BACKGROUND: The pathology underlying recurrent implantation failures (RIF) is not clear and treatment options proposed are generally not evidence based. Although the effect of heparin on trophoblast biology has not been studied extensively, given the available data suggesting a possible beneficial effect of heparin on embryo implantation, we decided to undertake this pilot study.

METHODS: One hundred and fifty women with ≥2 failed assisted reproduction treatment cycles were included in this randomized open-label pilot trial. Participants underwent controlled ovarian stimulation with the long protocol and were randomly allocated to receive 1 mg/kg/day low molecular weight heparin (LMWH) or no treatment in addition to routine luteal phase support (LPS) on the day after oocyte retrieval. LPS and LMWH was continued up to the 12th gestational week in pregnant participants.

RESULTS: There were 26 (34.7%) live births in the LMWH group, and 20 (26.7%) in the control group (absolute difference 8.0%, 95% CI -4.2 to 24.9%, P = 0.29). There were 34 (45.3%) and 29 (38.7%) clinical pregnancies in the LMWH and control groups, respectively (absolute difference 6.6%, 95% CI -9.0 to 21.8%, P = 0.41). Implantation rates were 24.5 and 19.8% in the LMWH and control groups, respectively (absolute difference 4.7%, 95% CI -4.7 to 14.1%, P = 0.33).

CONCLUSION: Despite lack of statistical significance, observed relative increase by 30% in live birth rates with LMWH may be regarded as a clinically significant trend necessitating further research on the use of empirical LMWH in women with RIF and possibly in all women undergoing assisted reproduction treatment. Failure to demonstrate statistical significance of the observed treatment difference may be due to limited sample size of this pilot study.

Clinicaltrials.gov registration number: nCT00750451.

Key words: in vitro fertilization / assisted reproduction / recurrent implantation failure / heparin / randomized controlled trial

Introduction

The efficacy of assisted reproduction therapy (ART) in terms of live birth rates has remained relatively constant showing only gradual improvements over the years (Andersen et al., 2007). The rate-limiting step appears to be implantation of the pre-embryo which is a complex process dependent upon many variables, most of which have not been adequately defined. Implantation requires orchestration of multiple events including trophoblast development and timely expression of numerous molecules playing roles in apposition, nidation and invasion of the embryo into the endometrium. Failure of implantation in couples undergoing assisted reproduction is a relatively common occurrence despite the transfer of seemingly good quality embryos. Implantation failure occasionally may be a recurring phenomenon leading to despair in couples and frustration in their caregivers. Implantation failure has been attributed to many factors; however, for most, the causal relationship is not established. Although not all studies concur, congenital and acquired coagulation defects have been found to be more prevalent in women with recurrent implantation failures (RIF) (Sher et al., 1998b; Martinelli et al., 2003a, b; Stern et al.,...
et al. (2003; Qublan et al., 2006; Stern and Chamley, 2006). The notion that coagulation disorders may lead to implantation failure led to the use of anticoagulants mainly heparin during the course of ART treatment. Several studies have examined the effect of heparin on the outcome of ART in women with antiphospholipid antibodies (Sher et al., 1994; Schenk et al., 1996; Kutteh et al., 1997; Sher et al., 1998a; Stern et al., 2003). Despite the heterogeneity of studies with regard to selection of participants and interventions, results suggest a potential for improvement in outcome with anticoagulant therapy.

More recently, heparin has been proposed to play a role in the complicated process of implantation beyond its anticoagulant effects (Fiedler and Wurfel, 2004; Nelson and Greer, 2008). Several lines of evidence from in vivo and in vitro studies suggest a beneficial effect of heparin on embryo implantation through interactions with several adhesion molecules, growth factors, cytokines and enzymes such as matrix metalloproteinases (Nelson and Greer, 2008).

Given the available data on heparin and embryo implantation we decided to undertake a pilot clinical trial with the aim to assess a possible beneficial effect of low molecular weight heparin (LMWH) on the outcome of ART in women with RIF but without hereditary or acquired thrombophilias. Couples with RIFs of unexplained etiology were randomly allocated to receive LMWH or no treatment in addition to routine luteal phase support (LPS) following oocyte retrieval (OR).

### Materials and Methods

#### Study design and study population

This was an open-label, randomized, controlled pilot trial to assess whether administration of LMWH in the luteal phase after ICSI and embryo transfer cycles would increase implantation and pregnancy rates in women with RIF. The study was undertaken in the American Hospital of Istanbul, Turkey. hCG 10 000 IU was administered intramuscularly to fertilize the oocytes. Cleavage stage embryos were graded according to the leading follicle reached 20 mm in the mean diameter when the leading follicle reached 20 mm in the mean diameter accompanied by ≥2 follicles of >16 mm. Stimulation protocols and the indications for hCG injection or cycle cancellation did not change throughout the study period.

OR was performed 36 h after the administration of hCG. ICSI was used to fertilize the oocytes. Cleavage stage embryos were graded according to Hardarson et al. (2001). Embryos were transferred on the third day after ICSI. A maximum of four embryos were transferred.

The luteal phase was supported with 90 mg vaginal progesterone gel (Crinone 8%, Serono, Serono, Bedfordshire, UK) starting from the day of oocyte collection. LPS was continued until the pregnancy test...
performed 12 days after embryo transfer. Women with a positive pregnancy test continued the vaginal progesterone gel until the 12th week of gestation.

The study group was administered LMWH (Enoxaparin Sodium, Clexane, Aventis Pharma) at a dose of 1 mg/kg/day starting on the day after OR. Pre-filled syringes that contained 100 mg enoxaparin sodium per 1 ml were included in trial medication packages. Patients’ weights were rounded to the closest multiple of 10 kg, and 0.1 ml/10 kg/day Clexane was self-administered subcutaneously by the participants. LMWH was discontinued if the pregnancy test 12 days after embryo transfer was negative, but continued up to the 12th week of pregnancy if the test was positive. The control group received no medication besides progesterone gel. In the study group platelet count was done on the day of OR and 1 week after commencement of LMWH treatment.

Women with high-order pregnancies at the end of the 12th gestational week were offered fetal reduction with transabdominal ultrasound guided intracardiac potassium chloride injection in the 13th gestational week.

**Outcome measures**

Pregnancy was confirmed by measuring serum beta-hCG levels 12 days after embryo transfer. Clinical pregnancy was defined as the presence of a fetus with a heartbeat at 6 weeks of gestation, a multiple pregnancy was defined as a gestation with more than one fetus, and ongoing pregnancy was defined as pregnancy proceeding beyond the 20th gestational week. Live birth was defined as delivery of one or more live infants. Preterm delivery was defined as delivery before completion of 37th week of gestation. Implantation rate was calculated separately for each participant as number of gestational sacs divided by number of transferred embryos multiplied by 100, and treated as a continuous variable to account for multiple implantations in a woman.

**Statistical analysis**

Sample size calculation

Ongoing pregnancy rate was the primary outcome measure. Assuming an ongoing pregnancy rate of 25% in the control group in accordance with previous figures achieved in women with ≥2 failed treatment cycles in our center, it was calculated that approximately 700 participants would be required to detect an absolute 10% increase with an alpha error level of 0.05 and beta error level of 0.2. Considering the difficulty of recruiting so many participants with RIF to a single center trial, the latest recruited participant that achieved an ongoing pregnancy, and women lost to follow-up before completion of the 20th gestational week but before delivery or expected completion of the 40th gestational week. Women lost to follow-up during the first period were considered not to have an ongoing pregnancy, and women lost to follow-up in the second period were considered not to have a live birth in the intention-to-treat analysis (Fig. 1).

Platelet counts did not change significantly in the LMWH group and three women in the control group were lost to follow-up before completion of the initially planned follow-up period (completion of the 20th gestational week for the latest recruited participant that achieved an ongoing pregnancy), and another two women in the LMWH group were lost to follow-up after completion of the 20th gestational week but before delivery or expected completion of the 40th gestational week. Women lost to follow-up during the first period were considered not to have an ongoing pregnancy, and women lost to follow-up in the second period were considered not to have a live birth in the intention-to-treat analysis (Fig. 1).

Platelet counts did not change significantly in the LMWH group during the study period. None of the patients experienced any adverse effects other than small ecchymoses around the LMWH injection sites. None of the participants in the LMWH group discontinued treatment due to pain or ecchymoses around the injection site. Ecchymoses that would be suggestive of LMWH self-administration were not observed in control group women during the physical examination undertaken on the day of the pregnancy test and again at the 6th week of gestation.

There were 34 (45.3%) and 29 (38.7%) clinical pregnancies in the LMWH and control groups, respectively ($P = 0.41$). Implantation rates were 24.5 and 19.8% in the LMWH and control groups, respectively ($P = 0.33$).

Four of five women with high-order multiple pregnancies opted for fetal reduction and their pregnancies were successfully reduced to

### Table I Characteristics of participants and assisted reproduction treatment cycles

<table>
<thead>
<tr>
<th></th>
<th>LMWH</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Female age in years</td>
<td>34.0 ± 5.0</td>
<td>34.8 ± 5.8</td>
</tr>
<tr>
<td>Etiology of infertility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulatory (%)</td>
<td>9 (12.0)</td>
<td>11 (14.7)</td>
</tr>
<tr>
<td>Tubal (%)</td>
<td>7 (9.3)</td>
<td>10 (13.3)</td>
</tr>
<tr>
<td>Endometriosis (%)</td>
<td>2 (2.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>33 (44.0)</td>
<td>29 (38.7)</td>
</tr>
<tr>
<td>Unexplained (%)</td>
<td>13 (17.3)</td>
<td>16 (21.3)</td>
</tr>
<tr>
<td>More than one factor (%)</td>
<td>11 (14.7)</td>
<td>9 (12.0)</td>
</tr>
<tr>
<td>Median number of previous failed cycles (interquartile range, minimum and maximum values)</td>
<td>2 (2–4, 2–14)</td>
<td>2 (2–4, 2–7)</td>
</tr>
<tr>
<td>Mean number of oocytes (standard deviation)</td>
<td>8.7 (4.5)</td>
<td>7.6 (4.7)</td>
</tr>
<tr>
<td>Mean number of MII oocytes (standard deviation)</td>
<td>6.4 (3.2)</td>
<td>5.3 (3.6)</td>
</tr>
<tr>
<td>Mean number of embryos transferred (standard deviation)</td>
<td>2.6 (0.7)</td>
<td>2.6 (0.8)</td>
</tr>
<tr>
<td>Mean number of grade I–II embryos transferred (standard deviation)</td>
<td>2.2 (0.9)</td>
<td>2.3 (1.1)</td>
</tr>
</tbody>
</table>

LMWH: low molecular weight heparin; MII: metaphase two.

* $p$-value $= 0.99$.

* $p$-value $= 0.54$. 

Three women in the LMWH group and three women in the control group were lost to follow-up before completion of the initially planned follow-up period (completion of the 20th gestational week for the latest recruited participant that achieved an ongoing pregnancy), and another two women in the LMWH group were lost to follow-up after completion of the 20th gestational week but before delivery or expected completion of the 40th gestational week. Women lost to follow-up during the first period were considered not to have an ongoing pregnancy, and women lost to follow-up in the second period were considered not to have a live birth in the intention-to-treat analysis (Fig. 1).
twins in the 13th gestational week. While one of these women was lost to follow-up after the 20th gestational week, three of them delivered live twins at 34th, 35th and 38th weeks. One participant in the LMWH group, with a quadruplet pregnancy refused fetal reduction and delivered quadruplets at 32nd week of gestation.

Number of live births were 26 (34.7%) and 20 (26.7%) in LMWH and control groups, respectively ($P = 0.29$). There were 28 (37.3%) ongoing pregnancies beyond 20th gestational week (initially defined primary end-point) in the LMWH group, and 20 (26.7%) in the control group ($P = 0.16$) (Table II, Fig. 2).

Numbers of preterm deliveries were 9 (34.6%) in LMWH and 6 (30.0%) in control groups ($P = 0.74$). Three women delivered in 32nd week (one set of quadruplets, one set of twins and a singleton, all in LMWH group), one woman (singleton in control group) delivered in 33rd week, four women delivered in 34th week (two sets of twins in LMWH group and two sets of twins in the control group), four women delivered in the 35th week (all twins, three and one in LMWH and control groups, respectively), three women delivered in the 36th week (one singleton in LMWH group and two sets of twins in the control group).

None of the infants had any congenital malformations. One boy (from the LMWH group) had a unilateral undescended testis, and another infant delivered at the 32nd week (from the LMWH group) underwent surgery due to necrotizing enterocolitis.

The administration of LMWH did not statistically significantly improve outcome parameters as compared with controls. Similar results were observed in cycles with high-order (≥3) previous implantation failures (Table III).

Figure 1 Flowchart of the study.
None of the women reported developing pre-eclampsia during the gestational period.

Discussion

To the best of our knowledge this is the first study assessing the efficacy of empirical LMWH administration throughout the luteal phase of ART cycle in women with RIF but without any evidence of thrombophilia. The results of this pilot study demonstrate a trend towards increased embryo implantation and live birth rates with empirical administration of LMWH in couples with unexplained RIF. Despite a lack of statistical significance, the observed relative increase of 30% in live birth rates with LMWH may be regarded as a clinically significant trend necessitating further research on the use of empirical LMWH in ART. Lack of statistical significance might be due to low statistical power associated with the small sample size of the current trial. A sample size of 150 would enable demonstration of an absolute difference of 24% up from a baseline event rate of 25% in the control group as statistically significant with the same alpha and beta error levels of 0.05 and 0.2. A difference of such magnitude corresponds to a relative increase by 100%, which is obviously not a realistic expectation. The present trial was defined as a pilot trial, in order to note limitations of its size. On the other hand, it should be considered that the observed differences might have been reduced had more participants been recruited.

Couples with laboratory or clinical findings of thrombophilias were excluded in order to ascribe any observed beneficial effect of LMWH on the outcome to its non-anticoagulant effects. The rationale behind selective inclusion of women with previously failed treatment cycles was 2-fold: firstly couples with previously failed treatment cycles generally demand a modification in their treatment protocols, and they were anticipated to be willing to participate in this pilot trial in the light of previous data suggesting a beneficial effect of heparin in RIF. The second reason was to evaluate the effect of LMWH on the outcome in this difficult to manage subset of patients with the aim to provide them another treatment option if found to be effective. Enoxaparin was chosen due to its established safety profile in

Table II  Overall outcomes

<table>
<thead>
<tr>
<th></th>
<th>LMWH</th>
<th>Control</th>
<th>Absolute difference, 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical pregnancy (%)</td>
<td>34/75 (45.3)</td>
<td>29/75 (38.7)</td>
<td>6.6% [−9.0% to +21.8%]</td>
<td>0.41</td>
</tr>
<tr>
<td>Multiple pregnancy (%) (twins, triplets, quadruplets)</td>
<td>12/34 (35.3) (9,1,2)</td>
<td>10/29 (34.5) (8,2,0)</td>
<td>0.8% [−22.8% to +24.4%]</td>
<td>0.94</td>
</tr>
<tr>
<td>Implantation</td>
<td>24.5%</td>
<td>19.8%</td>
<td>4.7% [−4.7% to +14.1%]</td>
<td>0.33</td>
</tr>
<tr>
<td>Ongoing pregnancya (%) (&gt;20 weeks)</td>
<td>28/75 (37.3)</td>
<td>20/75 (26.7)</td>
<td>10.6% [−4.2% to +24.9%]</td>
<td>0.16</td>
</tr>
<tr>
<td>Live birthb (%)</td>
<td>26/75 (34.7)</td>
<td>20/75 (26.7)</td>
<td>8.0% [−6.7% to +22.7%]</td>
<td>0.29</td>
</tr>
</tbody>
</table>

LMWH: low molecular weight heparin; CI: confidence interval.
aThree patients in each group were lost to follow up at the 20th gestational week.
bFive patients in the LMWH group and three patients in the control group were lost to follow up prior to delivery.

Table III  Outcome in high-order (>3) RIF

<table>
<thead>
<tr>
<th></th>
<th>LMWH</th>
<th>Control</th>
<th>Absolute difference, 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical pregnancy</td>
<td>16/37 (43.2)</td>
<td>13/34 (38.2)</td>
<td>5.0% [−19.0% to +28.1%]</td>
<td>0.67</td>
</tr>
<tr>
<td>Multiple pregnancy (%) (twins, triplets, quadruplets)</td>
<td>6/16 (37.5) (5,1,0)</td>
<td>4/13 (30.8) (3,1,0)</td>
<td>6.7% [−30.3% to +40.3%]</td>
<td>0.99</td>
</tr>
<tr>
<td>Implantation</td>
<td>21.6%</td>
<td>16.0%</td>
<td>5.6% [−7.9% to +18.9%]</td>
<td>0.41</td>
</tr>
<tr>
<td>Ongoing pregnancya (%) (&gt;20 weeks)</td>
<td>14/ 37 (37.8)</td>
<td>8/34 (23.5)</td>
<td>14.3% [−9.1% to +35.5%]</td>
<td>0.19</td>
</tr>
<tr>
<td>Live birthb (%)</td>
<td>12/37 (32.4)</td>
<td>8/34 (23.5)</td>
<td>8.9% [−11.9% to +29.7%]</td>
<td>0.40</td>
</tr>
</tbody>
</table>

LMWH: low molecular weight heparin; CI: confidence interval.
aOne patient in each group were lost to follow up at the 20th gestational week.
bThree patients in the LMWH group and one patient in the control group were lost to follow up prior to delivery.
pregnancy (Greer and Nelson-Piercy, 2005). Platelet counts were not routinely repeated after the first week of treatment, because of a relatively low risk of heparin induced thrombocytopenia with the administration of LMWH. None of the participants had clinical or laboratory findings suggesting a decrease in renal function that would prolong the half-life of the medication and increase the risk for thrombocytopenia.

The overall lost to follow-up rate is acceptable for a prospective trial (8/150, 5.3%). Although the sample size of the trial is limited, the outcomes in these patients are not expected to significantly alter the current results. Moreover, the present study is not a trial that was aimed at providing definitive results on the subject, but rather it was designed as a pilot trial inquiring into the feasibility of further research on the issue. Recruitment of women with two rather than three previous failed cycles may be regarded as a limitation of the study. Although the primary goal of the present study was to assess the effect of LMWH on the outcome of ART in women with RIF, considering the proposed mechanisms of action discussed below, LMWH can be expected to affect all women undergoing ART in a similar manner. Thus, it was decided to include women with two previous failed cycles, rather than sticking to the traditional definition of RIF in order to facilitate timely collection of data in this single center pilot trial. Non-blinded design may be regarded as another limitation of the current trial; however, the outcome parameters are binary variables that do not allow for subjective evaluation. Hence the foreknowledge of treatment allocation is not expected to alter the outcome assessment. Moreover, physicians were not aware of treatment allocation at the time of embryo transfer. Participants were randomized to treatment arms after OR by the nurse coordinator, and previous treatment procedures were administered uniformly for all participants. Therefore, treatment bias was eliminated as well.

Regulation of trophoblast invasion is integral to successful implantation and maintenance of pregnancy. Women who are carriers of congenital or acquired thrombophilia are at increased risk of implantation failure and pregnancy complications such as spontaneous miscarriages, fetal growth restriction, stillbirth and pre-eclampsia. Heparin with its anticoagulant effects has been used successfully in carriages, fetal growth restriction, stillbirth and pre-eclampsia. Effects of heparin beyond anticoagulation have led to its unsupported use in women who are carriers of these thrombophilic mutations. Effects of heparin beyond anticoagulation have led to its unsupported use in the absence of thrombophilias in women undergoing ART cycles, as well as in women with a bad obstetric history (Tzafetts et al., 2002; Nelson and Greer, 2008).

Different mechanisms of action besides anticoagulation can be suggested for the beneficial effects of heparin on implantation (Nelson and Greer, 2008). These include the following.

E-cadherin is an intercellular adhesion molecule that has been shown to be involved in trophoblast differentiation and invasiveness (Xue et al., 2003). Expression of E-cadherin has been shown to be modulated by unfractioned heparin as well as enoxaparin in rat pregnancies (Erden et al., 2006) and by membrane-bound heparin-binding epidermal growth factor (HB-EGF) in human pancreatic cell lines (Wang et al., 2007). Shih le et al. (2002) reported decreased trophoblast motility and invasiveness in the presence of E-cadherin expression in implantation-site intermediate trophoblastic cell lines. These findings suggest that LMWH may facilitate implantation process by down-regulating E-cadherin expression in the decidua. However, there are other in vitro studies with conflicting results with regard to effect of heparin and LMWH on trophoblast motility and invasiveness (Quenby et al., 2004; Bose et al., 2005; Di Simone et al., 2007; Ganapathy et al., 2007).

HB-EGF is considered to play a role in the implantation process. In mouse studies, HB-EGF was found to improve blastocyst formation and promote trophoblast outgrowth (Das et al., 1994). HB-EGF expression in luminal and glandular epithelium from human endometrial biopsy specimens was shown to be maximized during the peri-implantation period, and presence of HB-EGF on the surface of pinopodes was demonstrated (Stavreus-Evers et al., 2002). Tighty regulated expression of HB-EGF in luminal epithelium and of EGF-receptor in the blastocyst around the time of implantation suggests an important function for this ligand-receptor signaling in embryo-maternal cross-talk for implantation (Paria, 1993; Hamatani et al., 2004). Heparin, via heparan sulfate proteoglycans or HB-EGF may be involved in blastocyst adhesion and invasion (Fiedler and Wurfel, 2004; Nelson and Greer, 2008). Furthermore, HB-EGF was shown to enhance in vitro extravillous trophoblast differentiation (Leach et al., 2004). The authors showed that human EGF receptor-mediated autocrine and paracrine signaling by HB-EGF or other EGF family members induced cytotrophoblast differentiation to an invasive phenotype. Other studies have reported similar effects with LMWH (Quenby et al., 2004; Di Simone et al., 2007).

Enhanced trophoblast migration and invasiveness due to a LMWH induced increase in free insulin-like growth factor 1 is another proposed mechanism for a beneficial effect of LMWH on the implantation process (Lacey et al., 2002; Moller et al., 2006; Nelson and Greer, 2008).

Possible interactions between heparin/LMWH and cytokines and matrix metalloproteinases that can modulate the potential for successful implantation have been assessed by Nelson and Greer in a review of the role of heparin in assisted reproduction (Nelson and Greer, 2008).

In conclusion, heparin and its derivatives seem to affect at least some of the many variables that are involved in the complex process of implantation. The results of this pilot study suggest a potential beneficial effect of LMWH on the clinical outcome of ART in women with RIF. This finding, however, has yet to be corroborated in larger trials. Despite in vivo and in vitro evidence in favor of the alleged beneficial effect, wholesale adoption of this strategy into routine practice should be avoided until further research confirms the presence of such an effect and reveals its exact mechanism of action. Even if the proposed beneficial effect is confirmed, the subsets of patients in whom LMWH would be most effective and the appropriate dosing and duration of LMWH administration needs to be determined before unselectively exposing women and their embryos to the potential side effects of the medication.

Author’s Role

The author’s contributions are as follows; B.U.: design and institution of the study protocol, drafting, review and final preparation of the article, approval of the final version; B.A.: design and institution of the study protocol, collection and analysis of data, drafting, review and final preparation of the article and approval of the final version; K.Y., C.A., S.A., R.M. and B.B.: institution of the study protocol, review and approval of the final version.
Acknowledgement

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