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The effect of low-molecular-weight heparin on immune balance of patients with repeated implantation failure during the implantation window

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ABSTRACT

This study investigates the effect of low-molecular-weight heparin (LMWH) on cytokines TNF-α, IFN-γ, IL-2, IL-4, IL-6, and IL-10 in peripheral blood of patients with repeated implantation failure during the implantation window. From May 2019 to March 2021, we enrolled 32 patients with recurrent implantation failure (RIF group) and 30 patients with successful pregnancy after the first frozen embryo transfer (control group) in the Reproductive Medicine Centre of Wuxi Maternity and Child Health Care Hospital. During the implantation window, the following features were compared between two groups and between different time points using ELISA: the status of immune cytokines in peripheral blood; Th1 cytokines (TNF-α, IFN-γ, and IL-2) and Th2 cytokines (IL-4, IL-6, and IL-10) in peripheral blood. The levels of Th1 cytokines in the RIF group before treatment were higher in comparison with the control group. In the RIF group, the LMWH treatment can inhibit the expression of Th1 cytokines and enhance the expression of Th2 cytokines. Using LMWH during the implantation window can improve the immune imbalance of patients with repeated implantation failure, which makes it a potential treatment strategy for patients with abnormal cellular immunity.

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Introduction

Pregnancy is a major challenge to the maternal immune system. In normal pregnancy, there is a physiological balance between Th1 and Th2 cell responses, that the maternal Th2 cells are in the predominant status and exert an immune-protective role to maintain pregnancy (1). While the repeated implantation failure (RIF) population exhibits a Th1/Th2 imbalance, the secretion of Th1 cytokines increase and the immune damage is enhanced, leading to implantation failure (2). Many unexplained RIFs may be related to abnormal immune tolerance at the maternal-fetal interface, which makes the theory of immune tolerance deficiency possible. Therefore, some scholars have suggested that the immune abnormality that corrects this Th1/Th2 imbalance is a potentially modifiable cause of RIF. This study aimed to investigate the effect of LMWH on peripheral blood tumor necrosis factor (TNF-α), γ-interferon (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), etc., and explore the efficacy of LMWH on the treatment of abnormal cellular immunity in the implantation window period in the RIF population.

Materials and Methods

Patients

This study was approved by the reproductive ethics committee of the hospital. Overall, 32 patients with RIF (the RIF group) were recruited from May 2019 to March 2021 in Wuxi Maternal and Child Health Hospital, Jiangsu Province. In addition, 30 cases of successful pregnancy were included as the control group. The RIF group was further divided into the clinical pregnancy group and the non-clinical pregnancy group. The included patients have signed the informed consent form according to the informed consent of medication.

The inclusion criteria for the RIF group were as follows: (I) At least 4 high-quality embryos were transferred in at least 3 fresh or frozen cycles, but pregnancy was not achieved (implantation failure or pregnancy loss; (II) Age < 40 years old and basic FSH<10U/L; (3) If the above conditions were met, the frozen-thawed embryo transfer was performed again, and at least one high-quality blastocyst was transferred.

The inclusion criteria for the control group were as follows: (I) Age <40 years old and basal FSH <10U/L; (II) Patients who successfully obtained pregnancy after the first frozen embryo transfer and had no history of adverse pregnancy.

The exclusion criteria were as follows: (I) LMWH contraindications, such as thrombocytopenia, liver and kidney insufficiency, hemophilia, peptic ulcer, cerebrovascular hemorrhage, etc.; (II) Failed to treat according to the doctor's advice; (III) Disease history: Anatomical abnormalities, intrauterine adhesions, endometriosis, adenomyosis, uterine malformation, endometrial abnormalities, hydrosalpinx, chromosomal abnormalities of one or both couples, diabetes mellitus, thyroid disease and autoimmune disease; (IV) Endometrial thickness < 7mm on the day of transplantation; (V) Contraindications to assisted reproductive technology or combined with other diseases

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that have a major impact on pregnancy; (VI) Refused to sign the informed consent form.

Intima preparation

All enrolled patients have prepared endometrium in the replacement cycle. From the 3rd to 5th day of the menstrual cycle, oral administration of estradiol valerate (4-8 mg/day), and the ultrasound detection of endometrial thickness were performed one week after medication. Then the dose was adjusted according to the thickness of the endometrium (if the thickness of the endometrium is <7mm, the dose can be gradually increased to 8-12mg/d), and the endometrial thickness was monitored by every 3 days until the endometrial thickness was ≥ 8mm.

Luteal phase support program

Before treatment and during the treatment cycle, vaginal administration of progesterone gel (90mg, qd) plus dydrogesterone (20mg, Bid) was performed.

LMWH treatment

In the RIF group, Enoxaparin sodium (Nanjing Jianyou Biochemical Pharmaceutical Co., Ltd., 4000 IU, once a day, subcutaneously) was given since the day of luteal transformation. If the pregnancy continued, it was used until 28-30 days after embryo transfer, or else the treatment was terminated.

Th1 and Th2 analysis

Instruments and reagents Flow cytometer (Mindray BriCyte E6), Th1/Th2 subgroup detection kit (Saiji Biotechnology Co., Ltd.).

Around 3 mL of venous blood was collected on the 6th day of luteal transformation. The blood was centrifuged at 3000 r/min for 10 minutes, and the serum was collected and stored in a refrigerator at -80°C until testing. The Mindray BriCyte E6 flow cytometer was used to measure the levels of Th1 and Th2 cytokines, applying the Th1/Th2 subgroup detection kit (Saiji Biotechnology Co., Ltd.). All operations were strictly carried out according to the instructions.

Pregnancy criteria

Clinical pregnancy was considered if a gestational sac was detected by vaginal ultrasonography 28 days after transplantation. Early miscarriage was regarded if the natural pregnancy termination occurred within 12 weeks of gestation. The criteria of no clinical pregnancy included: (I) blood HCG < 5mIU/ml during 12-14 days after transplantation; (II) biochemical pregnancy (blood HCG ≥ 5mIU/ml but no gestational sac detected by clinical B-ultrasound).

Cytokine indicators

In the peripheral blood, levels of the Th1 associated cytokines (TNF-α, IFN-γ, and IL-2) and Th2 associated cytokines (IL-4, IL-6, and IL-10) and the ratios of some Th1-cytokine/Th2- cytokine (ie., TNF-α/IL-4, TNF-α/IL-10, IFN-γ/IL-4, IFN-γ/IL-10, IL-2/IL-4, IL-2/IL-10) were recorded.

Statistical analysis

Data were calculated by the SPSS software (Version 25.0), and the measurement data were expressed as Mean ± standard deviation and compared using the t-test. The category data were expressed as numbers (percentage), and the $\chi 2$ test was used to compare the rate between groups. A $P<0.05$ was considered statistically significant.

Results

First, there were no statistically significant differences in age, years of infertility, basal endocrine, body mass index (BMI), and anti-Mullerian hormone (AMH) between the LMWH group and the control group (Table 1). Before treatment, levels of Th1 and Th2 cytokines in peripheral blood were significantly different between RIF and control groups, including increased TNF-α, IFN-γ, and IL-2 levels, and decreased IL-4, IL-6, and IL-10 levels (Table 2). After LMWH treatment, the levels of Th1 and Th2 cytokines were significantly altered (Table 3), with decreased TNF-α, IFN-γ, and IL-2 levels and increased IL-4, IL-6, and IL-10 levels. And the cytokine levels after treatment were similar to the control group. Finally, we compared serum cytokine between the clinical-pregnancy and no-pregnancy groups. There were 17 clinical pregnancies in the 32 RIF cases of the LMWH group, as well as 15 no-pregnancy cases. The ratios of Th1/Th2 (e.g., TNF-α/IL-4, IFN-γ/IL-10, IL-2/IL-4) in the peripheral blood before treatment were higher in the clinical-pregnancy group vs the non-pregnancy group (Table 4), which suggests that those with significant abnormal Th1/Th2 cytokines are more likely to benefit from LMWH treatment.

Table 1. General information on patients in the control and RIF groups.

Features	RIF (n=32)	Control (n=30)	P value
Age (year)	30.16±2.59	29.3±2.74	0.210
Infertility years	4.67±2.77	4.11±2.31	0.390
bFSH (IU/L)	7.63±1.59	7.26±1.46	0.343
bLH (IU/L)	5.59±3.16	6.17±2.93	0.456
bE2 (pg/ml)	39.71±17.5	46±15.6	0.140
PRL (ng/ml)	13.21±5.56	15.8±6.76	0.106
T (ng/ml)	0.47±0.25	0.56±0.17	0.101
AMH (ng/ml)	5.99±5.25	7.0±3.23	0.363
TSH (uIU/ml)	2.11±0.82	2.03±1.11	0.749
BMI (kg/m2)	22.30±2.79	22.37±3.00	0.924

Table 2. Th1/Th2-related cytokines in peripheral blood before treatment.

Cytokines	RIF (n=32)	Control (n=30)	t	P
TNF-α	1.51±0.47	1.32±0.18	2.127	0.040
IFN-γ	2.28±0.95	1.44±0.19	4.898	0.000
IL-2	1.95±0.69	0.58±0.29	2.782	0.008
IL-4	0.98±0.38	1.25±0.41	-2.685	0.009
IL-6	2.78±1.75	1.79±0.52	3.059	0.004
IL-10	2.44±0.47	3.27±0.26	-8.674	0.000
IL-2/IL-4	1.89±1.08	0.48±0.22	7.227	0.000
IL-2/IL-10	0.97±0.38	0.37±0.16	8.29	0.000
TNF-α/IL-4	2.85±0.32	1.51±0.32	16.478	0.000
TNF-α/IL-10	0.58±0.50	0.35±0.21	2.387	0.022
IFN-γ/IL-4	2.62±2.20	1.45±0.64	2.881	0.007
IFN-γ/IL-10	1.41±1.02	1.03±0.26	2.038	0.049

Table 3. The effects of LMWH on Th1/Th2-related cytokines.

Cytokines	RIF (n=32)	Control (n=30)	t	P
TNF-α	1.51±0.47	1.25±0.78	1.615	0.112
IFN-γ	2.28±0.95	1.55±0.32	4.119	0.0002
IL-2	1.95±0.69	0.36±0.21	12.471	0.000
IL-4	0.98±0.38	1.18±0.21	-2.606	0.012
IL-6	2.78±1.75	1.23±0.41	4.878	0.000
IL-10	2.44±0.47	3.16±0.33	-7.092	0.000
IL-2/IL-4	1.89±1.08	0.52±0.43	6.667	0.000
IL-2/IL-10	0.97±0.38	0.31±0.17	8.969	0.000
TNF-α/IL-4	2.85±0.32	1.57±0.27	17.294	0.000
TNF-α/IL-10	0.58±0.50	0.35±0.14	2.51	0.017
IFN-γ/IL-4	2.62±2.20	1.31±1.06	3.035	0.004
IFN-γ/IL-10	1.41±1.02	0.99±0.35	2.203	0.034

Table 4. Comparison of pre-treatment Th1/Th2-related cytokines in peripheral blood between the clinical-pregnancy and non-pregnancy sub-groups.

Cytokines	RIF (n=32)	Control (n=30)	t	P
IL-2/IL-4	1.38±0.21	0.83±0.34	7.60	0.000
IL-2/IL-10	0.48±0.24	0.22±0.57	2.31	0.026
TNF-α/IL-4	2.48±0.47	1.10±1.56	4.79	0.000
TNF-α/IL-10	0.61±0.42	0.22±0.50	3.31	0.002
IFN-γ/IL-4	5.86±2.34	2.68±0.21	7.65	0.000
IFN-γ/IL-10	3.46±1.08	0.56±0.37	14.32	0.000

Discussion

Although assisted reproductive techniques have been widely used in the field of infertility in recent years, implantation failure remains a major obstacle (3,4). In recent years, a large number of clinical studies have been conducted on the relationship between RIF and immunological factors, but most of them have focused on local endometrial or animal experiments. There is still a lack of clinical data about the expression of immune cells in peripheral blood during the implantation window. In this study, we focused on 6 Th1/Th2 associated cytokines, which play an important role in embryo implantation and examined their expression in peripheral blood during the implantation window.

The mechanism of RIF is similar to that of allogeneic transfer rejection, in that a large influx of immune cells may induce maternal immune tolerance to protect the embryo from rejection. In this process, helper T(Th) cells play an important role in maternal-fetal immune regulation and are involved in the pathogenesis of RIF (5). Depending on the cytokines they secrete, they can be divided into Th1 and Th2 cells. Th1 cells mainly secrete inflammatory cytokines, such as IL-2, IFN-γ, and TNF-α, which contribute to cell-mediated immune and are associated with inflammation and tissue damage; Th2 cells mainly secrete IL-4, IL6, and IL-10, which are regarded as anti-inflammatory cytokines and contribute to humoral reactions; they can suppress immune inflammation and prevent excessive damage (6,7). When the immune homeostasis is disrupted, Th1 cytokines may be overexpressed and Th2 cytokines may be suppressed, the risk of organismal inflammation and tissue damage, and embryonic cytotoxicity are increased, which may cause implantation failure (8). Endometrial immune dysfunction was reported in 81.7% of RIF patients and over-activation of the immune system in 56.6% of RIF patients (9). The results of the present study showed that the ratios of pro-inflammatory cytokines IL-2 /IL-10, IFN-γ/IL-10 and TNF-α/IL-10 in the peripheral blood of patients in the RIF group (before treatment) were significantly higher than that of the control group. The overall trend in RIF is toward increased levels of cytokines with pro-inflammatory activity (Th1 type) and decreased levels of Th2 type anti-inflammatory cytokines. Therefore, modulating the immune system to improve the Th1/Th2 imbalance may improve fertility and pregnancy in patients with RIF.

LMWH is a multimeric molecular fragment produced by enzymatic or chemical cleavage of standard heparin, which belongs to ATIII-dependent lazy thrombin inhibitors. In addition to the antithrombotic effects, it has various biological activities such as anti-inflammatory and immunomodulatory. In recent years, it has been increasingly used in assisted reproduction. The higher levels of IL-10 and IL-4 in the treatment cycle in after LMWH treatment also indicate its effect on the regulation of immune homeostasis. Some immunomodulatory mechanisms of LMWH may be involved in the physiological process of embryo implantation. It has been suggested that heparin-binding epidermal growth factor (HB-EGF) plays an important role in embryo implantation in the early placental development stages and that HB-EGF is expressed at high levels in the trophectoderm during the first trimester of pregnancy, inducing an invasive trophoblast phenotype with a decreased apoptosis level; while the binding and activation of EGF and its receptor require heparin (10). Previously, it has been reported that an elevated level of Th2 cytokine is associated with successful implantation, while a Th1-dominated environment may lead to failure of implantation (11-14). Our study found a significant decrease in Th1-related cytokines (as well as the Th1/Th2 ratio) after LMWH treatment, suggesting that RIF patients with higher Th1/Th2 ratios may be candidates for LMWH treatment. However, further studies with large samples are needed to determine the threshold value of this ratio as an indicator of LMWH therapy.

In conclusion, using LMWH during the implantation window can improve the immune imbalance of patients with repeated implantation failure, which makes it a po-

tential treatment strategy for patients with abnormal cellular immunity.

Ethics approval and consent to participate
Not applicable to this type of manuscript.

Competing interests
We declare no competing interests exist.

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