Acquired and inherited thrombophilia: implication in recurrent IVF and embryo transfer failure

Hussein S.Qublan1,7, Suhair S.Eid2, Hani A.Ababneh3, Zouhair O.Amarin4, Aiman Z.Smadi1, Farakaid F.Al-Khafaji5 and Yousef S.Khader6

1Department of Obstetrics and Gynecology, 2Department of Coagulation and Hematology, 3Department of Immunology, Royal Medical Services, Jordan Armed Forces, 4Department of Obstetrics and Gynecology, Jordan University of Science and Technology, 5Department of Clinical Chemistry, Royal Medical Services, Jordan Armed Forces and 6Department of Public Health and Community Medicine, Jordan University of Science and Technology, Irbid, Jordan

BACKGROUND: The objective of this study was to determine the incidence of undiagnosed thrombophilic factors and its relation to IVF and embryo transfer failure in women who have had three or more previous IVF–embryo transfer cycles. METHODS: The study group comprised of 90 consecutive women with three or more previously failed IVF–embryo transfer cycles (group A). Two control groups were enrolled: group B (n = 90) included women who have had successful pregnancy after their first IVF–embryo transfer cycle, and group C (n = 100) included women who conceived spontaneously with at least one uneventful pregnancy and no previous history of miscarriage. All women were tested for the presence of inherited [factor V Leiden (FVL) mutation, prothrombin mutation, methylenetetrahydrofolate reductase (MTHFR) mutation and deficiencies in proteins S and C and antithrombin III] or acquired (lupus anticoagulant and anticardiolipin) thrombophilic factors. RESULTS: An increase in the incidences of FVL, MTHFR and antiphospholipid antibodies was found in the study group compared with the two control groups. At least one inherited or acquired thrombophilic factor was detected in 68.9% of women with repeated IVF failure compared with 25.6 and 25% in the groups B and C, respectively (P < 0.01). Combined thrombophilia (two or more thrombophilic factors) was significantly higher in women who have had repeated IVF failure as compared with the two control groups (35.6 versus 4.4 and 3%) (P < 0.0001). CONCLUSION: Thrombophilia has a significant role in IVF–embryo transfer implantation failure. Women with repeated IVF–embryo transfer failure should be screened for thrombophilia.

Key words: implantation failure/IVF/thrombophilia

Introduction

About one-third of women undergoing IVF and embryo transfer will achieve an ongoing pregnancy. Thus, failure to achieve pregnancy implies failure of the pregnancy at implantation or at a time shortly thereafter. Several factors have been recognized to affect either success or failure rate of IVF–embryo transfer. Such factors include age, parity, previously successful pregnancy, basal hormonal levels, number of antral follicles before stimulation, endometrial thickness, embryo grading, position and length of uterus and technique of embryo transfer (Templeton et al., 1996; Egbase et al., 2000; Ng et al., 2000; Surrey and Schoolcraft, 2000; Schoolcraft et al., 2001; Alkande et al., 2002; Kovacs et al., 2003; Qublan et al., 2005). Inherited [factor V Leiden (FVL) mutation, methylenetetrahydrofolate reductase mutation (MTHFR) (C677T), prothrombin gene mutation (G20210A), protein C deficiency, protein S deficiency and antithrombin III (ATIII) deficiency] or acquired [lupus anticoagulant (LA) and anticardiolipin (ACL)] thrombophilia have been recently implicated in early pregnancy loss and IVF implantation failure, by impairing the initial vascularization process occurring at implantation, which is necessary for a successful pregnancy (Geva et al., 1995; Grandone et al., 2001; Azem et al., 2004; Kujovich, 2004). There are limited data in the literature associating thrombophilia and repeated IVF–embryo transfer failure (Grandone et al., 2001; Martinelli et al., 2003; Azem et al., 2004; Coulam et al., 2006). We conducted this study to determine the incidence of undiagnosed thrombophilic factors and its relation to IVF–embryo transfer failure in women who have had three or more failed previous IVF–embryo transfer cycles.

Materials and methods

Ninety consecutive women with a history of at least three previously failed IVF–embryo transfer treatments, presenting to the infertility clinic between January 2001 and August 2005, were included in this
study (group A). Women’s age ranged from 23 to 44 years (mean ± SD, 31 ± 4.2). Two control groups were enrolled: group B (n = 90) included women who had had successful pregnancy after their first IVF–embryo transfer cycle. Their ages ranged from 22 to 40 years (mean ± SD, 30 ± 3.1). The second group (group C) consisted of 100 women who had conceived spontaneously with at least one uneventful pregnancy and no previous history of miscarriages. Women’s age in this group ranged from 17 to 41 years (mean ± SD, 30 ± 2.8). Women with endometriosis, hydrosalpinx, abnormal uterine cavity on the hysterosalpingogram and history of thromboembolic disease and those who were receiving hormonal treatment were not included in the study group. All women were investigated for the presence of inherited (FVL mutation, prothrombin mutation, MTHFR mutation and deficiencies in proteins S and C and ATIII) or acquired (LA and ACL) thrombophilic factors. In the IVF–embryo transfer cycles, only cycles in which grade 1 and 2 embryos were transferred were included in the study group. Indications for IVF treatment included anovulation, unexplained infertility and male and tubal factor.

All patients in the IVF programme received a standardized protocol of controlled ovarian stimulation using a combined regimen of GnRH agonist (triptorelin, Ipsen Pharma Biotech, Signes, France) and human menopausal gonadotrophin (Menogon, Ferring GMBH, Wittland II, Germany), as described elsewhere (Qublan et al., 2005).

Serum samples were obtained 5–7 days before the commencement of the ovarian stimulation from women in groups A and B and at least 2 months after delivery or abortion in group C. Peripheral blood was collected in Na₂ EDTA vacuum tubes (Becton Dickinson L, Rutherford, NJ, USA). The genotype of the three mutations—FVL mutation G1691A, prothrombin mutation (PTH) G20210A and MTHFR—was detected by genomic DNA isolation from 300 ml of buffy coat using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). Biotinylated primers were used for PCR amplification. This was followed by hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes that were immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin–alkaline phosphatase and colour substrate. All genotypes of the three mutations were detected on the same strip according to a predetermined staining pattern.

Functional protein C levels were measured using a chromogenic assay (Diagnostica Stago, Paris, France). Protein C deficiency was diagnosed when functional AT levels were <80% of normal range, 80–120%). Immunoglobulin M (IgM) and IgG ACL antibodies were assayed using validated enzyme-linked immunosorbent assay (ELISA) calibrated against international standards, as described elsewhere (Coulam et al., 1997). ACL antibodies were reported in international units (positive when >10 IU/ml). Screening for LA was performed by the kaolin cephalin clotting time utilizing sensitive reagents and by the dilute Russell’s viper venom time with a neutralization procedure using frozen–thawed platelets. Results for LA were expressed as positive or negative. Serum levels of homocystine were determined using a fluorescence polarization immunoassay (Abbott Laboratory, Abbott Park, IL, USA). Normal values for homocystine ranged between 4 and 11 μmol/l.

With the level of significance of 0.05 for testing one-sided hypothesis of the difference between proportions reported by Grandone et al. (2001), our sample size yielded a power of 90.7%.

The results of statistical analysis are presented as means ± SD and percentages. Chi-square test, Fisher’s exact test and unpaired Student’s t-test were used as appropriate. Bonferroni’s correction was used to adjust the P-values by multiplying the observed P-values from the significance tests by the number of tests, K; any K times that exceeded 1 was ignored. Then if any KP was < 0.05, the two groups were considered significantly different at the 0.05 level. The Statistics Package for Social Sciences software (version 11.5, SPSS, Chicago, IL, USA) and Microsoft Office Excel 2003 were used for data processing and data analysis.

Results

The clinical data of the study and control groups are summarized in Table I. Age was similar in the three groups. There were no significant differences between groups A and B in the duration and aetiology of infertility. Table II shows the frequency of the thrombophilic factors in the three groups, including the mutations in FVL, C677T MTHFR, FII G20210A, proteins S and C, ATIII and antiphospholipid antibodies (APLA) (LA and ACL antibodies). At least one inherited or acquired thrombophilic factor was detected in 68.9% (62/90) of women with repeated IVF failure compared with 25.6% (23/90) and 25% (25/100) in the successful IVF treatment and healthy controls, respectively. This difference was statistically significant (P < 0.01). The incidence of FVL mutation was higher in the study group compared with the two control groups. In women with repeated IVF failure, homozygosity for FVL mutation was found in 4.4% (4/90) of patients compared with none in the other two control groups. Similarly, 10% (9/90) of women in the study group were heterozygous for FVL mutation versus 1.1 and 2% in control groups B (P = 0.108).

Table I. Clinical data of the study and control groups

<table>
<thead>
<tr>
<th></th>
<th>Study group</th>
<th>Control group</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A (n = 90)</td>
<td>Group B (n = 90)</td>
<td>Group C (n = 100)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>31 ± 4.2</td>
<td>30 ± 3.1</td>
<td>30 ± 2.8</td>
</tr>
<tr>
<td>Duration of infertility (year)</td>
<td>6 ± 2.1</td>
<td>5.7 ± 2.4</td>
<td>0.336</td>
</tr>
<tr>
<td>Aetiology of infertility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anovulation</td>
<td>16 (17.8)</td>
<td>18 (20)</td>
<td></td>
</tr>
<tr>
<td>Unexplained infertility</td>
<td>28 (31.1)</td>
<td>26 (28.9)</td>
<td></td>
</tr>
<tr>
<td>Tubal factor</td>
<td>12 (13.3)</td>
<td>15 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Male factor</td>
<td>34 (37.8)</td>
<td>31 (34.4)</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD and percentages.
Frequency of thrombophilic factors in the study groups

<table>
<thead>
<tr>
<th>Thrombophilic factors</th>
<th>Study group (n = 90)</th>
<th>Control group (n = 90)</th>
<th>Control group (n = 100)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B</td>
<td>Group C</td>
<td>A versus B</td>
</tr>
<tr>
<td>Factor V Leiden</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>9 (10)</td>
<td>1 (1.1)</td>
<td>2 (2)</td>
<td>0.108</td>
</tr>
<tr>
<td>Homozygous</td>
<td>4 (4.4)</td>
<td>0</td>
<td>0</td>
<td>0.049</td>
</tr>
<tr>
<td>Methylene tetrahydrofolate reductase (C677T) mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>7 (7.8)</td>
<td>8 (8.9)</td>
<td>9 (9)</td>
<td>1.00</td>
</tr>
<tr>
<td>Homozygous</td>
<td>13 (14.4)</td>
<td>3 (3.3)</td>
<td>2 (2)</td>
<td>0.046*</td>
</tr>
<tr>
<td>Prothrombin G20210A gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>5 (5.6)</td>
<td>3 (3.3)</td>
<td>3 (3)</td>
<td>0.720</td>
</tr>
<tr>
<td>Homozygous</td>
<td>1 (1.1)</td>
<td>1 (1.1)</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>2 (2.2)</td>
<td>1 (1.1)</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>3 (3.3)</td>
<td>2 (2.2)</td>
<td>3 (3)</td>
<td>1.00</td>
</tr>
<tr>
<td>Antithrombin III deficiency</td>
<td>1 (1.1)</td>
<td>0</td>
<td>1 (1)</td>
<td>1.00</td>
</tr>
<tr>
<td>Lupus anticoagulant</td>
<td>8 (8.9)</td>
<td>2 (2.2)</td>
<td>2 (2)</td>
<td>0.294</td>
</tr>
<tr>
<td>Anticardiolipin</td>
<td>9 (10)</td>
<td>2 (2.2)</td>
<td>3 (3)</td>
<td>0.222</td>
</tr>
<tr>
<td>Combined thrombophilia</td>
<td>32 (35.6)</td>
<td>4 (4.4)</td>
<td>3 (3)</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

P-Values are adjusted using Bonferroni’s correction. Values within parentheses are percentage.

Significant at α = 0.05.

and C (P = 0.162), respectively. MTHFR C677T mutation was found in 22.2% (20/90) of women in the study group compared with 12.2 and 11% in control groups B and C, respectively. Furthermore, 65% (13/20) of women in the study group were homozygous for C677T MTHFR mutation compared with 27.3 and 18.2% in either of the control groups. In contrast, heterozygous C677T MTHFR mutation with hyperhomocystinaemia was similar in the three groups. In women with C677T MTHFR mutation, hyperhomocystinaemia was found to be significantly higher in the study group [60% (12/20) of women] compared with each control group [9.1% (1/11) each] (P < 0.0001). Homozygous C677T MTHFR mutation with hyperhomocystinaemia was found in 55% (11/20) of patients in the study group compared with none in both control groups (P < 0.042). The incidence of heterozygous C677T MTHFR mutation with hyperhomocystinaemia was similar in the three groups.

Regarding the other thrombophilic factors, ACL antibodies and LA antibodies were more common in women with repeated IVF failures (18.9%) compared with 4.4 and 5% in either of the control groups. However, these differences were not statistically significant. Furthermore, there were no statistically significant differences between group A and group B and between group A and group C in the prevalence of G20210A mutation and deficiencies of ATIII and proteins C and S. Finally, combined thrombophilia (two or more thrombophilic factors) was significantly higher in women who had repeated IVF failure as compared with the control groups (35.6 versus 4.4 and 3%) (P < 0.0001).

Discussion

This study showed that at least one thrombophilic factor was detected in 68.9% of women who have had previously three or more IVF–embryo transfer failures. This incidence is higher than that reported by others (Geva et al., 1995; Grandone et al., 2001; Martinelli et al., 2003; Azem et al., 2004). There are many possible explanations for this discrepancy. First, all patients in our study were of the same ethnic origin. Differences in the prevalence of thrombotic mutations according to ethnicity are likely to result in various strengths of associations in different populations. Second, strict criteria for selecting patients in our study were applied. All women were apparently healthy with no previous history of thyroid disease, diabetes mellitus or thromboembolic disease. Furthermore, women with endometriosis, hydrosalpinx and abnormal uterine cavity on the hysterosalpingogram and those who were receiving hormone treatment were not included in the study group. In the IVF–embryo transfer cycles, only cycles in which grade 1 and 2 embryos were included. Such a selection may be responsible for the increased prevalence of thrombophilia in our study. The polymorphism of MTHFR C677T represented the most common inherited thrombophilic factor in our study (22.2%). This finding is consistent with previous reports. Martinelli et al. (2003) evaluating 162 women with failed IVF/ICSI treatment reported an incidence of 19%. Moreover, Azem et al. (2004) who investigated 45 women with a history of four or more failed IVF cycles reported an incidence of 17.8%. Homozygosity for the thermolabile variant of MTHFR predisposes to the development of hyperhomocystinaemia (Engbersen et al., 1995). It has been reported that homozygosity for a common C677T mutation in the 5,10-MTHFR gene that was associated with hyperhomocystinaemia leads to a 3-fold increase in risk for early miscarriages (Unfried et al., 2002). Heby (1995) reported that elevated maternal plasma homocysteine levels were associated with impaired DNA methylation and gene expression that lead to defective chorionic villous vascularization with subsequent early embryonic death. Moreover, investigating the chorionic villous vascularization by both histopathology and an image analysis system in spontaneous miscarriage tissues from 19 women with recurrent early pregnancy losses, Nelen et al. (2000) observed significantly smaller median area, perimeters and diameter per chorionic vascular element in women with elevated total homocysteine levels.

FVL is a genetic disorder that is characterized by an impaired anticoagulant response to activated protein C. The distribution of FVL varies greatly in ethnic groups (De Stefano.
et al., 1998). Mutation in FVL gene increases thrombin generation, with a 4- to 8-fold increased risk of thrombosis in the heterozygous mutation and 80-fold increased risk in the homozygous mutation (Kujovich, 2004). In a recent meta-analysis, 3- to 4-fold increased risk of recurrent early pregnancy loss was found in women with FVL mutation (Rey et al., 2003). Vascular placental insufficiency has been suggested as a potential cause of these early pregnancy losses. Results of our study showed that the prevalence of FVL mutation was higher in women with repeated IVF failure (14.4%) than was found in women with successful IVF–embryo transfer (1%) and the healthy controls (2%). However, these differences did not reach statistical significance. This finding is consistent with that reported by Grandone et al. (2001), who found an incidence of 11.1% in women with greater than or equal to three IVF failures compared to none in the control groups. Others have failed to find such association (Martinelli et al., 2003; Azem et al., 2004).

Moreover, Gopel et al. (2001), investigating 102 mother–child pairs who had had successful IVF, reported improved implantation rate as an advantage of having FVL mutation. Further research in this area is required to reconcile these different findings.

Elevated plasma prothrombin levels with 2- to 4-fold increased risk of venous thrombosis were found in association with a single-nucleotide substitution (G20210A) in the 3’ untranslated regions of the prothrombin gene (Kujovich, 2004). In the meta-analysis of Rey et al. (2003), mutation in prothrombin gene was associated with a 2- to 3-fold increased risk of recurrent early miscarriages. The prevalence of prothrombin gene mutation in our series was similar in the study and the control groups, a finding that is in contrast to that of Grandone et al. (2001) and in agreement with that of Azem et al. (2004) and Martinelli et al. (2003). The disagreement with Grandone et al. (2001) might be related to the smaller sample size of their series, where they investigated 18 women with greater than three IVF failures and 24 with successful IVF–embryo transfer, comparing them with 216 women who conceived spontaneously.

In Jordan, the background genotype frequency for FVL is 15%, for FII G20210A 2% and for MTHFR C677T 24% (Eid and Rihani, 2004). This is not in accordance with what we have found in the two control groups. The reason for this discrepancy may be that most of the study subjects of the cited paper were males. These gene mutation frequencies were significantly higher among thrombotic Jordanian patients (25.7% for FVL, 6% for FII G20210A and 31.7% for MTHFR C677T) (Eid and Shubeilat, 2005).

Deficiencies in the natural anticoagulant proteins C and S and ATIII occur much less frequently. Several studies have evaluated the potential role of these deficiencies in recurrent early pregnancy losses (Sanson et al., 1996; Raziel et al., 2001). Conflicting results were reported in this regard. This might be related to the rarity of these deficiencies and the smaller-size studies. Only one study has examined the prevalence and the role of these deficiencies in women with repeated IVF failure (Azem et al., 2004). Azem et al. (2004) found no significant association between these deficiencies and repeated IVF failures, which is consistent with the findings of our study.

APLAs are a nonorgan-specific antibodies directed against the membrane phospholipids or phospholipid-binding plasma proteins (Galli et al., 1990). The importance of these antibodies as an aetiological factor of implantation failure after IVF–embryo transfer is now well established (Birkenfeld et al., 1994; Kaider et al., 1996; Coulam et al., 1997). It has been suggested that APLAs reduce the levels of Annexin V, which is a potent anticoagulant, leading to thrombosis and possible implantation failure or early pregnancy loss (Lockwood and Rand, 1994; Matsubayashi et al., 2001). In our study, 18.9% (17/90) of women with repeated IVF failure were positive for APLAs compared with 4.4% and 5% in either of the control groups, but this difference was not statistically significant. This is not in agreement with previous reports (Birkenfeld et al., 1994; Kaider et al., 1996; Coulam et al., 1997). Birkenfeld et al. (1994) evaluated 56 women with implantation failure and 14 women who successfully conceived after IVF–embryo transfer. They found that 32% of women with implantation failure versus 10% in the control group were positive for APLAs (P = 0.02). Moreover, Kaider et al. (1996), investigating 42 women with IVF failure and 42 who had conceived successfully after IVF–embryo transfer, found that 26.2% of women in the study group compared with 4.8% in the control group were positive for APLAs (P = 0.01). These authors recommended testing for the presence of APLAs in all patients undergoing IVF before initiating treatment. More recently, Coulam et al. (1997), in a larger-size study, found that 69 women of 312 (22%) with implantation failure after IVF treatment were positive for APLAs compared with five women of 100 (5%) fertile control group (P < 0.01).

The results of our study showed that the combination of two or more thrombophilic factors was significantly higher in women who had repeated IVF failures compared with the controls. These findings are in agreement with those reported recently by Coulam et al. (2006), who observed that 74% of women with recurrent implantation failure after IVF–embryo transfer had three or more gene mutations compared with 20% of healthy fertile controls.

In summary, the results of our study indicate that thrombophilia may have a significant role in IVF–embryo transfer implantation failure. Women with repeated IVF–embryo transfer failure should be screened for thrombophilia. Prospective randomized controlled interventional studies with large numbers are needed to determine the effect of thromboprophylaxis in such cases.

References

Submitted on March 23, 2006; resubmitted on April 20, 2006, May 2, 2006; accepted on May 10, 2006