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Prevalence of Alloantibodies and Autoantibodies in Transfusion Dependent Thalassemia Patients

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ABSTRACT

Background: The development of anti-red blood cell alloantibodies remains a major problem in transfusion of blood in thalassemia major patients. Also, Autoantibodies can result in clinical hemolysis and difficulty in cross-matching blood. We studied the frequency of red blood cell alloimmunization and autoimmunization among thalassemia patients who received regular transfusions in Ilam province of Iran.

Methods: This study was carried out on 110 multiply transfused patients with thalassemia major. The saline method, Albumin method, direct/indirect coombs' and Three cell panel test used for detection of red blood cell alloantibody/ autoantibody.

Results: 12 patients out of total 110 patients (10.9 %) developed alloantibodies and 2 (1.81 %) developed autoantibodies. Rh and Kell blood group system alloantibodies were most commonly found, with the majority of patients being transfused with blood matched for ABO and D antigens only.

Conclusion: This study suggests screening RBC antigens prior to transfusion. Our findings accentuate the necessity of antigen typing of supposed to be transfused red blood cells and screenings tests before the first transfusion, at least for Rh (Rh system) and Kell (Kell system) antigens.

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Introduction

Red blood cell (RBC) alloimmunization is an immune response against foreign RBC antigens; this generally occurs after sensitization due to blood transfusions and pregnancies.[1] Rh, Kell, Kidd and Duffy alloantibodies have a high clinical importance; they react at 37oC and cause hemolysis in transfused patients, fetuses and newborns.[1-3] Thalassemia is the most common genetic disorder worldwide and is the most common hereditary hemolytic anemia have been considered.[4,5] The disease has a high prevalence in the Mediterranean in the Middle East (Iran), India, and Southeast Asia is seen.[6,7] There are many forms of thalassemia due to decreased synthesis or lack of synthesis occurs in one or more chains of hemoglobin molecules.[8,9] The expansion of anti- RBC alloantibodies and autoantibodies can significantly make difficulties transfusion therapy. Various alloantibodies are hemolytic and may cause, however not regularly, hemolytic transfusion reactions and limit the availability of further safe transfusion. Others are clinically insignificant. Erythrocyte autoantibodies appear fewer commonly, but they can result in clinical hemolysis and in difficulty in cross-matching blood. Patients with autoantibodies may have a higher transfusion proportion and often require immunosuppressive drugs, a splenectomy, or alternative managements. Life-long RBC transfusion remnants the chief treatment for major thalassemia.[10-12] In spite of the recognition of autoantibodies as transfusion-related

threats, little is known about the extent and reasons of these occurrences among thalassemia patients or the suitable inhibition procedures. Methods for inhibition or management of alloimmunization are under discussion. They variety from providing of RBCs matched for all the major antigens associated with clinically significant antibodies to blood matched only for antibodies that have already been made. Aims for controversy as to the best approach lay in the fact that numerous alloantibodies are not harmful, and costly inhibition procedures may therefore benefit only some patients.[13-15] Conventional therapy is transfusion life-long regular blood which reduces the severe complications of anemia, maintain growth and increase the survival.[8,9,16,17] However, transfusion will seek out the complications includes iron overload, infections and alloantigens and autoantibodies formed against antigens from red blood cells.[5,6,17] Repeated blood transfusion in these patients, different antigens on red blood cells inter into the patient's body and stimulate the patient's immune system to produce alloantibodies and autoantibodies. Since when choosing a transfusion blood just ABO and RH groups controlled, most alloantibodies formed against other blood group systems which cause delayed hemolytic reactions and limits further safe transfusion and cross-matching is also faced with the problem. Some alloantibodies also are clinically insignificant.[5,9,18] Knowing the side effects of blood transfusion, is a major problem in the treatment of patients with thalassemia. The purpose of this study was to determine the prevalence of alloimmunization and erythrocyte autoimmunization among thalassemia patients who received regular transfusions in Ilam province of Iran.

Materials and Methods

After obtaining consent from all patients, clinical and transfusion informations of 110 transfusion dependent thalassemia patients (range: 10-35 years) who received regular transfusions were studied. The transfusion informations of all patients were studied for the presence of alloimmunization or autoimmunization, and antibody specificity. 110 patients who initially had negative results for RBC Alloantibodies/autoantibodies, were enrolled in the study. All patients were questioned and filled a questionnaire comprising of kind of thalassemia, date of the first blood transfusion, transfusion blood type (standard, washed, leucoreduced), hematocrite/hemoglobin level, reticulocyte count, direct and indirect Coombs test, total and direct bilirubin, hemoglobinuria and the type of alloantibody, as well as signs and symptoms like infection, dyspnea, chill, jaundice and pain (Table 1).

Sample from patients who received regular transfusions was drained in tubes containing acid citrate dextrose 21 days after the patients received a packed RBC transfusion and just before their next transfusion. Blood group typing done by Anti-A, Anti-B anti-D reagent (Iranian Blood Research and Fractionation Holding Company, Tehran-Iran) in Tube test procedure for each patient. Prior to each transfusion for detection of new Alloantibodies/autoantibodies to RBC antigens, serum was analyzed by means of standard blood bank procedures. The serum was mixed with saline-suspended red cells with the addition of low ionic strength saline (LISS) and incubated at 37°C for 15 minutes. Alloantibody screening and identification was done using 3-cell antigen panel in Ilam blood transfusion organization (Iran blood transfusion organization, Iran, Lot No: 119405), and an anti-IgG reagent was used for the antiglobulin phase (synagen, Lot No: m4051). The panel test which determines 18 blood groups for the following antigens "D, C, E, e, c, K, k, M, N, S, s, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b and P". Briefly, four drops of patient's serum add to be tested to each labeled tube. Then, two drop of thoroughly mixed reagent RBC add to the appropriate labeled tube. Subsequently, two drops of Albumin 22% added to each tube (Refining and blood research, Lot No: 89.BSA05). After mixing the contents of each tube, incubate all tubes at room temperature and 37ºC (±1ºC) for 15-30 minutes. Then proceed directly with the antiglobulin test phase following incubation. Wash tubes a minimum of 3 times with isotonic saline. Completely decant saline after final wash to obtain a "dry" red cell button. Add two drops anti-IgG to each tube. Centrifuge tubes in 3000 RPM for 1-3 minute. Immediately re-suspend the cells by gentle agitation; examine the tubes macroscopically for agglutination. If the screen was positive, an extended panel to recognize the antibody and a direct antiglobin (DAT) was implemented. Absorption techniques were employed in patients presenting with a new autoantibody. In cases of a positive DAT, additional study using specific reagents to detect IgG, IgM, or a complement were approved according to manufactory method in room temperature and 37C. When an antibody was detected, eluates were prepared and confirmed against common sample RBCs. polyethylene glycol was used to enhance the reactivity.

Table 1: Post-transfusion clinical and laboratory investigations in thalassemia patients with alloimmunization.

Post transfusion assessments	Percent
Positive direct coombs test	82
Increased reticulocyte count	58
Hemoglobinuria	52
Headache	63
Backache	66
Jaundice	63
Chill	62
Infection	42
Dyspnea	41

Results

There were 48 (43.7%) females and 62 (56.3) males. The mean age of patients was 22.5 (1SD±9.5). The mean age for the first blood transfusion was 5 (1SD±3.6) months for thalassemia major. 12 patients out of total 110 patients (10.9%) developed alloantibodies and two (1.81%) developed autoantibodies. The identified alloantibodies contains: D, C, E, c, K, Jk^a, Fy^a and Le^a (Table 2).

Table 2: Prevalence of alloantibodies in patients

Blood Groups	Number (%)
RH system	7 (58.3)
D	4 (33.4)
C	1 (8.3)
E	1 (8.3)
c	1 (8.3)
Kell system	2 (16.6)
Kidd system	1 (8.3)
Duffy system	1 (8.3)
Lewis system	1 (8.3)

Anti-D and anti-Le^a, was the most prevalent clinical significant, and non-significant antibodies, respectively. There was not any significant relationship between sex and prevalence of alloantibody ($p > 0.05$). There was not any significant relationship between age and prevalence of alloantibody ($p > 0.05$) (Table 3).

Prevalence of alloantibody in this study was 10.9% (12 patients) and 89.1% (98 patients) showed no alloantibody in their serum. Of 12 alloimmunized patients, 7 patients (11.3% of male patients) were male and 5 patients (10.4% of female patients) were female. The identified autoantibodies contains: P and N. Anti-P and anti-N were not clinical significant. Results of this study indicate low frequency of RBCs alloimmunization. The incidence of alloimmunization according to blood group was not confirmed. The alloantibody of 4 patients (33.33%) was recognized within six days of the transfusion; of 3 (25%) within ten days; of 2 (16.66%) within Thirty days; of 2 (16.66%) within 35 days and 1 (8.3%) within three months. The autoantibody of 1 patient (50%) was recognized within two months of the transfusion and 1 (50%) within four months. The highest titer was 1:128 (for anti-D) and lowest titer was 1:8 (for anti-Jk^a). There was no significant correlation between alloimmunization rate and patients who regularly used washed/leukoreduced packed cell. Out of 12 patients with alloantibodies 10 patients were found with two antibody, and 2 had three antibodies. The antibody screening panel test was positive in 39 patients (35.45%). There was not any significant relationship between sex and prevalence of autoantibody ($P>0.05$). There was not any significant relationship between age and prevalence of autoantibody ($P>0.05$) (Table 4). For patients who were on iron chelator (desferrioxamine), the pre-transfusion hemoglobin level was less than 8 gm/dl and post-transfusion hemoglobin level was 13 gm/dl. Patients who were not on iron chelator, the pre transfusion hemoglobin level was less than 8 gm/dl and post transfusion hemoglobin level was 11 gm/dl.

Patients with O negative blood group were the most frequent alloimmunized subjects, while patients with B negative and AB negative blood groups had the lowest frequent of alloimmunized subjects. Nevertheless, no significant risk or safety was well-known by the OR/p-value; the existence of alloimmunization in relation to blood group was not confirmed (Table 5).

Discussion

In our study, prevalence of alloantibody was 10.9%. The most prevalent antibody was against D antigen. Transfusion of red blood cells (RBCs) is a public method in the management of patients with major thalassemia for two reasons: (1) transfusion of packed cells increases the oxygen-carrying capacity of the blood in the patients with major thalassemia , and (2) the replacement of the non-functional RBCs with functional ones may improve the symptoms or avoid the complications of the disorder. Alloimmunization against blood groups occurs following transfusion, pregnancy and transplantation. In patients who are transfused regularly such as thalassemia and sickle cell anemia patients the frequency of alloimmunization is high.[19] The rate of alloimmunization in the other parts of Iran has been reported as following: 7.4% (Tehran),

Table 3: Correlation between sex and age with prevalence of alloantibody

Characteristics	Total	Non-alloimmunized		Alloimmunized		OR (95% CI)	P value
		n	(%)	n	(%)		
Number of Patients, (%)	110	98	(89.1)	12	10.9))	-	-
Age 8-19 years	73	66	(90.41)	7	9.58))	0.909 (0.309-2.495)	0.999
≥20 years	37	32	(86.48)	5	(13.51)		
Sex						0.925 (0.318-2.582)	0.999
Male	62	55	(88.7)	7	11.3))		
Female	48	43	(89.6)	5	(10.4)		

Fisher's Exact Test; OR: Odds Ratio

Table 4: Correlation between sex and age with prevalence of autoantibody

Characteristics	Total	Non-autoimmunized		Autoimmunized		OR (95% CI)	P value
		n	(%)	n	(%)		
Number of Patients, (%)	110	108	(98.18)	2	(1.81)	-	-
Age 8-19 years	73	73	(100)	0	-	0.800 (0.056-11.512)	
≥20 years	37	35	(94.6)	2	(5.40)		1.000
Sex						0.800 (0.056-11.512)	1.000
Male	62	62	(100)	0	-		
Female	48	6	(95.9)	2	(4.1)		

Fisher's Exact Test; OR: Odds Ratio

Table 5: Incidence of post-transfusion alloimmunization in patients with major thalassemia in relation to the ABO/RhD blood group

Blood group	Total	Non-alloimmunized		Alloimmunized			OR (95% CI)	P value
		n	(%)	n	(%)	Antibodies		
O positive	42	37	88	5	12	Anti-C Anti-K Anti-Lea Anti-Fya	0.701 (0.102-4.989)	0.826
O negative	13	9	70	4	30	Anti-D (x 3) Anti-K	0.356 (0.051-2.098)	0.124
A positive	16	15	94	1	6	Anti-c	0.413 (0.401-1.950)	0.928
A negative	7	6	86	1	14.3	Anti-D	0.572 (0.015-11.201)	0.999
B positive	19	17	89.5	2	10.5	Anti-K Anti-E	0.345 (0.078-8.107)	0.819
B negative	4	4	100	0	0	-	0.417 (0.008-3.207)	0.999
AB positive	7	6	86	1	14.3	Anti-JKa	0.978 (0.008-0.189)	0.0504
AB negative	2	2	100	0	0	-	0.347 (0.123-1.206)	0.0321

Fisher's Exact Test; OR: Odds Ratio

5.34% (Fars province), 18.7% (Southwest Iran) and 2.87% (Northeast Iran).[7,19] Furthermore, the alloimmunization frequency in other countries is 30% (Kuwait), 19.5% (Egypt), 7.4% (Hong Kong), 5% (Italy), 8% (India), and 3.7% (Greece).[7,18,20-22] Compared with Kuwait and Egypt, Results of this study point out low incidence of RBCs alloimmunization. This low alloimmunization frequency indicates that there is homogeneity of red cell antigens in blood donors & recipients. The antibody screening panel test was positive in 45 patients (40.9%). In another study in Southwest Iran (Ahvaz), the antibody screening panel test was positive in 42 patients (32.06%).[19] Also, two patients (4.1%) had autoantibody, while in another study in Southwest Iran (Ahvaz) was 12.7%.[19] Keikhaei, B., et al. Showed that the predominant pattern of alloimmunization was alloantibodies against RH sub-groups system in 55 percent of patients and 33% of patients had alloantibodies against Kell system.[19] In this study, we demonstrated that the predominant pattern of alloimmunization was alloantibodies against RH system (D, C, E and c) in 58.3 percent of patients and 16.6 percent of patients had alloantibodies against Kell system. One cause for the high incidence of anti-D among patients may mostly be due to lack of enough knowledge and education about weak D, partial D and Del• phenotypes in blood bank staff. So it is necessary for blood bank professionals and other healthcare employees involved in blood transfusion, to be well informed about these phenotypes. The D antigen is composed of numerous epitopes (designated by "epD"). Subsequent studies with monoclonal antibodies defined 30 or more epitopes designated as "epDl" to "epD9".[23] Amino acid changes in intracellular regions of the protein may alter D epitopes. Altered D is organized into four groups: weak D, partial D (including category D), Del• and nonfunctional RHD.[24-27] One of the most important reasons for alloimmunization is the transfusion of some red blood cells with rhesus D incompatible with thalassemia patients due to false negative results in D typing of blood donors. Traditionally, weak D red cells were defined as having a reduced amount of D antigen (formerly called "D^u") that required an indirect antiglobulin test (IAT) for detection. Weak D types are the result of an SNP that encodes a single amino acid change predicted to be located in the intracellular or transmembrane region of the protein, rather than on the outer surface of the red cell. The amino acid changes may affect the insertion of the protein in the membrane and, thus, reflect the reduced number of D antigen sites on the red cells.[25] A weak D phenotype can occur with many partial D phenotypes, Ce in trans with suppression of RHD, in the Rh_{mod} phenotype, and via autosomal recessive inheritance of two weak RHD alleles. The latter accounts for the majority of weak D phenotypes present in the general population. Because all the mutations are intramembranous or intracellular, it is assumed they do not significantly alter the presentation of D epitopes on the extracellular loops. This may explain why most weak D individuals do not make alloanti-D when transfused with D positive blood.[25,28] Partial D antigens are RHD proteins with missing D epitopes. Although they type as D-positive, persons with partial D antigens can make alloanti-D antibodies reactive with allogeneic, but not autologous, RBCs. The alloanti-D produced by these individuals recognizes D-specific epitopes missing on their own RBCs. In contrast to weak D types, partial changes are predicted to be located on the exterior membrane surface. Some partial D types are detected only by the IAT. Red cells that express extremely low levels of D antigen that cannot be detected by routine serologic methods are designated as "D-elution" or D_{el}. Del cells are found in 10% to 30% of D-negative people of Asian ancestry. It is worth noting that the identification of waek D phenotype in blood recipients is not necessary because they do not develop anti-D, after receiving D-positive blood. But partial D phenotypes need to be identified because transfusion of D-positive blood to partial D subjects will lead to alloimmunization. Hence, it is recommended to consider a separate refrigerator for storing products supposed to be given to patients with major thalassemia. We also recommend to do following experiments on the blood that is supposed to be transfused to patients with major thalassemia:

1- Using anti-D reagents combine a monoclonal IgM, which causes direct agglutination at room temperature, with a monoclonal or polyclonal IgG that is reactive by

IAT for the determination of weak D.

2- Typing Donors for D: The aim of D typing of RBC donors, including the identification of units with weak D or partial D types, is to prevent anti-D immunization of recipients. A unit labeled Rh negative must be confirmed by testing an integrally attached segment before transfusion. Weak D testing is not required. The RhD type of the units labeled Rh positive do not require confirmatory testing.

3- Typing Patients for D: When the D type of a patient is determined, a weak D test is not essential except to assess the red cells of an infant whose mother is at risk of D immunization. Monoclonal IgM reagents type numerous samples as being D positive in direct testing that would have previously been identified only by IAT. Consequently, some of the concerns regarding the unnecessary use of Rh-negative blood and RhIG have been addressed.

4- D typing discrepancies should always be inspected and resolved. An Rh-negative blood transfusion is an appropriate option for patients needing immediate transfusion, but a thorough clerical and serologic investigation should be performed. *RHD* genotyping is also useful to resolve D typing discrepancies. Because donor centers test for weak D, a donor who is correctly classified as Rh positive may be classified as Rh negative as a recipient. This discrepancy should not be considered problematic but, rather, should be communicated to the patient and health-care staff and be noted in the patient's medical record.

5- Inappropriately, serologic reagents cannot certainly be used to differentiate persons with partial D that is reactive only with improved techniques and procedures from D-positive persons. Many partial D red cells type as strongly D positive in direct tests and are known only after the patients produce anti-D. Guidelines regarding D typing techniques and choice of blood components for transfusion should be based on the patient population, risk of immunization to D, and limited supply of D-negative blood components. These guidelines should statement what should be done when a partial D type is found before the patient makes anti-D. Anti-D is a clinically significant antibody, and preventing immunization in females of childbearing potential is important to avoid the complications of HDFN.

6- RH genotyping: RH genotyping is an effective attachment to serologic testing for the typing of transfused patients, RHD zygosity determination, noninvasive fetal *D* typing, determination of D status, and identification of antigen-matched blood for patients with major thalassemia.

7- Typing multi-transfused patients: In patients receiving chronic or massive transfusions, the existence of donor red cells in the peripheral blood often makes red cell phenotyping by agglutination incorrect or problematic. Genotyping overcomes this restriction because blood grouping can be determined with DNA prepared from a blood sample, though the sample was collected after transfusion.

8- D-negative, first-time donors are screened for RHD to detect red cells with very weak D.

By several factors, alloimmunization is limited in the traditional practice of transfusion of blood. Many of the antigens present on RBCs uncommonly give rise to alloimmunization even when injected into patients lacking the antigen.[29] The factors for alloimmunization are complex and involve at least 3 main contributing features: The RBC antigenic difference between the blood donor and the recipient; the recipient's immune status; and the immunomodulatory effect of the allogeneic blood transfusions on the recipient's immune system. A low rate of alloimmunization may be expected when there is homogeneity of RBC antigens between the blood suppliers and recipients.[18] The ability to respond to alloantigens differs importantly from person to person. Some individuals will not become immunized to any antigens in spite of continuous transfusion, whereas others will become immunized, when transfused, to many of the antigens that they lack.[30] Blood banks will provide enough compatible blood for patients with thalassemia. Ameen et al. have reported Anti-K in 72% of patients and anti-E in 45.6%[31] and in our study Anti K, anti-E, anti-C, anti-c and anti D were found in 16.6%, 8.3%, 8.3%, 8.3% and 33.4 of patients respectively. In another study, Karimi et al. detected high prevalence of antibodies against Rh system (47.7%).[32] In Europe and the United States, the most commonly reported alloantibody in alloimmunized patients were alloantibody against Rh and Kell antigens. In a study in Minnesota, also anti-E and anti-K had higher rate than the other.[33] RBCs alloantibody formation was not influenced by gender, age at start of transfusions and number of packed cells received.

Conclusion

This study suggests screening RBC antigens prior to transfusion. Our findings accentuate the necessity of antigen typing of supposed to be transfused red blood cells and screenings tests before the first transfusion, at least for Rh (Rh system) and Kell (Kell system) antigens.

Conflict of Interest: None declared.

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