Int J Clin Exp Pathol 2017;10(1):688-694
www.ijcep.com /ISSN:1936-2625/IJCEP0040646

*Original Article*

# The study of the prognostic value of mixed lymphocyte reaction blocking factor (MLR-Bf) for the pregnancy outcome after lymphocyte immunization in different kinds of patients with unexplained recurrent spontaneous abortion

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Received September 12, 2016; Accepted September 28, 2016; Epub January 1, 2017; Published January 15, 2017

**Abstract:** The study was conducted to access the prognostic value of mixed lymphocyte reaction blocking factors (MLR-Bf) for the pregnancy outcome after lymphocyte immunization (LIT) in different kinds of patients with unexplained recurrent spontaneous abortion (URSA). Following LIT, the patients were divided into two groups according to the time when the miscarriage occurs (group 1: before the demonstration of embryonic cardiac activity; group 2: after the demonstration of embryonic cardiac activity) and the times of URSA (patients with two URSA and three or more URSA). We respectively compared the success rate in immunized patients who showed MLR-Bf with control in each group. For patients with two URSA, there was no significant difference between treatment group and control in each group; for patients with three or more URSA, the pregnancy outcome was significantly improved in treatment group in group 1 (67.2% *vs* 53.1%, *P*=0.0004). Whereas no significant difference was detected in group 2 (60.3% *vs* 56.6%, *P*=0.5684). The obtained data demonstrated that the presence of MLR-Bf is a good prognostic criterium for the pregnancy outcome in patients with three or more URSA who had never had the demonstration of embryonic cardiac activity after LIT.

**Keywords:** MLR-Bf, habitual abortion, immunotherapy

## Introduction

Recurrent spontaneous abortion (RSA) is a common complication of pregnancy affecting about 1-3% couples [1], which is defined as two or more clinical miscarriages documented by ultrasonography or histopathologic examination (not necessarily consecutive) by the American Society for Reproductive Medicine (ASRM) [2]. Factors such as genetic impairment, structural or functional abnormalities in genital organs, hormonal deficiency, infectious disease, metabolic disorder, autoimmune abnormalities, abnormal pre-thrombotic state are thought to be associated with the RSA [3]. However, in most women who suffered from RSA, no cause can be identified. The factors inducing miscarriage are still unclear. Alloimmune mechanisms have been proposed as the cause of some or all of these losses which prevent mothers from developing immunological responses essential for the survival of the embryo.

The embryo is thought to be a semi-allograft to the mother, so the immunological recognition of paternal alloantigen is critical for the maintenance of the fetus, inadequate recognition of fetal antigens might lead to the abortion of the women with RSA [4, 5]. For decades, on the basis of studies of human organ transplant survival and animal models of abortion [6-8], LIT has been employed as a popular treatment for unexplained recurrent spontaneous abortion (URSA). Some authors reported that this therapeutic approach can significantly increase the

rate of successful pregnancy [9-13], while some researchers considered it had no benefit at all [14, 15]. The results from clinical trials have been problematic [9, 10, 14, 15]. In previous studies, M LR-Bf is the most common used parameter for monitoring the treatment effectiveness of URSA in the world. While the prognostic value of it was controversial [14, 16, 17].

Before an effective treatment can be instituted, the reason of RSA must be determined. The causes for RSA of different gestational weeks are various. The first trimester miscarriage is hypothesized that this may due to chromosomal abnormalities, endocrinological disorders, immunological abnormalities and abnormal pre-thrombotic state. The main reasons for miscarriage occurring between 12-28 weeks includes abnormal pre-thrombotic state, infections, abnormal fetal appurtenances and so on. So in our study, to investigate the efficacy of LIT for URSA, we selected patients who had miscarriages in first trimester. The different time when the miscarriage occurs indicate that they may have different causes. In previous studies, we noticed that no one has evaluated the efficacy of LIT by dividing the patients into different groups according to the time when the miscarriage occurs, which may suggest the presence of specific cause. So in our study, we classified the patients into the following two groups according to the time when the miscarriage occurs: group 1: before the demonstration of embryonic cardiac activity; group 2: after the demonstration of embryonic cardiac activity. At the same time, as is known that the success rate of untreated patients with only two URSA is very high, the patients involved in each group were divided into two subgroups for detailed analysis. The first group consisted of women with only two URSA, the second one included women with three or more URSA. We respectively compared the success rate in immunized patients who showed positive MLR-Bf with control in each group, and reassess the prognostic value of MLR-Bf.

## Materials and methods

### *Patients*

A total of 1993 patients were recruited at our center between July 2007 and July 2015. The selection criteria were: 1. Each participant cohabiting with a single partner had experienced two or more confirmed first trimester (i.e.before 14 weeks of gestation) miscarriages that were not of chromosomally abnormal fetuses; 2. No MLR-Bf detected; 3. Age 40 years or younger at the time of recruitment; 4. Each participant's partner was negative for hepatitis B surface antigen (HbsAg), human immunodeficiency virus (HIV), Hepatitis C Virus (HCV) and syphilis; 5. No identifiable causes for the previous miscarriages including chromosomal abnormalities, structural functional abnormalities in genital organs, endocrinological disorders. Our study center had approval from the institute ethical committee, and all patients gave their consent to participate in the study.

### *Design and procedures*

According to the time when the previous miscarriage occurs, the patients were divided into the following two groups: group 1: before the demonstration of embryonic cardiac activity; group 2: after the demonstration of embryonic cardiac activity. URSA patients had underwent two or more miscarriages, so some patients might have a complicated pregnancy history, for example one patient's first miscarriage occurred after the demonstration of embryonic cardiac activity, second miscarriage occurred before the demonstration of embryonic cardiac activity; these kinds of patients were excluded from the investigation. The sufficient information of the effect of LIT was given to every participant. The patients who request LIT were considered to be the treatment group. Whereas the other patients who did not desire it were considered as controls. In this study, no other concomitant therapies for recurrent miscarriage were used. Then we counted the patients who gave birth to normal healthy children.

### *Analysis of chromosome of aborted villi*

Chromosomal analysis of aborted villi of the patients were conducted by G-banding karyotyping and array comparative genomic hybridization (array CGH).

### *G-banding karyotyping*

Conception tissues were transferred to a sterile glass container, after maternal tissue was removed, 15-30 mg chorionic villi were selected to add into 4-5 ml RPMI 1640 medium at

**Table 1.** Characteristics of Patients in the treatment group and control in each group

| | | No of patients | Age (mean, SD, range) | No of abortions (mean, SD, range) |
|---|---|---|---|---|
| Group 1 | Treatment | 387 | 30.6 (5.83, 19-40) | 3.4 (0.83, 2-6) |
| | Control | 364 | 30.3 (5.86, 19-40) | 3.2 (0.86, 2-6) |
| Group 2 | Treatment | 170 | 31.0 (5.50, 19-40) | 3.3 (0.89, 2-6) |
| | Control | 138 | 30.9 (5.43, 20-39) | 3.4 (0.80, 2-5) |

There were no significant differences of the age and number of spontaneous abortions in group 1 and 2.

37°C, in a 5% $CO_2$ incubator. They were harvest one week later. Metaphase chromosomes were prepared according to standard cytogenetic protocols. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature 2013 (ISCN 2013).

*Chromosomal copy number analysis by array CGH*

Conception tissues were rinsed in normal saline solution for three times. Then 10 mg of the tissue was selected to obtain genomic DNA (Tiangen, China). Genomic DNA samples were fluorescently labelled and competitively hybridized to CytoChip Focus Constitutional microarrays (Illumina, USA) with a normal male control gDNA in an array CGH experiment format. A laser scanner InnoScan w710AL (Innopsys, France) was used to excite the hybridized fluorophores and read and store the resulting images of the hybridization. Scanned images were then analyzed and quantified by an algorithm with fixed settings in BlueFuse Multi Software (Illumina, USA) (available protocol at www.cytochip.com).

*The detection of MLR-Bf*

The detection of MLR-Bf was conducted by the method provided by Khonina NA [16].

*Lymphocyte immunotherapy*

Twenty mL fresh peripheral blood was drawn from the blood donor, then peripheral blood lymphocytes were obtained by density gradient centrifugation. After two washing steps (using normal saline), lymphocytes were suspended in 2 mL normal saline and was injected intramuscular on the upper arm for 6 sites. The immunization was carried out once every three weeks for five times. MLR-Bf was measured 2 or more weeks after the last immunization. Then they were advised to conceive within 12 months. In each groups, the failed ones whose fetus chromosome were abnormal or unsuccessful were excluded from the study.

*Statistical analysis*

A t test was performed to analyze whether there is a significant difference in the age and the number of spontaneous abortions between the treatment group and control in each group. Success rates were compared by chi-squared analysis. Values of $P<0.05$ were considered to be statistically significant.

## Results

There were no significant differences of the age and number of spontaneous abortions between the treatment group and control in group 1 and group 2 (**Table 1**).

The patients recruited were shown in **Figure 1**. Among 1993 URSA patients, 1025 had pregnancy history without demonstrated cardiac activity (group 1), 456 had pregnancy history with the demonstrated cardiac activity (group 2), 512 had a complicate pregnancy history (group 3). The details of the patients are in shown in **Figure 1**. In group 1, following LIT, 387 showed positive MLR-Bf (we had excluded the ones with abnormal karyotypes). In group 2, 170 showed positive MLR-Bf (we also had excluded the ones with abnormal karyotypes).

*Comparison of success rate in treated patients with positive MLR-Bf and control in group 1 and 2*

As shown in **Table 2**, the pregnancy outcome was significantly improved in the treatment patients with positive MLR-Bf than control in group 1 (68.5% vs 56.6%, $P=0.0008$). Unfortunately, no statistically significant differences were detected in group 2 (62.4% *vs* 59.4%, $P=0.5997$).

*Comparison of success rate of patients with two URSA in each group*

For patients with two URSA, patients with positive MLR-Bf showed no significantly higher success rate as compared to control (**Table 3**).

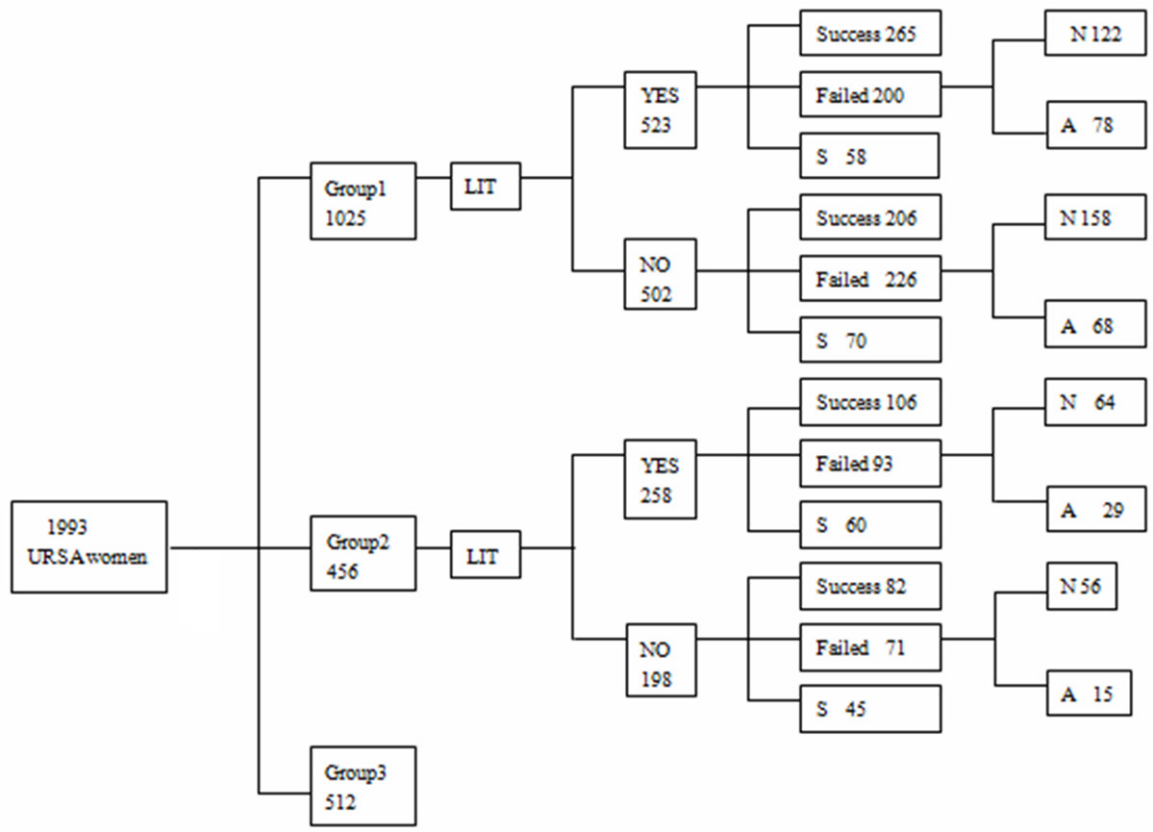


**Figure 1.** The details of patients. The 1993 URSA patients were dividing into three groups. In group 1, 265 immunized ones who showed positive MLR-Bf delivered healthy children, 122 aborted again. 206 unimmunized patients had improved pregnancy outcome, 158 did not. In group 2, 106 immunized ones with positive MLR-Bf had advanced pregnancy outcome, 64 did not. 82 unimmunized patients delivered healthy children, 56 did not. We had excluded the patients whose karyotype analyses were unsuccessful or abnormal; the immunized ones with negative MLR-Bf were also excluded from the study. S: Patients who were negative for MLR-Bf and did not get pregnant within 12 months. N: Patients who had normal chromosome karyotype. A: Patients who had abnormal or unsuccessful chromosome karyotype.

**Table 2.** Comparison of pregnancy outcome in group 1 and 2

| | | Success rate | P | OR | 95% CI |
|---|---|---|---|---|---|
| Group 1 | Treatment | 68.5% 387 (265) | 0.0008 | 0.6002 | 0.4454~0.8088 |
| | Control | 56.6% 364 (206) | | | |
| Group 2 | Treatment | 62.4% 170 (106) | 0.5997 | 0.8841 | 0.5580~1.4007 |
| | Control | 59.4% 138 (82) | | | |

The pregnancy outcomes were significantly improved in treatment group in group 1 (68.5% vs 56.6%, *P*=0.0008). Whereas no statistically significant differences were detected in group 2 (62.4% *vs* 59.4%, *P*=0.5997).

**Table 3.** Comparison of pregnancy outcome in patients with two URSA in group 1 and 2

| | | Success rate | P | OR | 95% CI |
|---|---|---|---|---|---|
| Group 1-1 | Treatment | 74.6% 67 (50) | 0.4719 | 0.7650 | 0.3683~1.5886 |
| | Control | 69.2% 78 (54) | | | |
| Group 2-1 | Treatment | 69.2% 39 (27) | 0.8082 | 0.8889 | 0.3432~2.3023 |
| | Control | 66.7% 39 (26) | | | |

There was no significant difference between the treatment group and control in patients with two URSA.

*Comparison of success rate of patients with three or more URSA in each group*

As is shown in **Table 4**, in patients who had underwent three or more URSA, we can see the pregnancy outcome was significantly improved in group 1-2 (67.2% *vs* 53.1%, *P*= 0.0004), but not in group 2-2 (60.3% *vs* 56.6%, *P*= 0.5684).

## Discussion

In our study, to investigate the prognostic value of MLR-Bf for URSA, we select-

**Table 4.** Comparison of pregnancy outcome in patients with three or more URSA in group 1 and 2

| | | Success rate | P | OR | 95% CI |
|---|---|---|---|---|---|
| Group 1-2 | Treatment | 67.2% 320 (215) | 0.0004 | 0.5540 | 0.3986~0.7699 |
| | Control | 53.1% 286 (152) | | | |
| Group 2-2 | Treatment | 60.3% 131 (79) | 0.5684 | 0.8572 | 0.5048~1.4557 |
| | Control | 56.6% 99 (56) | | | |

The success rates were significantly increased in treatment group in group 1-2 (67.2% *vs* 53.1%, *P*=0.0004), but not in group 2-2 (60.3% *vs* 56.6%, *P*=0.5684).

ed the URSA patients who had miscarriages in first trimester, we divided the patients into two different groups according to the time when the miscarriage occurs. Although we followed an identical protocol by adopting the same selection criteria, the same technical detail and the same immunotherapy procedure in the different groups, the outcome was still different in each group. In group 1, the success rates were significantly higher among immunized patients than the ones who did not request for it in group 1 (68.5% vs 56.6%, *P*=0.0008). Whereas no significant differences were detected in group 2 (62.4% *vs* 59.4%, *P*=0.5997). After we divided the patients into two sections according to the times of URSA in each group, we found that there was no significant difference in success rate between the treated and untreated patients with only two URSA in each group. However for patients who had underwent three or more URSA, the pregnancy outcomes were significantly improved in treatment group in group 1-2 (67.2% *vs* 53.1%, *P*=0.0004), but not in group 2-2 (60.3% *vs* 56.6%, *P*=0.5684).

Thus, the study can provide an useful clue to find an explanation for the previous conflicting results about LIT: they didn't divide the URSA patients into different groups according to the different status of the embryo when the miscarriage occurs [14-16]. Meanwhile, because of the definition of the URSA is different in different country, the patients involved in different study might be different. ASRM defines URSA as two or more clinical miscarriages documented by ultrasonography or histopathologic examination(not necessarily consecutive) [2], while Royal College of Obstetricians and Gynaecologists (RCOG) defines it as three or more consecutive pregnancy loss before 24 weeks of gestation [18]. There also may be other reasons for the discrepancy between their data and ours: 1. Lymphocyte cells they used were stored overnight, rather than freshly prepared cells, so the cells might lose the transfusion-related immunodulation, which can enhance the growth and survival of the feto-placental unit via CD200 [19, 20]. 2. Some people didn't use any criteria such as mixed lymphocyte reaction-blocking factor (MLR-bf), anti-HLA antibody, anti paternal lymphocyte antibodies (APLA) and antipaternal cytotoxic antibodies (APCA) for patient selection for immunotherapy, the treatment may be given to somebody who didn't need it at all [14]. 3. The procedure of the immunotherapy they adopted were different. Immunotherapy was performed only once or twice before pregnancy in most of the controversial studies, whereas in most successful studies, immunotherapy was performed three to four times or more at the regular intervals of 3 to 6 weeks [21], 4. The technical details of the treatment were different, such as the immunizing cell dose, number of treatment, route of lymphocytes delivery.

In former studies, there are many researchers used anti HLA antibody as the prognostic criteria for evaluating the efficacy of LIT. Previous studies showed that anti HLA antibodies are present in approximately 33% of normal successful pregnancies [22-24], however for patients with primary RSA, not more than 10% of them showed positive anti HLA antibody [25-27]. The difference fostered a hypothesis that a failure to produce HLA-antibodies was part of the underlying cause of URSA [25]. However, there is a disputable view that anti HLA antibodies in pregnant women are considered to be the consequences instead of causes of successful pregnancies [28]. Nowadays reduced MLR-Bf production is always considered to be a possible cause of URSA [16], and MLR-Bf is the most commonly used indicator for evaluating the efficacy of LIT in the world, while the prognostic value of it was controversial. So in our study, a study was conducted to explore the prognostic value of MLR-Bf for evaluating the efficacy of LIT. As we know, LIT is believed to promote a favorable environment for the paternal alloantigen-bearing embryos by modulating the immunity of the patients [29], as it could

provide the protect factors APCA and MLR-Bf which may protect the fetus from the toxic effect of the mother's immune system [5], reduce the NK cell activity [30] and perform the shift from Th1-type reactivity to Th2-type reactivity [31], but the precise mechanism of it is still under investigation. From my point of view, MLR-Bf was a kind of antibodies which emerged after LIT. The different numbers, cell doses and routes of the immunization brings different changes to the immune environment of the patients. In our study, following this immunizing cell dose, route and number, at the time the immunized ones developed MLR-Bf, the difference of the immune environment brought by the immunization can significantly improved the pregnancy outcome of the ones with three or more URSA who had never had the demonstration of embryonic cardiac activity, but not for patients with three or more URSA who had had the demonstration of embryonic cardiac activity and the ones with only two URSA.

In this study, although we followed an identical protocol in the different groups, the treatment effect was still different. Using MLR-Bf as the indicator that the patients could be ready for pregnancy, the pregnancy outcome was significantly improved in patients who suffer the miscarriage early in the pregnancy, but not in patients who had miscarriages later in pregnancy. In my opinion, there are two reasons responsible for it: 1. The inadequate protecting factors and the poor immune condition may be not the main reason for the miscarriage later in gestation. There may be other reasons responsible for the miscarriage. 2. Maybe there are other indicators for evaluating the efficacy of LIT in patients who had ever had demonstration of embryonic cardiac activity, which need further investigation.

In this study, the production of MLR-Bf is a good prognostic criterium for patients with three or more URSA who had never had the demonstration of embryonic cardiac activity, but not for patients with three or more URSA who had had the demonstration of embryonic cardiac activity and the ones with only two URSA.

## Acknowledgements

We thank the patients and the colleagues participating in the study. The study was accomplished in Medical Genetics Institute of Henan Provincial People's Hospital. This study was funded by the Medical Science Research Projects of Henan Province (No. 201303162); National Natural Science Foundation of China (NO. 81450018 and NO. 81501336).

## Disclosure of conflict of interest

None.

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