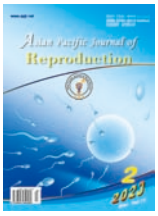

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Effect of heparin on recurrent IVF-ET failure patients

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ABSTRACT

Objective: To elucidate the possible role of unfractionated heparin in patients with failed repeated in *in vitro* fertilization and embryo transfer (IVF-ET) and thrombophilia.

Methods: This case control study evaluated the efficacy of the unfractionated heparin in increasing the pregnancy and implantation ratio in women with recurrent IVF-ET failures. Eighty-six women received *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) with a record of three or more previous IVF-ET failures. Participants were randomly distributed into two groups. Group A (n=43) received unfractionated heparin 5 000 IU twice daily, and group B (n=43) did not take any antithrombotic drugs. Coagulation abnormalities such as factor Ⅴ Leiden (FVL) mutation, methylene tetra hydro folate reductase (MTHFR) mutation and prothrombin mutation (FⅡ) were evaluated. Age, body mass index, basal follicular stimulating hormone, basal estradiol, duration of infertility, and number of IVF-ET failures were compared between two groups.

Results: 45.0% and 17.4% of women were pregnant with and without MTHFR and prothrombin mutation, respectively, when they received unfractionated heparin treatment. The implantation rate was more in group A (12.5%) than group B (4.3%) and differences in the fertilization rate of the two groups were observed (27.7% *vs.* 35.9%). The clinical pregnancy rate per cycle was remarkably more in group A (30.2%) than group B (14.0%).

Conclusions: Heparin is a safe and valuable treatment for patients with repeated IVF-ET failures. The clinical pregnancy and implantation rates are higher in the heparin-treated group in contrast with the control group.

Trial registration: The trial registration was done with clinical registration number of " IRCT138807202575N1".

KEYWORDS: Heparin; Recurrent implantation failure; Thrombophilia; MTHFR C677T; Prothrombin A20210G; Factor Ⅴ Leiden

1. Introduction

Infertility is now the third most serious disease in the world after cancer and cardiovascular disorders[1]. Infertility is defined by the World Health Organization as the inability to get pregnant after 12 months or more of continuous, unprotected intercourse[2-4]. Although infertility is not life-threatening, its negative effects on individuals can lead to poor quality of life, emotional trauma, anxiety, and despair[5]. These individuals could benefit from assisted reproductive techniques[6,7]. An option seems to increase the *in vitro* fertilization (IVF) success rate is anticoagulant therapy.

To date, there is no clear definition of recurrent pregnancy loss. Most reproductive experts concur that recurrent implantation failure is described as a failure to gain clinical pregnancy in ≤40-year-old

Significance

Heparin can help to increase the fertility rate in IVF-ET failure patients. Considering the fact that people may respond differently to medicine by their gene profile, this study examines the relevance between heparin administration and the increased probability of positive pregnancy in women with thrombophilic-related mutations. The study revealed that patients who have thrombophilia mutations and receive heparin will have better results than patients who do not have mutations and receive heparin.

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women[8] after three or more high-quality embryo transfers in at least three fresh or frozen cycles[9–11]. About 10% of women endure recurrent miscarriage in childbearing age[10,12]. Mother and embryo are two significant factors in successful or failed implantation[13]. The maternal risk factors for recurrent implantation failure are divided into uterine abnormalities, endocrine dysfunctions, prevalent diseases such as thrombophilia and abnormal immune response that might result in dysfunctional implantation. In addition, embryonic anomalies can be caused by paternal factors or oocytes[9,14,15].

Despite progress in success rate and meaningful increase in assisted reproductive techniques, live birth rate due to these techniques has plateaued yet[16,17]. Today, more focus on experimental strategies to promote the quality of implants and implantation rate. The implantation failure mechanism in the thrombophilic condition is considered a decrease in blood flow to the endometrium and the placenta, which can prevent the normal endometrial acceptance resulting in abortion[18–20]. It was reported that several inherited coagulation abnormalities such as factor Ⅴ Leiden (FVL) mutation, methylenetetrahydrofolate reductase (MTHFR) mutation, protein S, C deficiency and prothrombin mutation (FⅡ) make patients predispose to thrombophilia[21–24].

A higher live birth rate in patients with anticoagulant therapy compared to others has been reported in some studies[25,26]. It has been hypothesized that heparin could play a prominent role; these molecules have a physiological role during hatching and implanting[27,28]. Although the correlation between thrombophilia and recurrent pregnancy loss is familiar for both hereditary and acquired thrombophilia[29,30], the link between thrombophilia and unexplained female infertility is still discussed, especially for women with recurrent IVF failure and embryo transfer (ET) procedures[30,31]. Since the debate on using heparin and the increase in the live birth rate remains indistinct, our strategy is to use heparin as an agent that may have a significant effect on the live birth ratio in women with thrombophilia. This study seeks to shed more light on this subject.

2. Subjects and methods

2.1. Study participants

This prospective randomized control trial evaluated the efficacy of the unfractionated heparin in increasing pregnancy and implantation rates in women with recurrent IVF-ET failures. The experiment was conducted from March 2017 to May 2019 at Yazd Medical University's Fertility Research and Clinical Center. A total of 86 women with these criteria: 19-35 years old, basal follicle-stimulating hormone (FSH) less than 10 IU/L, body mass index (BMI) less than 29 kg/m^2 , the existence of both ovaries, history of three or more previous IVF-ET failures and proper quality embryos for transfer, entered into this study. And patients with polycystic ovary syndrome, hydrosalpinx, chronic systemic disease, and patients who were barred from prescribing heparin therapy were excluded.

2.2. Treatment protocol

All subjects were handled with a long protocol of ovarian stimulation. To reduce pituitary suppression, subjects were given 0.5 mg daily subcutaneous injection of buserellin from day 21 of the previous menstrual cycle (suprefact, Aventis, Frankfurt, Germany). In the event of desensitization, if plasma estradiol (E_2) levels were ≤50 pg/mL and transvaginal ultrasonography demonstrated the absence of ovarian cysts, the buserellin dose was reduced to 0.25 mg/day and continued until the day of human chorionic gonadotropin (hCG) administration. The controlled ovarian hyperstimulation was initiated with recombinant FSH (Gonal F, Serono, Aubnne, Switzerland) or human menopausal gonadotropin (HMG) (Menogon, Ferring, Pharmaceuticals, Germany) 150 IU/day on the day 2 of the menstrual cycle. Ovarian response was assessed by serial ultrasound examinations and evaluation of serum E_2 levels, and then gonadotropin dose adjustment was done as required. Urinary hCG (Pregnyl, Organon, Oss, the Netherlands) 10 000 IU was administered when at least three follicles showed a mean diameter of 18 mm. Oocyte retrieval was done by the transvaginal ultrasound guided approach, 34-36 h after hCG injection and conventional IVF or intracytoplasmic sperm injection (ICSI) was executed as clinically appropriate.

Under embryologist supervision, one to three good-quality embryos were transferred 48 h after oocyte retrieval under ultrasound guidance, with a CCD embryo transfer catheter (Laboratory C.C.D., Paris, France). At the same time, patients (n=86) were randomized into two groups using computer-generated randomization. Group A (n=43) contained the people who took unfractionated heparin (Heparin, Caspian Tamin, Rasht, Iran) 5000 IU twice a day through subcutaneous injection. Treatment continued for up to 14 days after the first day of embryo transfer. Unfractionated heparin injection continued until six weeks postpartum, if β-hCG was positive. Group B (n=43) did not take any antithrombotic drugs. Luteal phase support was divided into two groups, and 100 mg of progesterone in oil (Progesterone, Aburaihan Co., Tehran, Iran) was administered intramuscularly on the day of oocyte collection and continued until fetal cardiac activity was confirmed by ultrasound. Pregnancy was confirmed by measuring serum β-hCG levels 14 days after embryo transfer. Clinical pregnancy was considered two weeks after β-hCG positivity, which was confirmed by the presence of fetal cardiac activity on transvaginal sonography. Ongoing pregnancy was described a pregnancy procedure further than 12 gestational weeks.

The embryo score for high quality was 62.79% in group A and 53.48% in group B. The score for fair quality was 32.55% *vs.* 46.51% in groups A and B, respectively. Only two embryo (4.65%) had poor quality in group A and none in group B.

2.3. Genetic analysis

Peripheral blood samples were taken from cases (group A) and controls (group B) when they came for embryo transfer. DNA was extracted by Bio NEER DNA extraction kit (USA Bioneer, Inc.). Polymerase chain reaction (PCR) was performed using primers FVL, MTHFR and FⅡ, and amplification was confirmed by polyacrylamide gel electrophoresis. Restriction enzymes were Mnl1, HinfI, and HindⅢ to digest PCR bands of *FVL*, *MTHFR*, and *FⅡ* genes, respectively.

FVL gene has 140 nucleotides. The Mnl1 enzyme digests one restriction sites and produces two fragments (105 bp and 35 bp) after cleavage. The G1691A mutant allele restriction site between fragments 35 bp and 105 bp wanes.

MTHFR gene with a length of 198 bp contains no restriction site for the Hinf1 enzyme. However, C677T mutation causes one. Enzyme digestion makes two fragments (175 bp and 23 bp). And *FⅡ* gene, with a length of 345 bp, has no restriction site for the HindⅢ enzyme. G20210A mutation leads to forming a restriction site and dividing the gene into two-part with length of 322 bp and 23 bp after the enzyme has been digested (Table 1). Digested PCR products were separated by electrophoresis on agarose gels. Based on the size and number of bands, samples were classified as homozygous, heterozygous, or normal.

2.4. Statistical analysis

SPSS statistical package was used for data analysis (SPSS, version 22.0). Normality was assessed using Kolmogorov-Smirnov test. *t*-test, Mann-Whitney and *Chi*-square were handled for analysis as required. Data were expressed as mean and standard deviation (mean±SD), or median and interquartile range (IQR), or *n*(%). *P* values <0.05 were regarded as statistically significant.

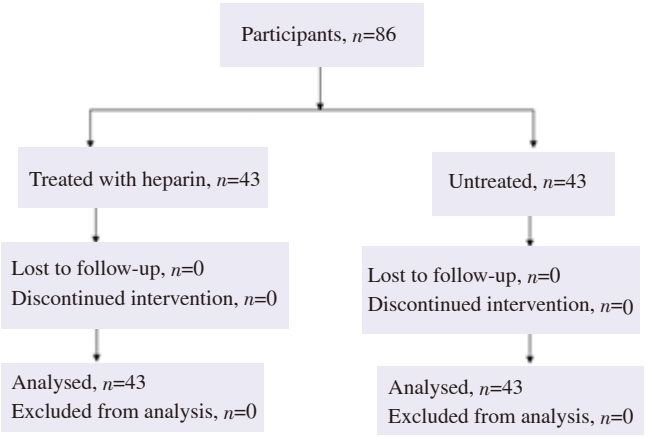

Figure 1. The study flow diagram.

2.5. Ethics statement

The study was approved by the local ethics committee of Yazd Research and Clinical Center for Infertility with the clinical registration number of "IRCT138807202575N1" and ethics approval number of "1378". All participants were informed with written consent before starting heparin treatment.

3. Results

3.1. Demographic and infertility characteristics

Of the 86 patients selected, 43 were in the study group A (5000 IU of unfractionated heparin administered subcutaneously twice daily) and 43 were in the control group B (luteal phase support only, no heparin). Not even one through 86 patients were deleted during this process. The study flowchart is seen in Figure 1. Demographic and

Table 1. Genotyping conditions of the studied genes.

Studied genes	Primer sequences (5'-3')	PCR condition	Bands after enzyme digestion and gel electrophoresis
MTHFR	F: TGAAGGAGAAGGTGTCTGCGGGA R: AGGACGGTGCGGTGAGAGTG	Initial denaturation at 95℃ for 3 min 35 cycles: Denaturation at 95℃ for 20 s Annealing at 59℃ for 20 s Extension at 72℃ for 20 s Extension at 72℃ for 5 min	CC:198 bp TT: 175 and 23bp CT: 198, 175 and 23 bp
FⅡ	F: TCTAGAAACAGTTGCCTGGC R: ATAGCACTGGGAGCATTGAAGC	Initial denaturation at 95℃ for 5min 35 cycles: Denaturation at 95℃ for 25 s Annealing at 55℃ for 25 s Extension at 72℃ for 40 s Extension at 72℃ for 5 min	GG: 345 bp AA: 322 and 23 bp GA: 345, 322 and 23 bp
FVL	F: CTTCAAGGACAAAATACCTGT R: GATGCCCAGTGCTTAACAAG	Initial denaturation at 95℃ for 5min 35 cycles: Denaturation at 95℃ for 25 s Annealing at 61℃ for 25 s Extension at 72℃ for 35 s Extension at 72℃ for 5 min	GG: 105 and 35 bp AA: 140 bp GA: 140, 105 and 35 bp

Table 2. Demographic and infertility characteristics of patients.

Variables	Group A	Group B	*P*-value
Age[#], years	30.1±4.2	31.5±3.5	0.101
BMI[&], kg/m^2	23.00(3.65)	24.85(3.25)	0.099
Basal FSH[&], IU/L	6.35(3.30)	6.55(3.63)	0.308
Basal E_2[#], pg/mL	58.73±3.99	56.62±4.54	0.420
Duration of infertility[&], years	8.00(6.38)	10.00(6.25)	0.489
No. IVF-failure[&]	3.00(0.00)	3.00(0.00)	0.493
Infertility causes[*]			
Male factor	33(76.74%)	23(53.49%)	0.055
Ovulatory	5(11.63%)	4(9.30%)	
Unexplained factor	3(6.98%)	9(20.93%)	
Tubal factor	0(0%)	2(4.65%)	
Mild endometriosis	1(2.32%)	0(0%)	
Mixed	1(2.32%)	5(11.63%)	
Thrombophilia associated genes[*]			
MTHFR gene mutation	19/43(44.2%)	16/43(37.2%)	0.510
FVL gene mutation	0/43(0%)	0/43(0%)	-
FII gene mutation	1/43(2.3%)	0/43(0%)	-

[&]Mann–Whitney *U* is used and data are expressed as median (IQR); [#]*t*–test is used and data are expressed as mean±SD; [*]*Chi*-square test is used and data are expressed as *n*(%). Group A (*n*=43) takes unfractionated heparin 5000 IU twice a day through subcutaneous injection; Group B (*n*=43) does not take any antithrombotic drugs. BMI: body mass index; FSH: follicular stimulating hormone; E_2: estradiol; IVF: *in vitro* fertilization.

Figure 2. Electrophoretic pattern of thrombophilic mutations after PCR-RFLP. (A) FVL, All lanes are wild type (G1691A). (B) FII, Lanes A-D are wild type, lane E shows 345 and 322 bp, indicating heterozygous FII mutation (G20210A). (C) MTHFR, Lanes A-D are wild type, lanes E and F show 198 and 175 bp, indicating heterozygous MTHFR mutation (C677T).

Table 3. Results of ovarian stimulation of the two groups.

Variables	Group A	Group B	*P*-value
No. Oocyte retrieval	10(7.75)	8(6)	0.188
No. Oocyte MII	7(7)	6(5)	0.138
No. Total embryo	5(4.75)	4(5)	0.229
No. Embryo transferred	2(1)	2(1)	0.927

Mann–Whitney *U* test is used and data are expressed as median (IQR). No.: number, MII: metaphase II.

infertility characteristics in both groups are shown in Table 2. Age, BMI, basal FSH, basal E_2, duration of infertility, number of IVF-ET failures and origin of infertility were compared between the two groups and there were no significant differences.

3.2. Outcomes of ovarian stimulation

The consequences of the ovarian stimulation and embryological characteristics are presented in Table 3. The number of retrieved oocytes {median (IQR): [10(7.75) *vs.* 8(6)], mature oocytes [7(7) *vs.* 6(5)], totally obtained embryos [5(4.75) *vs.* 4(5)] and transferred embryos [2(1) *vs.* 2(1)]} were not statistically different in both groups.

3.3. Gene mutation and pregnancy

None of the 86 patients were positive for G1691A mutation of *FVL*. Only one patient had a heterozygous G20210A *FII* (prothrombin) mutation and 35 women had a heterozygous C677T MTHFR mutation. Thirty-six (41.86%) of 86 patients showed mutations of three thrombophilic factors (Figure 2). Based on genetic analysis, positive pregnancy with mutations in group A was 45.0%, while in group B was 12.5% and positive pregnancy without mutations in groups A and B was 17.4% *vs.* 14.8%, respectively. There was a considerable relevance between pregnancy rate and these mutations in women who received unfractionated heparin (Table 4).

Table 4. Gene mutation and positive pregnancy in the two groups.

Gene mutation	Group A	Group B	*P*-value
Positive gene mutation	9(45.0%)	2(12.5%)	0.035
Negative gene mutation	4(17.4%)	4(14.8%)	0.266

Chi-square test is used and data are expressed as *n*(%).

Table 5. Outcome of cycle.

Variables	Group A	Group B	*P*-value
Fertilization rate	27.7% (114/411)	35.9% (109/304)	0.021
Implantation rate	12.5% (6/48)	4.3% (2/46)	0.042
Clinical pregnancy rate/cycle	30.2% (13/43)	14.0% (6/43)	0.034

Chi-square test is used and data are expressed as %.

3.4. Outcome of cycle

A significant difference was observed in the fertilitzation rate between group A and group B (27.7% *vs.* 35.9%, *P*=0.021). The implantation rate was more in group A than group B (12.5% *vs.* 4.3%, *P*=0.042). The clinical pregnancy rate per cycle was remarkably more in group A than group B (30.2% *vs.* 14.0%, *P*=0.034) (Table 5).

4. Discussion

Despite advances in assisted reproductive techniques procedures, the success rate in assisted reproduction techniques such as IVF has remained at 30%-40%[32]. This relatively low rate has led to further studies by researchers in this field. Although no definite consensus has been reached so far, heparin consumption has extensively studied, especially in patients with recurrent implantation failure. Besides, cases to investigate the effect of heparin on animal models have shown that heparin disaccharides could prevent rising tumor necrosis factor-α constructed by macrophages, resulting in possible increasing inflammation. Heparan sulfate proteoglycan and heparin-binding epidermal growth factor (EGF)-like growth factor are related heparin factors that have a part in a reproductive performance like the stimulation of trophoblast cell, blastocyst attachment to the uterine and blastocyst invasion[33,34]. During the first 3 months, heparin *via* α-poly-L-lysine has anti-apoptotic effects along with inhibition of trophoblast apoptosis and activation of growth factor receptors. Also, heparin by matrix metalloproteinase increases trophoblast invasion. In general, heparin can affect adhesions, apoptosis, and cell-to-cell contact during implantation, and also plays a role in fetal implantation[35-38].

There are conflicting results in this field. Jersak *et al* reported an interesting one with 15 failed IVFs. When enoxaparin was combined, the 16th cycle of IVF produced a normal male infant. The authors conclude that combination therapy, which consists of low molecular weight heparin (LMWH), can provide a prosperous IVF outcome[39]. A meta-analysis done by Potdar *et al* showed that, in women with recurrent implantation failure, use of LMWH along with IVF procedure significantly improved the livebirth rate by 79%[40]. Heparin treatment has dramatically improved implantation, as well as clinical pregnancy rates in subsequent IVF attempts for patients with recurrent implantation failure, diagnosed with thrombophilia[41,42].

In the Urman *et al*[43] and Qublan *et al*[42] studies, there was a significant rise in implantation ratio and pregnancy rates, with the intervention group receiving heparin, and they were introduced to LMWH as a safe drug during pregnancy. Our results are in line with these studies; furthermore, heparin is much cheaper. Seshadri *et al*'s studies displayed no remarkable difference in the live birth rate, clinical pregnancy rate, implantation and miscarriage rates in women taking heparin in contrast with placebo during IVF treatment[44]. In addition, in Hamdi *et al*'s study heparin had no statistically significant effect on pregnancy outcome[45]. In contrast, our results have shown meaningful differences in fertilization rate in the case and control groups.

At least one thrombotic factor was identified in 41.86% of patients with recurrent IVF-ET failure in this study. Azem *et al* found the incidence of thrombophilia 26.7% in repeated IVF failure[46]. Qublan *et al*[47] reported that 68.9% of women with repeated IVF failure had at least one thrombophilic factor. They found a frequency of homozygosity for the *FVL* mutation in 4.4% of patients and a frequency of heterozygosity for this mutation in 10% of patients, but not found in the present study. Both studies evaluated G1691A mutation in factor Ⅴ, which could not be found in the Iranian population.

This study showed a significant relevance between heparin administration and increased probability of positive pregnancy in women with thrombophilic related mutations. Pregnancy rate has been shown to be higher in women with thrombophilia treated with heparin. Pregnancy rate for mutation-free women treated with heparin was very close to the typical pregnancy rate at this center (30% per cycle). Various studies have shown that women with recurrent implantation failure may have thrombosis, but the specific cause has not yet been identified. The use of heparin in treatment has been evaluated in many studies with conflicting data. Many studies have shown that it may be effective in groups of patients with thrombotic mutations. Therefore, it may be necessary to identify subgroups of patients who may benefit[5,48].

Heparin seems to be helpful in IVF-ET failure patients. It prevents a thrombolytic accidents, increases cell-to-cell adhesion, trophoblast invasion and adhesion of blastocyst to the endometrium, which improves the pregnancy rate in women with repeated IVF failure[49,50].

This is the first study that investigated the effect of heparin on recurrent IVF-ET failure patients in the interest population (central Iran). We evaluated the prevalence of three genes' mutation: *FVL*, *MTHFR*, and *FⅡ* in studied patients. In the following, the effect of treatment with/without heparin in successful pregnant who had mutation/absence of mutation has been compared. Eventually, it seems that patients who had mutations and received heparin will

have better results than patients who did not have mutations and received heparin.

The limitation of the study was the identification of people who met the entry criteria and were willing to participate in this study. Furthermore, obtaining the necessary kits and enzymes was time-consuming and costly.

In conclusion, this study shows a significant relationship between the administration of heparin and the increased probability of pregnancy in women with thrombophilic mutations. With regards to a frequency rate of thrombophilia in IVF failure women, the use of unfractionated heparin, which is less expensive than LMWH and has not yet been reported with any pregnancy complications, is advised. According to the various results reports, and the small population of the present study, further studies with larger volumes are required.

Conflict of interest statement

All the authors declare that there are no conflicts of interest.

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Authors' contributions

Maryam Shirmohamadi performed the main steps of the manuscript and writing of the manuscript. Mehri Mashayekhy, Iraj Alipourfard, and Javad Fazeli collected the samples and helped to achieve restriction fragment length polymorphism. Nasrin Ghasemi, head of team, monitored and fixed technical errors during all study steps. The authors read and approved the final manuscript.

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