Antiphospholipid antibodies in women having in-vitro fertilization

M.A. Birdsall, G.M. Lockwood, W.L. Ledger, P.M. Johnson and L.W. Chamley

Antiphospholipid antibodies have an established association with pregnancy complications such as recurrent miscarriage, growth retardation, placental abruption and stillbirth but their mechanism of action is unclear. We have investigated whether antiphospholipid antibodies occur more frequently in women having in-vitro fertilization (IVF) and whether their presence is associated with the likelihood of failed implantation. We studied 240 women undergoing IVF treatment who were ≤38 years and had attempted fewer than three previous IVF cycles. Antiphospholipid antibodies (anticardiolipin and antiphosphatidyl serine immunoglobulin G and immunoglobulin M) were present in 36 out of 240 (15%) of the study population and were not associated with a failed IVF cycle or miscarriage. There was no association between the cause of infertility and the presence of antiphospholipid antibodies. Antiphospholipid antibodies were not detected more frequently in women with previous attempts at IVF compared with women having their first cycle, indicating that the high incidence of these antibodies is not due to the IVF treatment. There was a strong association between the presence of antiphospholipid antibodies and intrauterine growth retardation in singleton pregnancies (P < 0.005). We recommend routine screening for the presence of antiphospholipid antibodies in women having IVF in order to identify those pregnancies at increased risk of intrauterine growth retardation.

Key words: antiphospholipid antibodies/growth retardation/implantation/IVF

Materials and methods

IVF patients

The study group consisted of 240 women referred for IVF treatment. For entry into this study, women were required to be no more than 38 years old and to have attempted no more than three previous IVF cycles. Patients with markedly irregular cycles and markedly elevated follicular phase concentrations of follicle stimulating hormone (FSH, >20 IU/l) and patients with polycystic ovarian syndrome (confirmed by raised plasma luteinizing hormone (LH) and oligomenorrhea) were excluded.

The causes of infertility were defined as follows: significant endometriosis was held to be present if ovarian endometriomata had been noted at laparoscopy, and/or surgical or medical treatment required in the 6 months prior to IVF. Male factor infertility was deemed to be present if the estimated chance of conceiving was <15%, indicated by sperm parameters of <10 x 10^6 spermatozoa per

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ml and <40% motility. This was based on the experience gained with over 3000 cycles of IVF in the Oxford IVF Unit. Tubal disease was diagnosed if significant tubal adhesions or hydrosalpinges were present and the normal flow of dye was interrupted through observations at laparoscopy and dye insufflation.

The average age of women taking part in the study was 32.8 years (range 24.2–39.2) and the average weight was 62.2 kg (range 41.3–93.0). Tubal factor was deemed as the cause for fertility treatment in 161 out of 240 patients (67.8%), male factor in 47 out of 240 (19.6%), endometriosis in 26 out of 240 (10.8%) and mixed disorders accounting for six out of 240 (2.5%).

Pituitary suppression was achieved with the use of buserelin or nafarelin, started on day 21 of the cycle. A blood sample was taken after 14–21 days to check whether down-regulation had occurred (plasma oestradiol <40 pg/ml). Gonadotrophin therapy was then commenced at an individual dosage according to the patient's age, weight, previous response and early follicular FSH. Human chorionic gonadotrophin (HCG) was given as soon as there were three or more follicles >16 mm diameter. When these criteria had been met, the analogue and gonadotrophin therapies were discontinued and a single i.m injection of 10 000 IU HCG was administered.

Transvaginal oocyte recovery was performed 35 h after HCG administration. Oocytes were incubated for 5–6 h prior to insemination with freshly prepared semen or thawed cryopreserved semen, where the use of donor spermatozoa was required. Each oocyte was inseminated with 400 000 spermatozoa in 2 ml of medium and assessed 12–18 h later for the presence of two pronuclei.

On day 2, embryos were evaluated and the best were selected for transfer. A maximum of three embryos was transferred to the uterus transcervically 50–60 h after oocyte retrieval. Luteal support was provided by HCG injections for patients with low or average (<1000 pg/ml) oestradiol concentrations on the day of HCG injection or by progesterone injections for high responders. A pregnancy test was carried out using a monoclonal antibody-based urine test 2 weeks after embryo transfer. Those with a positive test were scanned 2 weeks later to detect a clinical pregnancy, i.e. fetal heart activity.

**Antiphospholipid antibody assays**

Blood was drawn for antiphospholipid antibody assays at the time of the down-regulation blood test, i.e. prior to commencing gonadotrophin therapy. Serum samples were then frozen in aliquots and stored at −20°C. The assays were not performed until all babies in the study were delivered. Anticardiolipin and antiphosphatidyl serine autoantibodies were measured blind by L.W.C. using an assay described previously (Chamley et al., 1991; Johns et al., 1994). Briefly, NUNC (Life Technologies, UK) 96-well enzyme-linked immunosorbent assay (ELISA) plates were coated overnight with 50 μl/well of an ethanolic solution of cardiolipin or phosphatidyl serine (50 μg/ml) and allowed to air dry at 4°C. Plates were blocked with 100 μl/well 10% newborn calf serum (Life Technologies) diluted in phosphate-buffered saline (PBS), pH 7.4 for 60 min at room temperature. The blocking solution was discarded and patients' samples or standards, diluted 1:100 in fresh blocking solution, were incubated on the plates for 60 min. The samples were discarded and the plates washed three times with PBS. 100 μl/well of horseradish peroxidase conjugated, affinity purified, antihuman immunoglobulin γ or μ chain antiserum (Caltag) diluted in 1:3000 in blocking solution incubated on the plates at room temperature for 60 min. The plates were again washed three times with PBS and 100 μl/well of a solution of o-phenylene diamine dihydrochloride (1mg/ml) incubated for 30 min in the dark to allow colour development. The reaction was stopped by the addition of 50 μl/well 10% HCl and the optical density at 492 nm determined using a Titretek Multiscan Plus automated ELISA reader (ICN Flow, UK). Standards used in these assays had been calibrated against standards purchased from Louisville APL Diagnostics, Inc. (Louisville, USA). The units MPL and GPL were used which indicated ~1 μg of immunoglobulin (Ig)G or IgM class antiphospholipid autoantibody respectively. Those samples containing five or more MPL or GPL units were considered to be positive in both the anticardiolipin and antiphosphatidyl serine antibody assays (Pattison et al., 1993). All serum samples were screened, batchwise, in duplicate on at least two occasions. Any sample found to be positive in the initial screen was retested using both an antigen coated plate and a blank plate to ensure binding was specific for the phospholipid tested.

Antinuclear antibodies were detected using Hep 2 cells immobilized on 8-well slides. Patient sera were diluted 1:40 in PBS and added to the slides for 60 min. The slides were then washed with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated antihuman IgG (Dako, UK) for 30 min. Following washing again with PBS, the slides were mounted and examined using a Leitz fluorescent microscope (Leitz UK Ltd., UK). Positive and negative controls were included on each slide. Samples identified as positive in the original screen were then titred on the Hep 2 cell slides.

**Statistical analysis**

The major endpoint in this study was pregnancy outcome. This was categorized as either a negative pregnancy test 2 weeks post-embryo transfer, or a biochemical pregnancy which was defined as a positive pregnancy test although no fetal heart activity was visible on a 6 week ultrasound scan, or an established miscarriage (i.e. a fetal loss prior to 20 weeks), or a livebirth. Secondary endpoints included causes of infertility, birthweight and gestational age. All these endpoints were compared to the presence or absence of aPL. Growth retardation was defined as birthweight <10th centile for locally derived data on singleton pregnancies (Yudkin et al., 1987). No such information for multiple pregnancies exists. Statistical comparison of proportions between groups was carried out using the χ² test. Student's t-tests were used to compare the mean values of groups. P < 0.05 was regarded as significant.

**Results**

Antiphospholipid antibodies of any type were demonstrated in 36 out of 240 (15%) of the women undergoing IVF in the study (Table I). Antiphosphatidyl serine antibodies (IgG and/or IgM) were present in 28 out of 240 (11.6%) and anticardiolipin antibodies (IgG and/or IgM) were present in 29 out of 240 (12.1%); 21 women had both anticardiolipin and antiphosphatidyl serine antibodies. Several large studies of unselected subjects have demonstrated the prevalence of both anticardiolipin and antiphosphatidyl serine antibodies to be in the range of 1–3% (El-Roeiy and Gleicher, 1988; Lockwood et al., 1987; Pattison et al., 1993). The study performed by Pattison et al. (1993) examined 1000 consecutively-booked antenatal patients. Using the same assay and laboratory personnel as in this study, nine out of 933 (1%) of women had anticardiolipin antibodies. Thus anticardiolipin antibodies appeared significantly over-represented in the current study population (P < 0.0001, 95% confidence interval (CI) 0.086–0.136). Antinuclear antibodies were detected in 15 out of 240 (6.3%) of the IVF study population, which was slightly above the prevalence of antinuclear antibodies in the general obstetric
Table I. Major outcome measures comparing women with antiphospholipid antibodies to those without antiphospholipid antibodies. Figures in parentheses are percentages

<table>
<thead>
<tr>
<th></th>
<th>Antiphospholipid negative n = 204</th>
<th>Antiphospholipid positive n = 36</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean no. oocytes (SD)</td>
<td>6.7 (3.9)</td>
<td>6.1 (3.2)</td>
<td>0.38</td>
</tr>
<tr>
<td>Fertilization rate (SD)</td>
<td>64 (35)</td>
<td>62 (37)</td>
<td>0.75</td>
</tr>
<tr>
<td>Mean no. embryos transferred</td>
<td>2.2 (1.2)</td>
<td>2.3 (1.4)</td>
<td>0.65</td>
</tr>
<tr>
<td>Negative pregnancy test</td>
<td>108/172 (62.8)</td>
<td>1630/533 (33.4)</td>
<td>0.43</td>
</tr>
<tr>
<td>No. women &gt;1 live births</td>
<td>52/204 (25.5)</td>
<td>13/36 (36.1)</td>
<td>0.30</td>
</tr>
<tr>
<td>Multiple pregnancies</td>
<td>22/52 (42.3)</td>
<td>8/13 (61.5)</td>
<td>0.47</td>
</tr>
<tr>
<td>Miscarriage</td>
<td>5/57 (8.8)</td>
<td>1/14 (7.1)</td>
<td>0.74</td>
</tr>
<tr>
<td>Biochemical pregnancy</td>
<td>7/204 (3.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failed fertilization</td>
<td>24/204 (11.8)</td>
<td>5/36 (13.9)</td>
<td>0.94</td>
</tr>
<tr>
<td>No embryo transfer</td>
<td>6/204 (2.9)</td>
<td>1/36 (2.8)</td>
<td>0.61</td>
</tr>
<tr>
<td>Withdrew</td>
<td>2/204 (1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There were 65 women in this study who completed a successful pregnancy, with 11% having triplets and 32% having twin births. There was no difference in the number of oocytes obtained, fertilization rate, number of embryos transferred, pregnancy rate, miscarriage rate, livebirth rate or multiple pregnancy rates between those women with and without aPL (Table I). Positive pregnancy tests occurred in 64 out of 172 (37.2%) women who were negative for aPL and in 14 out of 30 (46.7%) women who were positive for aPL (P = 0.43, 95% CI 0.28–0.09). No fetal or neonatal deaths occurred in this study. Only one miscarriage occurred in the aPL group, and overall the number of miscarriages in this IVF population was low at six out of 71 (8.5%). No ectopic pregnancies occurred in the study population.

Pregnancies occurred in 57 out of 204 (27.9%) of the aPL-negative women, of whom five out of 57 (8.8%) had early miscarriages with the remaining women delivering at least one live baby (52 out of 204; 25.5%). In women with aPL, pregnancies occurred in 14 out of 36 (38.9%). The only significant difference between women who were aPL positive and those who were not, was that intrauterine growth retardation occurred more frequently in the aPL-positive women with singleton babies (See Tables II and III) (P = 0.007, 95% CI 0.27–1.06). Antinuclear antibodies had no significant associations with any of the major outcome measures in the study.

We also determined whether aPL were detected more frequently in women who had previous unsuccessful attempts at IVF. Women who were aPL positive comprised 28 out of 211 of the first cycles (13.3%) and eight out of 29 subsequent attempts (27.6%), and there was not a significant difference (P = 0.08, 95% CI 0.282–0.004).

The presence of aPL was compared to the cause of infertility. There were no differences observed in the causes of infertility between the women with aPL and those who were negative for aPL (Table IV). Nor was there any difference in the outcome measures when women with anticardiolipin and/or phosphatidyl serine IgG autoantibodies were compared to those of the IgM isotype. Women having two aPL specificities (n = 21) were compared with those with only one aPL specificity (n = 15) and no significant differences in outcome measures were observed between these two groups. Failed fertilization occurred in four out of 21 (19%) of those with two aPL compared with one out of 15 (6.7%) of those with only one aPL. This difference was not statistically significant (P = 0.5, 95% CI 0.11–0.35); however, only small numbers were involved.

Discussion

This study makes three important findings. Firstly, aPL are found in significantly more women needing IVF treatment compared with a general population. Secondly, aPL do not appear to interfere with the initial implantation process as pregnancies occurred with the same frequency in women with aPL as those without aPL. Thirdly, an association has been demonstrated between the presence of aPL at the time of IVF treatment and the occurrence of singleton growth-retarded babies conceived during that cycle. Antinuclear antibodies, studied as a control, had no influence on IVF outcome.

This IVF population would be expected to have an excellent pregnancy rate given the young age of women and high proportion of couples having their first attempt at IVF. It was surprising, therefore, to find such a high number of women who were aPL-positive. This begs the question as to whether the presence of these antibodies can interfere with normal conception and whether this effect might be overcome by the techniques of IVF? The association of aPL with the various causes of infertility was examined to help answer this question; however, women with aPL were divided evenly between the various causes of infertility. We did not find a higher number of women suffering from endometriosis, as has been reported by others (Kennedy et al., 1989). We also did not find that women with previous IVF attempts had a higher incidence of these antibodies (Geva et al., 1995). Previous researchers have suggested that the IVF process itself and, in particular, the egg collection, may stimulate the production of anticardiolipin antibodies (Fisch et al., 1991). This study has found a high prevalence of aPL in women prior to the commencement of IVF treatment, indicating the high incidence of aPL in women requiring IVF does not result from tissue damage caused by oocyte collection. This finding also adds weight to our hypothesis that aPL may interfere with normal conception.

How could aPL interfere with normal conception? We speculate that aPL may bind to phospholipids (or phospholipid binding proteins) in the ovary, preventing formation/release of a functional ovum from the ovary. Alternatively, aPL may bind to either the surface of the egg or spermatozoon and interfere with fertilization. The process of IVF, in which oocytes are collected, the cumulus stripped away and many more spermatozoa are exposed to each oocyte than are present in vivo, may overcome such interference and hence improve pregnancy rates. Apparently supportive data for such a specula-
be interrupted before conception can occur and that IVF may to support this hypothesis but suggests that the process may be one reason for the conflicting results. It has been suggested that unexplained infertility may be due to recurrent early loss et al, 1995) and this could recurrent miscarriage study (Rai et al, 1994) and the findings of Birkenfeld et al, 2004). Antiphospholipid antibodies would appear from our study, aggressive side-effects. Nevertheless, it would appear that screening all women having IVF treatment would be useful in order that high risk pregnancies may be identified and cared for in the appropriate setting with close antenatal and intrapartum monitoring. The association of growth-retarded babies and the preconceptual presence of aPL has not been previously reported in an IVF population. Babies born following IVF have a higher risk of growth retardation (MRC Working Party, 1990). Initially this was thought to be purely the influence of multiple pregnancies; however studies on singleton babies born after IVF have confirmed this finding (Tan et al, 1992). This study suggests that the presence of maternal aPL may be a contributor to the problem of small babies following IVF. Growth retarded babies have a higher risk of operative delivery, preterm birth, asphyxial brain damage, necrotizing enterocolitis, perinatal death, later learning difficulties and cerebral palsy. In women with aPL, growth retardation is not necessarily associated with hypertension. The attending clinician thus should be aware of these autoantibodies in order that appropriate pregnancy surveillance is instituted. The question arises whether all women having IVF should be screened for these autoantibodies. There is no consensus as to the optimal treatment for women with aPL (Sher et al, 1994) and treatments, such as corticosteroids, may have harmful side-effects. Nevertheless, it would appear that screening all women having IVF treatment would be useful in order that high risk pregnancies may be identified and cared for in the appropriate setting with close antenatal and intrapartum monitoring. In summary, aPL are a common finding in women having IVF treatment and, although they do not appear to influence the outcome of the fertility treatment, they do have a significant impact on intrauterine growth should a successful pregnancy occur. Consideration should therefore be given to routine screening for the presence of these autoantibodies in women undergoing assisted conception.
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References


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