al. measured Protein C levels before transfusion and 1 hour after transfusion and found a significant improvement in the value of Protein C after transfusion.[13]

Protein C is a serine protease that is dependent on vitamin K, which has a Gla domain that will interact with negatively charged phospholipids on the cell surface and interacts with its endothelial receptor (EPRC). The function of aPC is to inactivate two important factors in the coagulation cascade, namely factors V / Va and factor VIII / VIIIa. This process is modulated by the presence of other factors namely Ca^{2+} ion, phospholipid and Protein S as cofactors. Protein S has the Gla domain and EGF-like domain but does not have a serine protease domain. Protein S also has a broad C terminus domain and has a role as a cofactor in the activation of Protein C or C4BP.[11] Protein S directly binds to the negative phospholipids on the cell surface of erythrocytes and serves as a "bridge" to the charge macrophages for phagocytosis.[9]

The procoagulant effect of thalassemia red blood cells originates from increased expression of anionic phospholipids on the cell surface, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS), which normally increases in old red blood cells.[5] Protein C and Protein S directly bind to the negative phospholipids on erythrocytes or endothelial cell surfaces and act as activators to prothrombinase complex, resulting in increased production of thrombin.[9] In this study, it can be seen that there was a significant decrease in Protein C and Protein S levels in the patient group when compared with controls.[10] Splenectomy also caused more consumption of Protein C. Protein C levels in two splenectomized patients were 36.2 % and 29.5 %, where both of these values were lower compared with the average values in the patient group. Hasan et al. also showed a lower Protein C level in patients who have been splenectomized.[10]

AT-III levels in the patient group also appeared to decline even with insignificant differences. This may be due to the differences in the properties of AT-III, which are not directly activated by negatively charged phospholipids on the surface of the erythrocyte cells of thalassemia patients. Therefore, unlike Protein C and Protein S, AT-III can be found to be less affected by changes in the erythrocyte structure.[12] Similarly, higher D-dimer levels were found in the patient group, which showed increased fibrinolysis in thalassemia patients as a result of hypercoagulation conditions.

The improvement of anticoagulation protein is more often caused by the mixing of thalassemia erythrocytes with normal erythrocytes so that the effect of vascular destruction is reduced. Vascular destruction is caused by abnormalities of rigid thalassemia erythrocyte membranes that tend to clot and have a stronger adherence to the endothelial cells. In addition, the presence of normal red cells on systemic thalassemia patients will also reduce the hypoxic conditions that cause endothelial damage, when compared with the condition that only relies on thalassemia methemoglobin erythrocytes that bind oxygen more strongly than normal hemoglobin.[5] Similarly, with the existence of normal red cells, the interaction

of Protein C with negatively charged phospholipids on the surface of thalassemia erythrocytes is reduced, resulting in improved levels of Protein C. For this reason, the efforts of doctors to plan more frequent transfusion therapy, accompanied by adequate chelation therapy, are very important. Likewise, patient compliance must be a concern and always confirmed to patients and their families.

Ferritin level > 2500 ng / ml is associated with the prognosis of poor long-term survival. This is caused by the buildup of iron in erythrocytes and other tissues such as the endothelium, liver, and heart into a complex condition that encourages the creation of hypercoagulation in thalassemia patients. Consequently, frequent transfusion, without the right and regular chelation therapy, will instead become a "second disease" for major thalassemia patients.[14]

In general, the amount of Protein S in thalassemia patients decreases when compared to controls. However, as shown in Table 3 and Table 4, the tendency of Protein S increases relatively to patients who rarely receive transfusions or patients with serum ferritin levels > 2,500 ng/ml. This may be due to the Protein S examination method in this study, which solely evaluated the free Protein S level so that the assessment of free Protein S and total Protein S distribution cannot be done. The proportion of Protein S increased when compared with total Protein S, which can be caused by conditions with active coagulation.[15]

This study can be a preliminary study for increasing the knowledge about hypercoagulation that occurs in patients with major β-thalassemia. This study can still be enhanced by increasing the number of patients included, especially those who have undergone splenectomy, or by conducting a study in intermedia β-thalassemia patients. Moreover, diagnostic tests, especially for Protein C, and the assessment of coagulation changes based on age groups are also needed.

CONCLUSION

In this study, significant changes in Protein C, S Protein, and D-dimer levels were found in major β-thalassemia patients compared to normal controls. From this study, there was also an improvement in the coagulation of proteins in patients who received transfusions more often.

ETHICAL CLEARANCE

This research has received ethical clearance from the Health Research Ethics Committee of the Faculty of Medicine, the University of North Sumatra with number 66/TGL/KEPK FK USU-RSUP HAM/2018 and the Health Research Committee in Haji Adam Malik General Hospital Medan with number LB.02.03/II.4/291/2018.

CONFLICT OF INTEREST

None of the authors have any conflict of interest.

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AUTHOR'S CONTRIBUTION

Jane Tetraulina Silitonga was the owner of the idea, writing the manuscript and revised the final manuscript. Jane Tetraulina Silitonga together with Adi Koesoema Aman and Bidasari Lubis established the study design, collected the clinical and laboratory data, analyzed and interpreted the results. Jane Tetraulina Silitonga and Adi Koesoema Aman did the laboratory tests, and Bidasari Lubis supervised the sampling process.

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