

Evaluation of failed immunotherapy among patients with negative APLA recurrent spontaneous abortion by serum anticardiolipin antibodies and mononuclear cell of Tim-1

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Abstract. – **OBJECTIVE:** The present study is aimed to analyze the expression of serum anticardiolipin antibody (ACA) and mononuclear cells of Tim-1 among the patients with negative anti paternal lymphocyte antibody (APLA) recurrent spontaneous abortion conducted by lymphocyte immunotherapy resulting in failure.

PATIENTS AND METHODS: 58 patients with negative APLA recurrent spontaneous abortion (RSA) who was diagnosed for the first time and received lymphocyte immunotherapy in our hospital were selected continuously. According to the therapeutic outcome, the patients were divided into success group with 31 cases and failure group with 27 cases. The positive rate of APLA and ACA in serum were compared, as well as the expressions of serum IL-6, IL-10, TNF- α , and IFN- γ were studied by the method of ELISA. Furthermore, the ratios of CD4+CD25+ regulatory T cells (Treg) were detected by flow cytometry, and mRNA expression levels of Tim-1 were tested by the fluorogenic quantitative RT-PCR method.

RESULTS: The positive rate of APLA after treatments of both the groups were compared, without any difference. The positive rate of ACA in failure group before treatment was higher than the data of after treatment, and the difference was statistically significant ($p < 0.05$). After treatment, serum IL-6 and IL-10 levels of two groups increased, TNF- α and IFN- γ levels were observed to be decreased in comparison with the data before treatment. Furthermore, the improvement in success group was greater than failure group, and the difference was statistical significant ($p < 0.05$). After treatment, Treg proportion of two groups increased compared with before, and the proportion of success group was greater than that of failure group; mRNA expression levels of Tim-1 in failure group before and after treatment were higher than that of success group, and the differences had statistical significance ($p < 0.05$).

CONCLUSIONS: High level of serum anticardiolipin antibody and mononuclear cell Tim-1 might lead to failed immunotherapy for the pa-

tients with negative APLA recurrent spontaneous abortion by influencing T lymphocyte immunity.

Key Words:

Anticardiolipin antibody, Tim-1, Negative APLA, Lymphocyte immunotherapy,

Introduction

Recurrent spontaneous abortion (RSA) refers to women with consecutive spontaneous abortions. There are multiple causes responsible for the above pathological state including corpus luteum insufficiency, genital tract malformation, chromosomal abnormality, microbial infections, uterus dysplasia, blood type mismatch between mother and infant, immune disorders and other uncertain factors¹. Further, genetic expression abnormality also plays an important role in the occurrence of RSA, with the observation indicating RSA and HLA immune response have some certain correlation. The positive rate of a closed antibody that is also known as anti paternal lymphocyte antibody (APLA) of patients is low, is the most prominent manifestation of immunologic derangement². Lymphocyte active immunotherapy is an important means of the same, and a large number of clinical practices confirm that the pregnancy success rate is 40-60%³. With the deep research of infertility, the function of serum anticardiolipin antibody (ACA) was brought to the forefront gradually, which can result in infertility by causing organism hypercoagulability, germ cell flaw, endometrium damage, intercellular information transfer disturbs and placenta blood supply impact⁴. Tim-1 gene is one of the members that were found earliest in TMI family, which mainly involved in the proliferation and differentiation of T

lymphocyte, influencing the balance between Th1 and Th2⁵. The present study is aimed to analyze the expressions of ACA and Tim-1 among the patients with negative APLA recurrent spontaneous abortion leading to novel ideas for the clinical management of the above diseased state.

Patients and Methods

Patients

58 patients with negative APLA recurrent spontaneous abortion who were diagnosed for the first time and who received lymphocyte immunotherapy from January 2013 to January 2016 from our hospital, were selected continuously in the present study. **INCLUSION CRITERIA:** 1) Continuous natural abortion occurred twice or above, within 28 weeks for pregnant. 2) APLA was diagnosed as negative for twice or 3 times continuously; 3) The karyotypes of both sides of husband and wife were normal and the routine inspection of husband's semen was normal as well. 4) The menstrual cycle and ovulation of patients were normal. **EXCLUSION CRITERIA:** 1) The age was above 40 years old. 2) The patients had reproductive tract infection and autoimmune disease. 3) The genital was malformation. 4) Husband suffered from infectious diseases, such as hepatitis, HIV, and syphilis. 5) Immune function drugs were taken within 3 months. According to the therapeutic outcome, the patients were divided into success group with 31 cases and failure group with 27 cases. The average age of success group was (28.6 ± 3.7) years old, with average abortion gestational weeks of (24.6 ± 2.7), average abortion times of (2.8 ± 0.6), and average body mass index (BMI) of (21.5 ± 2.3) kg/m²; The average age of success group was (29.7 ± 3.8) years old, with average abortion gestational weeks of (23.8 ± 2.6), average abortion times of (2.9 ± 0.7), and average body mass index (BMI) of (21.3 ± 2.5) kg/m²; the baseline data of two groups was comparable.

Extraction of Lymphocyte

Sterilization centrifuge tube was applied to dilute the heparin anticoagulation with normal saline to strike and blend repeatedly. Another centrifuge tube was taken, and 10 ml lymphocyte separation medium was put in it under aseptic conditions, with disposable sterilized straw absorbing 10 ml diluted anticoagulant to spread on the layer of lymphocyte separation medium slowly. Under the room temperature, lymphocyte

layer was extracted lightly and slowly by 3000 g centrifugal for 30 mins, which was transferred to another centrifuge tube. 10 ml sodium chloride solution of 0.9% was used to clean by 2000 g centrifuge for 10 mins, and the supernatant was abandoned. The final concentration of lymphocyte was adjusted to $(200-00) \times 10^5/\text{ml}$, with a cell suspension of about 1-2 ml.

Therapeutic Process

Qualified lymphocytes suspension was extracted with injector as standby application, which was injected subcutaneously with multipoint to the patients (each arm for three needles, six needles altogether). The injection site was epidermis of inner forearm, left arm for the first time and right arm for the right arm with alternate injection. The treatment was conducted every two weeks, and the patients received micro-lymphocyte cytotoxic test after twice treatment, if dead cells were above 10%, treatment would stop, and patients would be suggested to prepare pregnancy and conceive within 6 months. If dead cells were less than 10%, treatment would continue for twice after two weeks; then, the patients would be suggested to prepare pregnancy. When the gestational week is 5 weeks after pregnancy, the treatment is strengthened at the continuous rate of once every three weeks and it was continued until the 16th gestational week. Then pregnant women were preceded with B ultrasound on the abdomen at fixed period, if the examination of B ultrasound was normal when the gestation period was above 12 weeks, the treatment would be successful; if the embryo was diagnosed as non-development by B ultrasound or early spontaneous abortion occurred, the treatment would be a failure.

Observation Index and Detection Methods

The ELISA kit was bought from Sigma-Aldrich Company in St. Louis, MO, USA, and the operation was performed following the instructions. 2 ml was separated after lymphocyte chromatography and Treg-specific marks, and the flow cytometry was brought from Beckman Coulter. 2 ml was isolated by mononuclear cell, RNA extraction (kit was brought from Beijing Biyun-tian, China), concentration, purity and integrity detection, synthesis cDNA (reverse transcription kit was bought from Invitrogen, Carlsbad, CA, USA), PCR amplification (PCR amplification device was brought from Beijing Liuyi Factory, and primer design was finished by Sangon Technology co., Ltd. (Shanghai, China) amplification system,

Table I. Comparison of positive rate of APLA and ACA before and after treatment (%).

Groups	Case	APLA before treatment	ACA before treatment	ACA after treatment
Success group	31	25 (80.6)	9 (29.0)	3 (9.7)
Failure group	27	20 (74.1)	15 (55.6)	12 (44.4)
χ^2		0.358	4.185	9.098
p		0.549	0.041	0.003

and the parameter were set up according to the instructions), electrophoretic bands (electrophoretic apparatus was bought from Bio-Rad Company, Hercules, CA, USA), Image J software (Leica Co., NuBloch, Germany) was used to analyze and the results were presented by $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Software SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used to process data, and the recorded data was presented by mean $\pm$ standard deviation. Comparison between groups took independent-sample t -test, and comparison in one group applied paired t -test to detect. Enumeration data was showed by case or rate (%), and comparison between groups took χ^2 -test; $p < 0.05$ meant that the difference has statistical significance.

Results

Comparison of Positive Rate of APLA and ACA Before and After Treatment

The positive rate of APLA after treatment of two groups was compared, without any difference. The positive rate of ACA in failure group before treatment was higher obviously than the data of after treatment, and the difference was statistically significant ($p < 0.05$) (Table I).

Comparison of Expression Level of Serum IL-6, IL-10, TNF- α and IFN- γ

After treatment, serum IL-6 and IL-10 level of two groups increased, TNF- α and IFN- γ level decreased compared with the data before treatment, and the improvement of success group was greater than failure group's, difference with statistical significance ($p < 0.05$) (Table II).

Comparison of Serum Treg Proportion and mRNA Expression Level of Tim-1

After treatment, Treg proportion of two groups increased compared with before, and the proportion of success group was greater than that of failure group; mRNA expression level of Tim-1 in failure group before and after treatment was higher than that of success group, and the difference had statistical significance ($p < 0.05$) (Table III).

Discussion

Pregnancy is similar to allograft, so the mother body will not reject fetus under normal circumstances, and the immune cells can maintain the immune balance of maternal-fetal interface by the immune regulatory networks. Once the immune balance between mother and fetus is disturbed, immune adjustment disorder might lead to rejection reaction of fetus resulting in spontaneous abortion^{6,7}. Recurrent spontaneous abortion caused by negative APLA is usually handled with active immune therapy by adopting the lymphocytes of spouse or closed relative in blood relationship⁸. The mechanism of immune therapy includes injecting specific antigen into patient's body, as a kind of sensitogen to trigger sensitization response of patient.

The patients will generate specific immune factor by their own immune system response, which increases the immunoreactivity of patients to prevent fetus paternal antigen or embryo from identified and damaged by immune system of patients.

Table II. Comparison of expression level of serum IL-6, IL-10, TNF- α and IFN- γ (ng/L).

Groups	IL-6 Before treatment	IL-10 After treatment	TNF- α Before treatment	IFN- γ After treatment	Before treatment	After treatment	Before treatment	After treatment
Success group	23.6 $\pm$ 5.2	54.7 $\pm$ 7.6	13.8 $\pm$ 3.6	35.6 $\pm$ 4.2	62.3 $\pm$ 7.6	32.6 $\pm$ 4.5	54.9 $\pm$ 6.8	25.8 $\pm$ 3.9
Failure group	24.3 $\pm$ 5.3	28.5 $\pm$ 5.5	15.6 $\pm$ 3.7	19.7 $\pm$ 3.8	61.5 $\pm$ 7.3	53.2 $\pm$ 6.6	53.8 $\pm$ 6.5	46.9 $\pm$ 5.4
t	0.126	4.628	0.215	4.452	0.232	5.102	0.168	5.326
p	0.903	0.030	0.867	0.033	0.759	0.026	0.865	0.024

Table III. Comparison of serum Treg proportion and mRNA expression level of Tim-1.

Groups	Treg Proportion (%)		Tim-1 mRNA	
	Before treatment	After treatment	Before treatment	After treatment
Success group	12.4±3.5	33.6±4.2	0.3265±0.1025	0.2157±0.0638
Failure group	13.3±3.6	15.8±3.8	0.4452±0.1324	0.4265±0.1526
<i>t</i>	0.326	5.257	5.326	5.568
<i>p</i>	0.645	0.025	0.022	0.018

The CD4+CD25+ regulatory T cells in patients got elevated by immune therapy, thereby, helped in immunological tolerance on local maternal-fetal interface and hence maintained normal pregnancy process⁹. Furthermore, the ancillary cells of Th1 in patient's body could secrete TNF- α and IFN- γ , which could inhibit the normal secretion of endometrium, leading to immune injury for trophoblast cell, hampering growth of embryo finally¹⁰⁻¹². Lymphocyte active immunotherapy is also effective in suppression of auxiliary cell of Th1 leading to a successful pregnancy¹³.

The anticardiolipin antibody is often not identified by the immune system, but on its exposure, it would combine with the phospholipid composition on the surface of patient's uterus and placenta blood vessel endothelium leading to hypoxia which final cause spontaneous abortion¹⁴. It could also cause germ cell defects and endometrial damage leading to the occurrence of autoimmune diseases¹⁵. Further, ACA could influence the up-regulated expression of costimulatory molecules PD-L1 in the activation process of T cells generating lethal effect for embryo and fetus¹⁶. Tim-1 gene on CD4+T cell surface has selective expression that can promote the activation of CD4+T cell and secrete cytokine¹⁷. When the expression of TMI-1 is regulated up, the IFN- γ synthesis capacity of T cell would strengthen, and would generate immune injury for trophoblast cells, hampering the normal embryonic implantation and development¹⁸.

Conclusions

From this research, we can conclude that the positive rate of APLA after treatment of two groups was compared, without any difference. The positive rate of ACA in failure group before treatment was higher obviously than the data of after treatment. After treatment, serum IL-6, IL-10 levels and Treg of two groups decreased significantly in comparison to success group. On

the other hand, TNF- α and IFN- γ level increased, mRNA expression level of Tim-1 in failure group before and after treatment was higher than that of success group, the difference with statistical significance. Furthermore, besides APLA, high expression of serum anticardiolipin antibody and mononuclear cells Tim-1 could lead to immune treatment failure for the patients with negative APLA habitual abortion by affecting T lymphocyte immune, which might become a new intervention target to improve successful pregnancy for the patients with habitual abortion.

Conflict of interest

The authors declare no conflicts of interest.

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