Nitroblue tetrazolium test in patients with beta-thalassemia major

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ABSTRACT

الأهداف: تقييم وظيفة عدلة الكريات البيض لدى المرضى المصابين بالثلاسيميا الكبرى (TM)، ومقارنة ذلك مع مجموعة التحكم وللتعرف على العوامل المناسبة.

الطريقة: أجريت دراسة كتائبية نسيجية خلال الفترة مابين أكتوبر2007م وحتى فبراير 2008م، بمركز أبحاث الثلاسيميا – مستشفي بوعلي سيناء – مدينة ساري مازانداران –إيران. تكونت الدراسة من المرضى المصابين بالثلاسيميا الكبرى (TM) بمستشفى بوعلي سينا التعليمي. كانت طريقة العينات في مجموعة الحالة نظامية، وكانت هدفاً بناءً على مجموعة التحكم. تم تحديد حجم العينة بناءً على الدراسات السابقة. كان يتم تشخيص الثلاسيميا الكبرى (TM) وفقاً لإستشراد الهيموجلوبين الكهربائي وكانت مجموعة التحكم لدينا عبارة عن إخوان وأخوات بلغت أعمارهم حوالي 5 سنوات، وتختلف من مريض إلى آخر، ولكنهم كانوا من نفس الجنس. تم تجميع بياناتنا بناءً على المقابلة، الفحوصات، الصفات السكانية الجغرافية، والمعلومات الطبية من سجلاتهم الطبية. تم تقييم وظيفة عدلة الكريات البيضاء بواسطة أداء اختبار تراجع نيتروبلو تيترازوليوم (NBT). أجري الاختبار على المجموعتين وتم تحليل البيانات بواسطة الاختبارات المناسبة باستعمال طريقة التحليل الإحصائي (SPSS 13.0).

النتائج: في هذه الدراسة تمت مقارنة 39 مريضاً مع 39 شخصاً سليماً في مجموعة التحكم. بلغ متوسط العمر للمرضى 21.6±5.3 عاماً، وفي مجموعة التحكم الأصحاء 22.4±5.1 عاماً، (p=0.7). تبين وجود صلة ملحوظة بين نتائج الاختبار وعمر المرضى (p=0.008). كانت نتائج قصور اختبار (NBT) في مجموعة المرضى 36%، و 90% في مجموعة التحكم حيث كانت مختلفة بشكل ملحوظ. نشاط عدلة الكريات البيض بناء عُلى نتيجة اختبار NBT 89.9±11.6% و 93.7±2.51% في مجموعتي الحالة والتحكم على التوالي، (p=0.025).

خاتمة: في هذه الدراسة كانت مؤشرات وظيفة عدلة الكريات البيض لدى المرضى المصابين بالثلاسيميا منخفضة بشكل ملحوظ بالمقارنة مع مجموعة التحكم، خاصة لدى المرضى صغار السن. لذلك وبناء على هذه النتائج، فإنه من الضروري القيام بتقييم وظيفة عدلة الكريات البيض والالتهابات المتقيحة لدى المرضى المصابين بالثلاسيميا الكبرى (TM).

Objective: To assess the neutrophil function in thalassemia major (TM) patients and compare itwith the control group, and to recognize its relevant factors.

Methods: This was a retrospective cohort study, which was carried out from October 2007 to February 2008 in the Thalassemia Research Center in Boo Ali Sina Hospital in Sari, Mazandaran, north of Iran. The study population consisted of TM patients in Boo Ali Sina Teaching Hospital. The method of sampling in the case group was systematic, and it was target based in the control group. The sample size determined was based on previous studies. Thalassemia major was diagnosed based on hemoglobin electrophoresis (case group). The control group was their brothers and sisters, who had ±5 years of age difference, and were of the same gender as the patients. Data collection was based on interview, investigating demographic characteristics, and also obtaining medical information from the medical records of the patients. The neutrophil function was assessed by performing nitroblue tetrazolium (NBT) reduction test. The test was carried out on both groups, and the data were analyzed by software using SPSS version 13.0.

Results: In this study, 39 patients and 39 healthy controls were compared. The average age of the patients was 21.6±5.3 years, and it was 22.4±5.1 years in healthy controls (p=0.7). There was a significant correlation between the tests' results, and the patients' age (p=0.008). The rate of impaired NBT results in the patients was 36%, while it was 10% in controls, which were significantly different. The neutrophil activity based on NBT test was 89.9±11.6% in the case group, and 93.7±2.51% in the control group, (p=0.025).

Conclusion: This study indicates that neutrophil activity in thalassemic patients was significantly lower, compared to the normal control group, especially in young patients. Based on the results, evaluation of neutrophil function, and pyogenic infections in TM patients seems necessary.

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Thalassemia is a quantitative disorder of hemoglobin chain synthesis, and also one of the most frequent genetical disorders in the world.[1] Beta-thalassemia is so prevalent in Iran, specifically in Mazandaran (northern province).[2] In its major form, the disease manifests itself as a severe chronic hemolytic anemia (which requires blood transfusion), with growth failure and multiple organ dysfunction related to some mechanisms such as chronic hypoxia and iron overload.[3,4] The effect of thalassemia major (TM) on some organs such as liver, heart, pancreas, and hypothalamus is relatively known,[1-6] but regarding immune system, specifically neutrophils, there is insufficient and paradoxical evidence. As a proof, some studies indicated neutrophil dysfunction in severe forms of the disease,[7-12] while in other studies it did not.[13-20] According to the role of neutrophils in the immune system, and owing to the fact that TM patients are susceptible to infection due to other factors such as chronic hypoxia, and splenectomy, the neutrophil function is very critical and important in TM. Hence, the aim of this study was to assess neutrophil function in these patients, and its relevant factors.

Methods. This retrospective cohort study was carried out from October 2007 to February 2008 in the Thalassemia Research Center in Boo Ali Sina Hospital in Sari, Mazandaran, north of Iran. The study population were TM patients of the Thalassemia Center in Boo Ali Sina Teaching Hospital. The inclusion criteria include TM patients aged from 10 to 40 years, having sibling within their ages. All patients with acute febrile infections were excluded from the study (patients oral temperature >37.8^{0}C, and chills). All the patients and controls had participated in this study voluntarily, and signed a consent form. The approval was obtained from the local ethics committee prior to the commencement of the study. The sampling method was systematic in the patients, and target based in the control group. There were 39 patients in each group. This number was determined based on previous studies. The diagnosis of TM was based on the medical records and hemoglobin electrophoresis of the patients. The healthy controls were siblings of the patients with ±5 years of age differences, and of the same gender. The method of data collection was personal interview, and questionnaire filling. The variables included age, gender, diagnosis age, initiation age of transfusion, transfusion intervals, splenectomy age, dosage of Desferal, mean hemoglobin, and the last amount of ferritin, also complications (diabetes mellitus [DM], cardiovascular disease, hypothyroidism, hypoparathyroidism), duration and dosage of using hydroxyurea, and other drugs. The nitroblue tetrazolium (NBT) reduction test was performed to evaluate the neutrophils' function. The test was carried out as a simple NBT screening test: peripheral blood of normal and thalassemic donors was collected in tubes containing ethylene diamine tetra acetic acid, after making a mixture of NBT 1%, and phormyl minestric acetate), 50 ml of this mixture was exposed to 50 ml of fresh blood, and the mixture was incubated for 30 minutes in 37^{0}C, and after 30 minutes of centrifugation (2500 RPM), the hematological smear was prepared, and after Giemsa staining, respiratory bursts were counted. When the colorless dye of NBT, is added to normal phagocytozing leukocytes, the reactive oxygen species reduced the NBT creating purple formazan and normal neutrophils contain a cytoplasm full of reduced colors, which are counted. In normal subjects, the percentage of NBT-positive granulocytes is more than 90%. The test was performed the test in patients and control group, then we compared the results.

Statistical analysis was performed using chi-square and Mann-Whitney test, and Statistical Packages for Social Sciences software version 13.0 (SPSS Inc., Chicago, Illinois, USA). A $p<0.05$ was considered to be significant.

Results. A total of 39 patients and 39 normal controls were studied. The average age was 22.4 ± 5.1 in the control group, and 21.6 ± 5.3 in the case group. Gender distribution of the case and control group: 56.4% female in cases, and 61.5% female in controls. Among the patients 30 (76.9%) had face changes, 18 (46.2%) were splenectomized, and 13 (33.3%) had cardiovascular disease. There were 8 (20%) diabetic patients, and 6 (15.4%) hepatic disorders. No one was hypothyroid or hypoparathyroid. All patients receive Desferal, none on hydroxyurea, or any drug that significantly affects neutrophil function. The diagnosis age was 15 ± 19 (1-76) months, and the initiation age of transfusion was 19.1 ± 23 (1-115) months. The transfusion intervals were approximately 2.6 ± 0.7 (1-5) weeks. Splenectomy was performed in 17 patients within 12 ± 5.6 (6-24) years of age. The dosage of Desferal was 18.4 ± 5.6 (10-30) mg/kg/day. Mean hemoglobin level was 9 ± 0.9 (7.3-11.6) g/dl, and the last ferritin level was 2664 ± 1487 ng/ml (2635-7275). The severity of the disease was categorized to criteria as shown in **Table 1**. A total score of 0-3 is considered as mild, and 4-5 as severe disease. On the basis of such categories, out of 39 patients, 8 (20.5%) had severe disease, and 31 (80%) had mild disease. Evaluating neutrophil function based on NBT test and considering an NBT of >90% as normal, showed that 64% of the patients, and 90% of healthy controls had active NBT test indicating a significant difference between the 2 groups ($p=0.025$, odds ratio=3.75 [1.026-13.70], **Table 2**). An NBT result in the mild cases was 91.7 ± 2%, and in severe forms was 89.1 ± 0.9% without any significant difference ($p=0.17$).

This study did not find any significant relationship between NBT and gender (p=0.40), splenectomy (p=0.35), DM (p=0.17), hepatic dysfunction (p=0.31), and cardiovascular disease (p=0.16). Furthermore, there was no significant correlation between NBT results, and the use of drugs, hormones, and serum ferritin level. In evaluating the relationship between NBT results and the patients' age, it showed a significant correlation: in the impaired NBT group (≤90%) the mean age was 18.5 ± 3.4 years, while this was 23.2 ± 5.51 years in normal NBT group (p=0.008). Considering 21 years of age as a cut off point, the impairment of NBT in the above and under 21 years of age was significant (p=0.006, **Table 3**).

Discussion. In this study, the evaluation of neutrophil function in TM patients(which was assessed using NBT test), showed a significant decrease (p<0.02) in comparison to the 39 healthy controls. In another study, which was performed by Cantinieaux et al[7] in Brussels, polymorphonuclear neutrophils (PMN's) phagocytosis was shown to be decreased, and they considered this abnormality due to the cellular and serumal dysfunction due to iron overload. In another study, which was carried out by the same researcher in 1999, PMN's phagocytic action specially in neutrophils was impaired, and they considered that as a result of cellular and serumal abnormalities due to iron overload.[8] In other study, which was carried out by Matzner et al[9] in Pennsylvania, all of the TM patients except a single one, had chemotactic defects in their neutrophils, and all of them had a positive history of pyogenic infections. Palacios et al,[10] and Kutukculer et al[11] also found a chemotactic impairment in neutrophils in thalassemic sera, and concluded that the defect found might be caused by transfusion overload. Bassaris et al[12] also reported that adherence, and activity of PMN's in thalassemic patients are decreased, and they suggested that the results are related to a heat labile factor in thalassemic sera, whereas suspending PMN's in heated thalassemic serum resulted in an increase of adherence.

On the other hand, several studies reported different results. Falcao et al[13] in Spain, found a decrease in NBT results only in sickle cell anemia and splenectomized individuals who were otherwise healthy, however, the sickle cell beta thalassemic cases and most of the non-splenectomized patients had normal or near normal NBT scores, while all splenectomized patients exhibited decreased values. The authors considered the results as an indicator of a possible contribution of the spleen in the pathogenesis of such neutrophil dysfunction. Oren et al[14] in Turkey, studied the correlation between neutrophil apoptosis and frequency of infection episodes, serum ferritin levels, and neutrophil count in thalassemic patients, in which no significant correlation was found. They reported that neutrophil apoptosis could not be a possible cause for increased susceptibility to infections in the patients. In Deo et al's[15] study in Bombay, PMN's function, in both normal and thalassemic sera, was normal and did not show any decrease. Shaiegan et al[16] in Tehran also concluded that there was no significant decrease in PMN's function based on NBT results in major thalassemia as compared to normal controls. In another study performed by Speer et al[17], all aspects of neutrophil's activity in thalassemia such as adherence, migration, chemotaxis, and superoxide production were reported as normal. In Fritsche-Polanz et al's[18] study in Austria in 2004, which was carried out on patients with congenital hyperferritinemia-cataract syndrome, it was reported that this hyperferritinemia does not have any effect on neutrophils' function. Luyt et al[19] in 1993 in South Africa, measured pulmonary function tests and neutrophil function concurrently, all pulmonary volumes and capacities were reduced, but this reduction was not correlated with neutrophils' oxidants. In Donadi et al's[20] study in 1987, neutrophils' functions did not have any decrease in TM and sickle cell anemia.

Table 1- Factors for determining the severity of the disease in patients.

Rows	Relative factors	Score	
		0	1
1	Diagnosis age	>4 years	<4 years
2	Face deformities	-	+
3	Transfusion intervals	4-5 weeks	2-3 weeks
4	Splenectomy age	>10 years	<10 years
5	Mean last 3 hemoglobin	>8 gr/dl	<8 gr/dl

Table 2 - Comparison between impaired and normal nitroblue tetrazolium (NBT) results in case and control group.

Groups	Percentage of NBT	
	Impaired	Normal
Case	36	64
Control	10	90
P-value	0.025	

Table 3 - Comparison of nitroblue tetrazolium (NBT) results based on age in 39 thallasemia major patients.

Age	NBT		
	>90%	≤90%	*P* - value
≤21	14.3%	85.7%	0.006
>21	60%	40%	

The difference between the results discussed above and our results might be explained by some factors: first, our study population (39 case, 39 controls) was more than the previous studies. On the other hand, factors like treatment (such as blood transfusion), desired hemoglobin usage of iron chelator, and serum levels of ferritin are possible explanations of the results. In this study, all of our patients are using Desferal, and in addition to this, their PMN's function based on NBT was more impaired than their controls. As seen in Oren's study,[14] iron overload in thalassemic patients with or without chelation therapy, did not have any significant effect on neutrophil function, apoptosis and susceptibility to infection. This indicates that a probably higher level of serum ferritin does not cause impairment in the neutrophil's function. Also in other studies, any decrease in neutrophil function, is believed to be related to multiple transfusions, but not iron overload.[14] In this study, there was no significant difference between NBT results in splenectomized and non-splenectomized patients. Other complications such as cardiovascular impairment, diabetes mellitus, and hepatic disorders also, were not significantly effective on NBT results. These findings suggest that some other factors, (except chronic hypoxia, iron overload) are suspicious in causing neutrophil dysfunction in thalassemic patients. Age was the only effusive factor on NBT results in our study, the prevalence of impaired PMN's function was higher in younger ages, and that may be explained by the lesser contact of their immune system and neutrophils to foreign agents, and their lesser resistance toward invasion.

In summary, this study indicated that neutrophil function had a significant decrease in TM patients as compared to healthy controls, which was significantly more severe in the younger age. Further studies focusing on neutrophil function in thalassemic patients seems necessary. We assessed neutrophils' function only by one test (NBT), which is the limitation of this study. Undoubtedly, the results would be much more accurate if other tests were considered. Furthermore, it would be much better if the number of patients was greater, and the study was multi-centric in different areas. However, in comparison to other previous studies, our study had some new aspects. We compared each thalassemic patient with a normal person, who was their own sibling, within their age and gender; also they were in the same family, in the same place, and the same socioeconomic state. This condition makes the comparison more accurate. Also the range of the patients' age was wide, and too many relevant factors were evaluated.

Finally, we found that NBT results are significantly lower in patients as compared to the normal subjects, and also it was significantly lower in patients under 21 years of age. So, routine screening these patients for susceptibility to pyogenic infections seems necessary, and is recommended.

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