

Embryo implantation after assisted reproductive procedures and maternal thrombophilia

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Background and Objectives. Women undergoing assisted reproductive procedures, such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), fail to achieve pregnancy in approximately 70% of cases. Postulating that among the possible causes of failure of embryo implantation might be an impairment of the uteroplacental circulation due to hypercoagulability in the mother, we investigated the association between thrombophilia and failure to achieve pregnancy after IVF or ICSI.

Design and Methods. A case-control study was carried out in 234 women undergoing IVF or ICSI and in 234 women who, in the same period, conceived naturally. Thrombophilia due to mutations in genes encoding coagulation factor V (G1691A), prothrombin (G20210A), methylene-tetrahydrofolate reductase (C677T) and the presence of antiphospholipid antibodies was searched for.

Results. The prevalence of factor V, prothrombin and methylene-tetrahydrofolate reductase mutations was similar in the 162 women who failed to achieve pregnancy after IVF or ICSI and in control women (5% and 2% for factor V G1691A, odd ratio 2.4, 95% CI 0.8-7.4; 3% and 6% for prothrombin G20210A, odds ratio 0.5, 95% CI 0.2-1.5; 19% and 20% for homozygous methylene-tetrahydrofolate reductase C677T, odds ratio 1.0, 95% CI 0.6-1.6). Nor was any association found when women who failed to achieve pregnancy were divided according to the total number of assisted reproductive procedures, age, type of procedure and cause of infertility. Antiphospholipid antibodies were not detected in any of the women.

Interpretation and Conclusions. This study provides no evidence for an association between maternal thrombophilia and failure to achieve pregnancy after assisted reproductive procedures. Routine anticoagulant treatment in women undergoing assisted reproductive procedures is not warranted.

Key words: infertility, *in vitro* fertilization, intracytoplasmic sperm injection, blood coagulation.

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Assisted reproductive procedures, such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI), offer many otherwise infertile couples the opportunity to conceive. However, failure of implantation of the fertilized embryo in the endometrium remains a major problem, the likelihood of a live birth per embryo-transfer procedure being approximately 30 % with either IVF or ICSI.¹ The mechanisms responsible for the high rate of failure of the assisted reproductive procedures are largely unclear and involve unsuccessful implantation or placentation.¹ Abnormal placentation is responsible for various obstetric complications, such as pregnancy loss, *abruptio placentae* and pre-eclampsia.²⁻⁴ Recently, an association between these conditions and hypercoagulability due to the presence of thrombophilia has been observed,^{3,4} although the matter is still controversial.^{5,6} Determinants of thrombophilia are deficiencies of the naturally occurring anticoagulants antithrombin, protein C and protein S, point mutations in the genes encoding coagulation factor V (a substitution of guanine by adenine at nucleotide 1691, referred to as factor V Leiden) and prothrombin (a substitution of guanine by adenine at nucleotide 20210), and the presence of antiphospholipid antibodies.⁷ In addition, homozygosity for a cytosine to thymine substitution at nucleotide 677 in the gene encoding methylene-tetrahydrofolate reductase (a key enzyme in the conversion of homocysteine to methionine), often resulting in mildly elevated plasma concentrations of homocysteine, has been associated with obstetric complications,^{4,8} although the role of this mutation as a determinant of thrombophilia is still uncertain.^{7,9}

We hypothesized that hypercoagulability in the mother, leading to an impairment of the uteroplacental circulation, is among the possible causes of failure to achieve pregnancy after IVF or ICSI. An association between the hypercoagulable state due to the presence of antiphospholipid antibodies and unsuccessful embryo implantation has been observed, and women tested positive for antiphospholipid antibodies undergoing assisted reproductive procedures are commonly treated with heparin and aspirin.^{10,11} Although no clear evidence of an association between thrombophilia and infertility is available and data about the risks and benefits of anticoagulants in IVF/ICSI patients are limited, use of this therapy is becoming more and more common.¹² To determine whether or not thrombophilic conditions are associated with an increased probability of failure to achieve pregnancy, and therefore whether preventive anticoag-

ulant treatment is necessary in these cases, we carried out a case-control study of women with one or more failed cycles of IVF or ICSI.

Design and Methods

Study population

Consecutive women 40 years old or younger who were referred for either IVF or ICSI to two infertility clinics in Milan, Italy, between October 2000 and March 2002, were eligible for the study. Women were excluded if they had had hydrosalpinx or uterine fibroids, conditions that may negatively affect implantation. This study included only women in whom fresh embryos prepared with fresh sperm (not cryoconserved) were transferred. A score from 1 to 4 in decreasing order of quality was assigned to each transferred embryo according to its morphology, as previously described¹³ (1=blastomers of equal size - no cytoplasmic fragmentation; 2=blastomers of equal size - less than 20% of cytoplasmic fragmentation; 3=blastomers of distinctly equal size - less than 50% of cytoplasmic fragmentation; 4=blastomers of any size - more than 50% of cytoplasmic fragmentation). Women were followed until the time of uterine ultrasound examination, showing whether or not implantation had occurred. Controls were women who conceived naturally without hormonal ovarian stimulation therapy and gave birth to healthy infants. One control for each case was identified in the puerperium wards of the same hospitals to which the cases had been referred and in the same study period. Because of ethnic differences in the prevalence of mutations in factor V and prothrombin, only women of Caucasian origin were included.⁷ A complete obstetric and thrombotic history was obtained from all the women using a structured questionnaire.¹⁴ The study was approved by the institutional review boards of the participating hospitals, and all women gave their written informed consent to inclusion in the study.

Laboratory tests

Blood samples were taken at the time of the assisted reproductive procedure for cases and from one to five days after delivery for controls. Because both hormonal ovarian stimulation therapy and the post-partum period can influence plasma measurements of the naturally occurring anticoagulant proteins, antithrombin, protein C and protein S were not measured. The presence of the mutations adenine to guanine at nucleotide 1691 in the factor V gene, adenine to guanine at nucleotide 20210 in the prothrombin gene and cytosine to thymine at nucleotide 677 in the methylene-tetrahydrofolate reductase gene were determined by independent technicians unaware of the origin of the blood samples, according to previously described methods.¹⁵⁻¹⁷

Serum antiphospholipid antibodies were measured as lupus anticoagulant (including activated partial thromboplastin time assays, dilute Russel viper venom time, kaolin clotting time and silica clotting time) or anticardiolipin antibodies;¹⁸ titers of more than 20 IgG or IgM phospholipid units were considered to be a positive result.

Statistical analysis

Before starting the study, we calculated the sample size necessary in order to achieve a statistical power of 80%. Assuming a 3% prevalence of the factor V or prothrombin mutation in the general population, and an odds ratio of failure of assisted reproductive procedures increased by a factor of 4 in the presence of either mutation, 120 cases and 240 controls should have been included in the study. A 4-fold increased risk was assumed since the current literature reports that the mutations are associated with a 3- to 5-fold increased risk of various obstetric complications.^{3,4} Odds ratios and 95% confidence intervals (95% CI) adjusted for possible confounding factors such as age, gravidity and smoking status, were used as a measure of the association between failure of assisted reproductive procedures and the thrombophilia markers. Student's t-test was performed to compare the mean age of case and control women, the mean number of oocytes retrieved, the fertilization rate and the implantation rate of women with and without thrombophilia. χ^2 testing was used to compare the frequency of previous miscarriages and smoking status of case and control women, and to compare the prevalence of successful embryo implantation at first attempt in women with and without thrombophilia. Statistical analyses were performed with the Statistical Analysis Software package for Windows, version 8.1 (SAS Institute, Inc., Cary, North Carolina, USA).

Results

Of the 264 women undergoing assisted reproductive procedures, 26 were excluded from the study. Fourteen were non-Caucasian, 9 produced no oocytes after hormonal stimulation, and 3 were not tested because of inadequate blood samples. Of the remaining 238 women, 234 agreed to participate in the study. Infertility was unexplained in 37 couples, whereas reasons for the assisted reproductive procedure were male infertility in 86 couples, tubal abnormalities in 53, endometriosis in 18, and anovulatory cycles in 4. More than one cause was recognized in the remaining 36 couples.

The characteristics of women undergoing assisted reproductive procedures and control women are shown in Table 1. Case and control women were similar in terms of age, frequency of previous miscarriages and smoking status. One-hundred and forty-

four women (62%) underwent IVF and 90 (38%) ICSI. One hundred and forty-four women (62%) were at their first attempt. Only three women had had previous recurrent (3 or more) miscarriages (occurring within the first 12 weeks of gestation), and no woman had a prior late fetal death. Among the women who underwent assisted reproductive procedures, 72 (31%) became pregnant, for a pregnancy-rate of 20% after IVF and 48% after ICSI. The remaining 162 women failed to achieve pregnancy.

Table 2 shows that the prevalence of factor V Leiden, prothrombin mutation or homozygous methylene-tetrahydrofolate reductase gene mutation was similar in women who failed the assisted reproductive procedure and in control women. All mutations in factor V and prothrombin genes were heterozygous, and none of the women carried both mutations or showed the presence of antiphospholipid antibodies. Methylene-tetrahydrofolate reductase gene mutation coexisted with factor V Leiden in one woman and with prothrombin mutation in two women. No association between failure of assisted reproductive procedures and thrombophilia was observed after stratification of women according to the total number of procedures in their life; 3 of the 93 women (3%) at their first attempt carried factor V or prothrombin mutation, 4 of the 43 (9%) at their second attempt, 1 of the 19 (5%) at their third attempt, and 1 of the 7 (14%) at their fourth attempt. The prevalence of homozygous methylene-tetrahydrofolate reductase mutation was approximately 20% in each group. Likewise, no association was found when the study population was divided according to the median age; 4 of the 109 women (5%) aged 35 or less and 3 of the 52 (6%) aged more than 35 carried factor V or prothrombin mutation; homozygous methylene-tetrahydrofolate reductase mutation was present in 20% and 13% of cases, respectively. The prevalence of the mutations was similar among the 115 women who underwent IVF and among the 47 who underwent ICSI, and did not change according to the causes of infertility (*data not shown*). One hundred and twenty-six women had at least one embryo of score 1 transferred, while 108 had only score 2 and 3 embryos. The pregnancy rates were 27% and 35%, respectively, and the mutations were equally distributed among women who failed to become pregnant and those who did achieve a pregnancy, independently on the quality of the embryos. The mean number (\pm SD) of oocytes retrieved was similar in women with thrombophilia and in those without (10.7 ± 6.9 and 11.2 ± 5.6), as was the fertilization rate (74% and 68%) and the implantation rate (14% and 15%).

Of the 72 women who became pregnant after assisted reproductive procedures, 3 (4%) carried factor V Leiden, 4 (6%) the prothrombin mutation, and 10 (14%) the methylene-tetrahydrofolate reductase mutation. Fifty-one were at their first attempt and

Table 1. Clinical characteristics of the women undergoing assisted reproductive procedures and control women.

Characteristics	Women undergoing assisted reproductive procedures	Women with spontaneous conception
Number	234	234
Median age (range)	35 (24-40)	26 (16-40)
<i>In vitro</i> fertilization, no. (%)	144 (62)	—
First cycle	91 (63)	—
Second cycle	37 (26)	—
Third cycle	12 (8)	—
Fourth cycle	4 (3)	—
Intracytoplasmic sperm injection, no. (%)	90 (38)	—
First cycle	53 (59)	—
Second cycle	18 (20)	—
Third cycle	14 (16)	—
Fourth cycle	3 (3)	—
Fifth cycle	2 (2)	—
At least one successful pregnancy, no. (%)	15 (6)	234 (100)*
At least one previous miscarriage, no. (%)	53 (23)	40 (17)
Gravidity, ° mean (range)	0.5 (0-5)#	0.9 (0-4)#
Current smoking, no. (%)	56 (24)	54 (23)

*by definition; °including deliveries, spontaneous and provoked abortions, and extra-uterine pregnancies; #excluding the actual pregnancy.

Table 2. Prevalence of mutations in factor V, prothrombin and methylene-tetrahydrofolate reductase in the women undergoing assisted reproductive procedures and control women.

	Women in whom assisted reproductive procedures failed (n=162)	Women with spontaneous conception (n=234)	Odds ratio* (95% CI)
Factor V gene mutation	8 (5)	5 (2)	2.4 (0.7-8.3)
Prothrombin gene mutation	5 (3)	13 (6)	0.5 (0.2-1.6)
Methylene-tetrahydrofolate reductase gene mutation	31 (19)	46 (20)	1.0 (0.5-1.7)

*adjusted for age, gravidity and smoking status.

22 had had previous attempts. Among the former, the prevalence of factor V Leiden was 4%, that of prothrombin mutation 8% and that of methylene-tetrahydrofolate reductase mutation 14%; among the latter, the prevalence was 8%, 0% and 14%, respectively. Six of the 7 women (86%) with either factor V Leiden or prothrombin mutation were at their first attempt, versus 45 of 65 (69%) without mutations (χ^2 0.83, $p=0.2$).

Discussion

The average pregnancy rate per cycle for either IVF or ICSI is only 30% or less,¹ indicating that implantation of the embryo and normal placental develop-

ment are complex mechanisms that are largely unclear. Although data in the literature are still controversial,^{3-6,19} it has been observed that hypercoagulability due to the presence of thrombophilia plays a role in various complications of pregnancy, such as miscarriages and fetal losses, *abruptio placentae*, pre-eclampsia and intrauterine growth retardation, through placental thrombosis as a possible underlying mechanism.²⁻⁴ The most common causes of inherited thrombophilia are two point mutations in genes encoding factor V (factor V Leiden) and prothrombin, found altogether in approximately 20% of patients with venous thrombosis and in 6% of the general population.⁷ With this as a background, we surmised that an impaired uteroplacental circulation due to maternal hypercoagulability might influence embryo implantation. To assess the association between thrombophilia and lack of implantation, we chose to investigate women who failed to achieve pregnancy after assisted reproductive procedures.

This study shows that failure to achieve pregnancy after IVF or ICSI is not associated with an increased probability of having thrombophilia. Furthermore, no association was found when women who failed to achieve pregnancy were divided according to the total number of assisted reproductive procedures, age, type of procedure and cause of infertility. An association between thrombophilia due to factor V Leiden or prothrombin mutation and IVF implantation failure has been reported by Grandone *et al.*,²⁰ who investigated a small series of 42 women undergoing the procedure and 210 controls who conceived spontaneously. These findings needed confirmation, because if such an association were true, anticoagulant prophylaxis at the time of the assisted reproductive procedure could be indicated in order to improve the implantation rate. Such treatment has also been considered in women with antiphospholipid antibodies undergoing assisted reproductive procedures,^{10,11} although no relationship between this acquired cause of thrombophilia and implantation failure has been demonstrated.^{21,22} Other than inherited coagulation abnormalities, such as factor V Leiden and prothrombin mutation and the acquired thrombophilia due to antiphospholipid antibodies, we also investigated the homozygous methylenetetrahydrofolate reductase mutation, which is often associated with mild hyperhomocysteinemia, a cause of thrombophilia.⁹ Data in the literature on the role of this mutation in the pathogenesis of thrombosis⁹ and obstetric complications^{3,4,8,23} are conflicting. On the other hand, the mutation seems to play a role in the pathogenesis of male infertility, since in its homozygous form it decreases folate levels in the blood and increases plasma homocysteine, affecting sperm count and motility in some instances.²⁴ As in other settings,^{3,25} in this study we found a similar prevalence of homozygous methylenetetrahydrofolate reductase mutation in case and control women.

Our study has some limitations. First, the odds ratio of failure of embryo implantation in women carriers of factor V Leiden was not statistically significant.²⁴ This may suggest that the possible association between the mutation and failure of embryo implantation is weak, and therefore it should have been assessed in a larger sample size in order to obtain statistical significance. Second, the possibility that implantation failure might depend on hypercoagulability of the embryo cannot be ruled out, since embryos and male partners were not tested for thrombophilia. However, since the aim of the study was to assess whether or not thrombophilia was associated with implantation failure through an impairment of uteroplacental circulation, and since maternal thrombophilia is an independent risk factor for other complications of pregnancy, we limited this investigation to women only. Third, it could be argued that a more appropriate control group of our case women would be women who conceived after assisted reproductive procedures. However, since few (two to three out of ten) women undergoing the procedures have successful embryo implantation, it would have been impracticable to obtain controls and cases at the same time. We believe that the choice of our control group of women who conceived naturally is suitable, since it has been recently observed that thrombophilia may represent a selective advantage on embryo implantation after assisted reproductive procedures.²⁶ This hypothesis could not be tested in our study, since it was designed to assess a relationship between thrombophilia and failure of implantation, and therefore the analyses done in the subgroup of women who became pregnant after IVF or ICSI (31% of the whole cohort) are limited by the relatively small sample. A selective advantage of factor V Leiden on implantation was demonstrated, observing that this was more likely to occur at first attempt in carriers than in non-carriers of the mutation.²⁶ These data were not confirmed by other investigators.²⁷ We found a slightly, but not statistically significant, higher implantation rate at the first attempt in women with factor V Leiden or prothrombin mutation than in those without (86% and 68%).

In conclusion, this study shows that thrombophilia does not predispose to failure of embryo implantation. A possible weak role of factor V Leiden remains to be assessed in studies of very large sample size. The lack of association between thrombophilia and failure to achieve pregnancy after assisted reproductive techniques at present does not support the routine use of anticoagulant treatment in women undergoing IVF or ICSI.

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Pre-publication Report & Outcomes of Peer Review

Contributions

IM: conception and design, drafting the article, final approval; ET: analysis and interpretation of data, revising the article, final approval; GR: conception and design, revising the article, final approval; PL-S: conception and design, revising the article, final approval; SP: analysis and interpretation of data, revising the article, final approval; TB: conception and design, revising the article, final approval; CL: conception and design, revising the article, final approval; PMM: conception and design, revising the article, final approval. Primary responsibility for the publication and Tables 1 and 2: IM. We thank all the women who agreed to participate in this study. We also thank the nurses of the Obstetric Hospitals for their help in withdrawing blood samples.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received May 5, 2003; accepted June 3, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

The role of factor V Leiden in failure of embryo implantation is unclear.

What this study adds

This study suggests that carriers of factor V Leiden mutation are not at higher risk of implantation failure, so at present anticoagulant treatment is not warranted in women undergoing assisted reproductive procedures.