The Effect of HLA-DRB1 Sharing between the Couples with Recurrent Pregnancy Loss on the Pregnancy Outcome after Leukocyte Therapy

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ABSTRACT

Background: Miscarriage is a common phenomenon complicating more than half of pregnancies. Recurrent Pregnancy Loss (RPL) is defined as three or more pregnancies lost before the twentieth week of gestation. It is believed that abnormality in maternal immune reaction to fetus and sharing of HLA antigens might be associated with RPL. **Objective:** To investigate the effect of HLA-DRB1 sharing between the couples with recurrent pregnancy loss on the pregnancy outcome after leukocyte therapy. **Methods:** Sixty primary RPL women who were immunized and followed after therapy (30 successful and 30 unsuccessful) and their husbands formed the cases of this study. In addition, one hundred healthy women were considered as the controls. HLA-DRB1 genotypes of all the cases and controls were checked by PCR-SSP method. **Results:** HLA typing indicated that the prevalence of HLA-DRB1 sharing (defined as at least one allele sharing) between the couples with unsuccessful outcomes was significantly higher compared to those with successful outcomes (63.3% vs. 23.3%, $p<0.004$). Moreover, HLA DRB1*07:01 allelic group was significantly more frequent in the patients with unsuccessful outcome compared to the controls (18.3% vs. 8%, $p<0.04$). **Conclusion:** Our results confirmed the role of HLA sharing in RPL and revealed that HLA-DRB1 typing may be a valuable prognostic factor for the leukocyte therapy outcome.

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INTRODUCTION

Traditionally, Recurrent Pregnancy Loss (RPL) which affects about 1-2% of all pregnancies is defined as three or more pregnancies lost before the twentieth week of gestation (1). The percent of the affected couples will increase to 5% by using the recent classification that labels pregnancy loss as recurrent with only two or more sequential abortions (2). Although the etiology is unknown in the vast majority of cases, it is thought that reaction of maternal immune system against the semi-allogenic fetus may contribute to RPL. Several studies have highlighted the role of immunological disturbances as the major cause of RPL. Indeed, in the pregnancy period, in contrast to an allogenic transplant, maternal immune system must tolerate the fetus for nine months. Medawar for the first time described this discrepancy (3). During the pregnancy period, maternal immune system is in close contact with fetal antigens. Besides, maternal immune cells are able to recognize the semi-allogenic fetal antigens in all steps of a pregnancy from mating until delivery. In contrast to the old hypothesis emphasizing that "maternal immune system must be silent to the semi-allograft fetus to maintain the pregnancy", recent evidences have indicated that maternal immune system actively recognizes and responds to the fetal antigens during the pregnancy period. Indeed, the maternal immune system in the fetal–maternal interface deviates the immune system toward a protective and tolerogenic response (4). In response to an allograft transplant, on the other hand, the immune system is activated in a way that rejects the allograft (4). Despite the controversy about its effectiveness, leukocyte therapy is still used for treatment of a group of RPL women in several countries with very divergent success rates. In some trials, the efficiency of paternal leukocyte therapy has been reported to be about 75%, while other studies have demonstrated a treatment effect of less than 38% (5-10). Another issue requiring attention is the fact that heterogeneity in the inclusion criteria for selection of candidate women is one of the major sources for divergent results.

The exact mechanisms of leukocyte therapy with paternal leukocytes are yet to be elucidated. Nonetheless, it seems that injection of leukocytes, with an antigenic structure similar to that of trophoblast cells, activates the maternal immune cells in a way to tolerate the fetus (8).

To date, there is no reliable guideline for selection of the candidates or tests for evaluating the results of leukocyte therapy. Human Leukocyte Antigen (HLA) molecules are among the most polymorphic molecules in the immune system that direct the immune responses against allo-antigens. While HLA incompatibility is accounted as the main reason for allograft rejection, HLA sharing between the couples may affect the pregnancy outcome (11). Previous studies have indicated that certain HLA molecules, including HLA-I and HLA-II alleles, might be associated with the susceptibility to RPL (12-15). HLA-DR is the most polymorphic molecule among HLA-II antigens. Although few studies have directly assessed the relationship between HLA-DR sharing and RPL (16,17), the relationship between the effectiveness of leukocyte therapy and HLA-DR sharing has not been studied yet. Thus, the present study was conducted on a group of women with RPL in our center to evaluate the effect of HLA-DRB1 sharing between the RPL couples on the outcome of leukocyte therapy.

MATERIALS AND METHODS

Subjects. The present study cases included sixty primary RPL women between 22 and 38 years old who were immunized and followed after therapy and their husbands. Half of the cases were selected among the RPL couples with positive outcomes (live birth after therapy), while the remaining 30 were selected from those who did not benefit from leukocyte therapy and experienced another abortion before the 20^{th} week of gestation after therapy. Diagnosis of RPL was based on the clinical and laboratory findings. All the patients and their previous aborted fetuses had a normal karyotype pattern report. All the RPL patients were evaluated for normal clinical criteria by a gynecologist using laboratory tests to rule out the presence of anti-phospholipid antibodies (including anti-cardiolipin, lupus anticoagulant, and β2-glycoprotein antibodies) as well as anti-thyroid antibodies. Moreover, the control group included one hundred healthy women with at least two previous successful pregnancies without any history of pregnancy disorders, autoimmune diseases, and cancer. The cases and controls were age and ethnicity matched and was selected from the same geographical area (Fars province, southwest of Iran). Written informed consents were obtained from all the participants and the study was approved by the local Ethics Committee of Shiraz University of Medical Sciences.

Immunotherapy. All the patients were immunized with the same protocol as previously described (9,10). Briefly, approximately 50-100 million washed and re-suspended mononuclear cells were injected by I.V, S.C, and I.D route. Before the therapy, all the women and their husbands had negative WBC results (checked by serological method). The result of each injection was assessed by WBC cross matching between the couples after four weeks of injection. Immunization was also repeated in the fifth week to a maximum of 3 times if needed.

HLA-DRB1 Typing. DNA was extracted from 200 µl of whole peripheral blood by a column based extraction method using GenetBio extraction kit (Korea). Besides, HLA-DRB1 typing was performed using PCR-SSP method at low/intermediate resolution level (BAG healthcare Lich, Germany).

Statistical Analysis. All the statistical analyses were carried out using SPSS, version 17 for Windows (SPSS Inc., Chicago, IL, USA). Chi-square test with Yates correction or Fisher Exact test was used for comparison of the results. In addition, the couples were considered as HLA-DRB1 share when at least one HLA-DRB1 allele was shared between a woman and her husband. P values (two-tailed) less than 0.05 were considered as statistically significant.

RESULTS

HLA typing indicated that the prevalence of HLA-DRB1 sharing (defined as at least one allele sharing) was 43.3% among the RPL couples (26 out of 60). Interestingly, the HLA-DRB1 sharing between the couples with unsuccessful outcomes was significantly higher compared to those with successful outcomes (Table 1; 63.3% vs. 23.3%, OR=5.68, RR=2.71, 95% CI, $p<0.004$). Moreover, as shown in Table 2, the most frequent allelic group was HLA-DRB1*11 in the successful group (26.7%) and HLA-DRB1*15 in the unsuccessful group (21.7%); however, the difference was not statistically significant.

Table 1. Comparison of HLA-DRB1 allele sharing between the RPL couples.

	HLA-DRB1 Sharing[a]		
Outcome	**Positive Number (%)**	**Negative Number (%)**	**P Value**
Successful	7 (23.3)	23 (73.3)	<0.004
Unsuccessful	19 (63.3)	11 (36.7)	

[a]HLA-DRB1 sharing was defined as at least one allele sharing
* Calculated using Chi-Square test, OR=5.68 (1.62<OR<20.74), RR=2.71 (1.34<RR<5.48), X^2=8.21, P<0.004 (Yates corrected)

The frequency of HLA-DRB1*07:01 in the unsuccessful patients was about two times higher compared to the successful ones (18.3% vs. 10%); however, the differences was not statistically significant. No significant difference was observed between the successful and unsuccessful patients concerning the distribution rate of other allelic groups (Table 2).

Table 2. The relationship between HLA-DRB1 allele frequencies and pregnancy outcome in the RPL cases.

	Pregnancy outcome		
DRB1 allelic group	Successful Number (%)	Unsuccessful Number (%)	P value
*01:01	2 (3.3)	1 (1.7)	N.S
*03:01	3 (5)	1 (1.7)	N.S
*04:01	7 (11.7)	6 (10)	N.S
*07:01	6 (10)	11 (18.3)	N.S
*08:01	2 (3.3)	2 (3.3)	N.S
*09:01	0 (0)	1 (1.7)	N.S
*10:01	2 (3.3)	1 (1.7)	N.S
*11:01/02/03	16 (26.7)	10 (16.7)	N.S
*12:01	1 (1.7)	0 (0)	N.S
*13:01/02/03	5 (8.3)	6 (10)	N.S
*14:01	3 (5)	3 (5)	N.S
*15:01/02	7 (11.7)	13 (21.7)	N.S
*16:01	6 (10)	5 (8.3)	N.S

N.S= Not significant

Distribution of HLA-DRB1 allelic frequencies in RPL and healthy women is presented in Table 3.

Table 3. Distribution of HLA-DRB1 allele frequencies in the RPL cases and healthy women.

DRB1 allelic groups	RPL Number (%)	Control Number (%)	P value
*01:01	3 (2.5)	18 (9)	N.S
*03:01	4 (3.3)	21 (10.5)	N.S
*04:01	13 (10.8)	18 (9)	N.S
*07:01	17 (14.2)	16 (8)	<0.03*
*08:01	4 (3.3)	3 (1.5)	N.S
*09:01	1 (0.8)	2 (1)	N.S
*10:01	3 (2.5)	6 (3)	N.S
*11:01/02/03	26 (21.7)	44 (22)	N.S
*12:01	1 (0.8)	2 (1)	N.S
*13:01/02/03	11 (9.2)	9 (4.5)	N.S
*14:01	6 (5)	10 (5)	N.S
*15:01/02	20 (16.7)	24 (12)	N.S
*16:01	11 (9.2)	27 (13.5)	N.S

* Calculated using Chi-Square test, OR=2.36 (1.07<OR<5.19), RR=2.13 (1.12<RR<4.03), X^2=4.63, P<0. 03 (Yates corrected)
N.S= Not significant

As indicated in Table 3, HLA-DRB1*11 was the most frequent allelic group in both cases and controls (21.7% and 22%, respectively). In addition, the study results revealed a significant difference between the RPL patients and controls regarding the distribution of HLA-DRB1*07:01 allelic groups (Table 3). While 14.2% of the patients possessed HLA-DRB1*07:01 allele, the frequency of this allele was only 8% in the normal women (OR=2.36, RR=2.13, 95% CI, p<0.03). No other differences were found between the patients and controls concerning allelic distribution (Table 3). As indicated in Table 4, HLA DRB1*07:01 allelic group was significantly more frequent in the patients with unsuccessful outcomes in comparison to the controls (18.3% vs. 8%, OR=2.58, RR=2.29, 95% CI, p<0.04). Moreover, the frequency of HLA-DRB1*15 allelic group was higher while that of HLA-DRB1*01:01 and HLA-DRB1* 03:01 groups were lower in the patients with unsuccessful outcomes compared to the healthy controls with a trend toward a significant difference (p=0.09, p=0.09, and p=0.06, respectively) (Table 4). No significant difference was observed between the RPL patients with successful outcomes and the healthy controls regarding HLA-DRB1 allele frequency (data not shown).

Table 4. The difference between unsuccessful cases and healthy control women regarding HLA-DRB1 allele frequencies.

	Pregnancy outcome		
DRB1 allelic group	Control Number (%)	Unsuccessful Number (%)	P value
*01:01	18 (9)	1 (1.7)	<0.09
*03:01	21 (10.5)	1 (1.7)	<0.06
*04:01	18 (9)	6 (10)	N.S
*07:01	16 (8)	11 (18.3)	<0.04*
*08:01	3 (1.5)	2 (3.3)	N.S
*09:01	2 (1)	1 (1.7)	N.S
*10:01	6 (3)	1 (1.7)	N.S
*11:01/02/03	44 (22)	10 (16.7)	N.S
*12:01	2 (17)	0 (0)	N.S
*13:01/02/03	9 (4.5)	6 (10)	N.S
*14:01	10 (5)	3 (5)	N.S
*15:01/02	24 (12)	13 (21.7)	<0.09
*16:01	27 (13.5)	5 (8.3)	N.S

* Calculated using Chi-Square test, OR=2.58 (1.04<OR<6.36), RR=2.29 (1.13<RR<4.67), X^2=4.24, P<0. 04 (Yates corrected)
N.S= Not significant

DISCUSSION

Up to now, very few studies have directly assessed the effect of HLA-RDB1 sharing on the pregnancy outcome in RPL patients.

Christiansen *et al*. were among the first groups who studied the association between HLA antigens and PRL. They indicated that HLA-II antigens may predispose the Danish women to RPL (18). In the same line, Takakuwa *et al*. indicated a relationship between the possession of HLA-DRB1*1502 allele and susceptibility to RPL in a group of Japanese patients (12). Takakuwa and coworkers suggested that the compatibility of HLA class II antigens between the couples might be involved in occurrence of unexplained recurrent miscarriage (19). Kano *et al*. for the first time demonstrated the impact of HLA testing on prediction of the effectiveness of leukocyte therapy on the pregnancy outcome (16). The association between HLA-DRB1*03, HLA-DRB1*1501, and HLA-DRB1*1104 and RPL have already been reported (13-15). However, no reports have been published in this regard in Iran. Yet, the findings of the present study indicated that possession of HLA-DRB1*07:01 allelic group might predispose Iranian women to RPL (RR=2.13).

Although the association between possession of HLA-DRB1 alleles, including HLA-DRB1*03, 11, and 15, and susceptibility to RPL has been reported, this is the first report regarding the association between HLA-DRB1*07:01 and susceptibility to RPL. In line with a previous report from Japan, the frequency of HLA-DRB1*15 allelic group in our patients, especially in the RPL cases with unsuccessful outcomes, was higher compared to the normal controls and the RPL cases with successful outcomes; however,

this difference was not statistically significant (Table 4, $p<0.09$). The sample size and ethnicity of the patients both may be important reasons for this discrepancy. Moreover, the results of the present study indicated that the frequencies of two HLA-DRB1 allelic groups; i.e., HLA-DRB1*01:01 and HLA-DRB1*03:01, were lower among the Iranian RPL patients, especially those with unsuccessful outcomes with a trend toward statistical significance. This finding showed HLA-DRB1*01:01 and HLA-DRB1*03:01 as protective alleles in susceptibility to PRL and response to leukocyte therapy, a finding which is in need for confirmation in the future studies using larger sample sizes.
The most important finding of the present study was the correlation between HLA-DRB1 sharing in the RPL couples and the pregnancy outcome after leukocyte therapy. In the pregnancy period, the mother's immune system recognizes the fetus as foreign and activates in a way to regulate the immune responses to the semi-allograft fetus to maintain the pregnancy. This type of immune response is referred to as an allo-immune response (4). HLA molecules are among the most important antigens that activate allo-immune responses to the foreign antigens. When the father and mother share some of the same HLA antigens, allo-immune response does not take place properly thereby exposing the conceptus to a rejection process. It is believed that many repeated miscarriages and/or late pregnancy losses occur in such circumstances.
In conclusion, the results of this study suggest that the effectiveness of leukocyte therapy in RPL patients might be controlled at least in part by HLA-DRB1 sharing between the couples. Furthermore, HLA-DRB1 typing may be a valuable prognostic marker for the leukocyte therapy outcome.

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