HIGH FECUNDITY RATES FOLLOWING IN-VITRO FERTILIZATION AND EMBRYO TRANSFER IN ANTIPHOSPHOLIPID ANTIBODY SEROPOSITIVE WOMEN TREATED WITH HEPARIN AND ASPIRIN

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This study was undertaken to explore whether intervention with heparin and aspirin (H/A) in selected patients undergoing in-vitro fertilization (IVF) and embryo transfer could improve fecundity rates. Specifically, it explored the possibility that women diagnosed with organic pelvic disease who demonstrated antiphospholipid antibodies (APA) could benefit from H/A administration in a similar manner to that used in patients with recurrent pregnancy loss. We used an enzyme-linked immunosorbent assay for six different phospholipids to identify patients who expressed APA before they underwent IVF/embryo transfer. This study was confined to the first IVF/ embryo transfer cycle that followed assessment of APA status and accordingly, the number of IVF/embryo transfer cycles corresponds with the number of patients treated. APA seropositive patients were treated with aspirin, 81 mg orally q.d., and heparin 5000 IU s.c. b.i.d., beginning on day 1 of controlled ovarian stimulation. The endpoint for success was a live birth or an ultrasound confirming fetal cardiac activity (a viable pregnancy). The prevalence of APA in patients diagnosed with organic pelvic disease (53%) was much higher than in those without female pathology (14%). The administration of H/A to APA seropositive patients significantly ($P < 0.05$) improved the viable pregnancy rate (49%) compared to the untreated APA seropositive group (16%). The viable pregnancy rate for APA seropositive women treated with H/A was also significantly ($P < 0.001$) higher than for untreated APA seronegative patients (27%). We conclude that all women undergoing IVF/embryo transfer should be tested for APA prior to initiating ovarian stimulation, and those with APA seropositivity should be treated with H/A.

Key words: antiphospholipid antibodies/aspirin/fecundity/ heparin/in-vitro fertilization

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INTRODUCTION

One of the great endeavours facing reproductive medicine is to improve pregnancy rates following in-vitro fertilization (IVF) and embryo transfer. Patients undergo IVF and embryo transfer for infertility secondary to organic pelvic disease, ovarian dysfunction, male factor, and immunological and unexplained aetiologies. It has been well documented that fertilization rates by assisted reproductive technologies are high (Lopata *et al*., 1982), yet the North American national

IVF/embryo transfer birth rate for the year ending December 1991 was only 15.2% per egg retrieval (Society for Assisted Reproductive Technology, 1993). Autoantibodies have been detected in humans and other animals who have failed to conceive despite repeated attempts at IVF/embryo transfer. One such study has implicated antibodies to negatively charged phospholipids in unsuccessful IVF/embryo transfer cycles (Fisch *et al*., 199l). An increased prevalence of antiphospholipid antibodies (APA) has also been demonstrated in patients diagnosed with pelvic endometriosis (Gleicher *et al*., 1987). This has led to speculation that autoimmune processes similar to those known to be associated with recurrent pregnancy wastage may compromise embryo implantation following IVF/embryo transfer.

Several mechanisms have been proposed to explain how APA contribute to pregnancy wastage: platelet membrane and/ or endothelial cell wall damage may initiate the clotting cascade, and inhibition of prostacyclin and inability to activate protein C (an endogenous anticoagulant) may contribute to a hypercoagulable state (Harris *et al*., 1985). More recent work has demonstrated that several negatively charged APA, notably antiphosphoserine and antiphosphoethanolamine, interfere with the formation of syncytiotrophoblasts from cytotrophoblasts (Rote *et al*., 1992). This latter mechanism may also play a significant role in IVF/embryo transfer, as it emphasizes the function of phospholipids as adhesion molecules modulating the process of implantation. Interference with these adhesive properties may preclude implantation altogether or result in spontaneous abortions.

A study was undertaken to evaluate the effect of APA on women undergoing IVF/embryo transfer so as to determine (i) the prevalence of APA seropositivity in women with organic pelvic disease (i.e. post-surgical adhesions, endometriosis and/or pelvic inflammatory disease), compared to women whose infertility was not associated with tissue-damaging conditions (e.g. male factor and/or unexplained infertility), (ii) whether APA seropositivity adversely affects implantation and pregnancy rates with IVF/embryo transfer, and (iii) whether combined treatment with heparin and aspirin (H/A) improves ongoing clinical pregnancy rates in seropositive women who undergo IVF/embryo transfer.

MATERIALS AND METHODS

Patient population

We evaluated 429 women <40 years of age who underwent IVF/ embryo transfer at the Pacific Fertility Medical Centers in California, during the 30 month period commencing January 1, 1992 through June 30, 1994 in order to assess the relationship between APA seropositivity and the cause of infertility. The assessment of outcome following IVF/embryo transfer was confined to those cycles of treatment which immediately followed the diagnosis of APA status. Patients were divided into two groups: group 1 comprised 365 women with organic pelvic disease. Male factor infertility was absent in all cases. This group was further subdivided according to diagnosis as follows: group 1A comprised 79 women with endometriosis and of these, 52 (66%) women were APA seropositive; group 1B comprised 187 women who underwent IVF/embryo transfer for infertility due to pelvic inflammatory disease, and of these 85 (45%) women were APA seropositive; group 1C comprised 99 women who underwent IVF/embryo transfer for infertility due to abdominal/pelvic adhesions unassociated with prior pelvic inflammatory disease or endometriosis and 57 (58%) of these women were APA seropositive.

Group 2 comprised 64 women whose infertility was not associated with female pelvic pathology. The cause of infertility in this group was an 'isolated male factor' in 49 of these women and was 'unexplained' in 15 cases. Nine (14%) women in group 2 were APA seropositive.

The diagnosis of 'isolated male factor infertility' required the detection of $< 10 \times 10^6$/ml motile spermatozoa on semen analysis in the absence of any identifiable female cause for infertility. Organic pelvic pathology was diagnosed or excluded by any combination of hysterosalpingography, laparoscopy and/or laparotomy. All women in the study had normal uterine cavities as observed at hysteroscopy. Women with thyroid dysfunction or hyperprolactinaemia were treated appropriately prior to commencing IVF/embryo transfer cycles.

laboratory evaluation

All women were required to undergo serum follicle stimulating hormone (FSH) and oestradiol measurements (by radioimmunoassay) on the second or third day of a preceding menstrual cycle and were only included in this study if the FSH and oestradiol concentrations were < 15 mIU/ml and 40 pg/ml respectively. These women also underwent concomitant APA testing using an enzyme-linked immunosorbent assay for antibodies to six phospholipid epitopes (cardiolipin, phosphoserine, phosphoglycerol, phosphoethanolamine, phosphatidic acid and phosphoinositol), as previously described (Matzner *et al*, 1994). Cervical swabs and cultures were obtained tor *Ureaplasma urealyticum* as well as DNA probes for *Chlamydia* and *Gonococcus*. Male partners underwent semen evaluations which included sperm counts, motility, morphology and culture for pathogenic organisms. In addition, both women and men had sperm antibody serologies performed using the indirect immunobead test.

Treatment

Since women of a similar age with organic pelvic disease in the absence of male factor are expected to have comparable clinical pregnancy rates following IVF/embryo transfer attempts, evaluation of treatment with H/A was limited to patients in group 1. Antiphospholipid antibody serop-

ositivity was defined by the detection of any concentration of APA in the IgG, IgM and/or IgA immunoglobulin fraction. There were 194 IVF/embryo transfer cycles performed on APA seropositive women. Of these, a total of 169 women received heparin sulphate [Lyphomed, Deerfield, Illinois, 60015; (5000 units s.c.)] twice daily, along with aspirin [Bayer, Division of Sterling Winthrop Inc, New York, NY 10016; (81mg)] orally once a day and there were 25 APA seropositive women who did not receive H/A. Treatment with H/A commenced with the initiation of ovarian stimulation on cycle day 2 to be continued through the 34th week of pregnancy. A luteal phase pituitary gland 'down-regulation' protocol using a gonadotrophin-releasing hormone agonist in conjunction with gonadotrophins was employed to achieve optimal ovarian stimulation as previously described (Feinman *et al*., 1993). Heparin was temporarily withheld after the morning administration on the day prior to oocyte retrieval and aspirin was withheld 2 days prior to this procedure (i.e. from the day of human chorionic gonadotrophin administration). Both heparin and aspirin therapy were reinitiated immediately following transvaginal ultrasound-guided oocyte retrieval. All patients undergoing H/A therapy had normal activated partial prothrombin times, serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase concentrations and normal red blood cell and platelet counts prior to initiating treatment. These tests were repeated at 2 week intervals for 2 months and thereafter at monthly intervals until termination of the medication regimen.

Endpoint

The endpoint was defined as either a viable pregnancy or delivery. The diagnosis of a viable pregnancy was based on sonographic confirmation of fetal cardiac activity.

Statistical methods

Comparisons of individual proportions in any two treatment groups were carried out using Fisher's exact test. A collective test for treatment over several subgroups, such as groups 1A, 1B, and 1C in Table II, was made using the logarithmic regression model proposed by Cox (1972). The estimates and confidence limits obtained by this technique are computed from the complete data, and not simply from the marginal totals.

RESULTS

Table I illustrates that there was no evidence of systematic differences in the mean ages, ovarian stimulation protocols employed and the number of embryos transferred per IVF attempt among the group of women with organic pelvic pathology, male factor and unexplained infertility. There was, however, evidence that women in group l had a higher prevalence ($P < 0.001$) of APA seropositivity (53%) than women in group 2 (14%).

To determine whether APA seropositivity adversely affected IVF/ embryo transfer outcome, the pregnancy rates of untreated APA seropositive women were compared with those for APA seronegative women, following one IVF/embryo transfer cycle performed on group 1 patients, in the cycle that followed the diagnosis of the women's APA status (see Table II). However, there was no firm statistical evidence of differential pregnancy rates. The ratio of pregnancy rates (APA- /APA+) was estimated as 1.66 with 95% confidence limits of 0.58 and 4.72, which embraced the 'null hypothesis' value of 1.0. The small number of untreated APA + women no doubt contributed to this failure to find evidence of an effect.

There was statistical evidence that the pregnancy rate in H/A-treated APA seropositive women (49%) was significantly higher ($P < 0.05$) than for untreated APA seropositive women (16%). The logarithmic regression estimated the ratio of pregnancy rates (treated/untreated) as 3.02, with 95% confidence limits of 1.09 and 8.40); limits which do not embrace the 'null hypothesis' value of 1.0. The viable pregnancy rates per IVF/embryo transfer cycle, stratified by subgroups of organic pelvic pathology, are presented in Table II.

The pregnancy rate of the APA seropositive treatment group treated with heparin and aspirin (49%) was also significantly higher ($P < 0.001$) than that obtained from the APA seronegative group (27%). The ratio of pregnancy rates (APA + /APA-) was estimated as 1.79, with 95% confidence limits of 1.24 and 2.59, providing convincing evidence of the effect.

DISCUSSION

It has been suggested that ~ 20% of pregnancies spontaneously abort prior to clinical (ultrasound) confirmation, and that an estimated 33% of gestations fail to result in live births (Wilcox *et al*., 1988). The ability of IVF to produce oocyte fertilization rates of > 80% is well documented (Naaktgeboren *et al*., 1987), although the majority of morphologically normal embryos fail to implant (Lopata *et al*., 1982; Jones *et al*., 1983; Yovich *et al*., 1984; Grudzinskas and Nysenbaum, 1985; Acosta *et al*., 1988; Boyers, 1989).

Low embryo implantation rates following IVF/embryo transfer have been attributed to a number of factors including, but not limited to, age of the embryo recipient (Sher *et al*., 1986; Edwards and Steptoe, 1983; Lopata, 1983), the presence of uterine pathology (Sher *et al*., 1991, 1993), thickness and sonographic appearance of the late proliferative endometrial lining (sher *et al*., 1991, 1993;Glissant *et al*., 1985; Gonen and Casper, 1990; Smith *et al*., 1984), age of the oocyte provider (Feinman *et al*., 1993), the protocol of ovarian stimulation employed (Sher *et al*., 1986), use of cryopreserved/thawed versus fresh embryos (Edwards and Steptoe, 1983; Lopata, 1983), and possible adverse effects on the endometrium of ovarian stimulation

(Feinman *et al*., 1993; Edwards, 1993; Paulson *et al*., 1990; Sharma *et al*., 1990).

A large body of evidence is emerging which suggests that a series of complex immune mechanisms modulates implantation. It has been demonstrated that increased concentrations of prostaglandin (PG) E_2 and PGF_{2a} at the site of embryo implantation increase vascular permeability prior to implantation and are critical to the process (Malathy *et al*., 1986; Hoffman *et al*., 1984). Platelet activation factor (PAF), an ether-linked phospholipid, is produced by the blastocyst, by invading trophoblast and by adjacent decidua for a few days around the time of implantation (Johnston *et al*., 1987; O'Neill, 1987; O'Neill *et al*., 1985). PAF facilitates implantation by increasing local consumption of thrombocytes and by promoting the release of PGE2 (Holmes *et al*., 1989; van der Welden *et al*., 1991).

Phospholipids function as adhesion molecules in the formation of myoblasts and syncytiotrophoblasts (Sessions and Horowitz, 1981, 1982). Exposure of surface phospholipids (especially phosphoserine and phosphoethanolamine in the hexagonal phase II form) creates an immunogenic state leading to delayed syncytialization of the trophoblast. This mechanism could play an important role in the pathogenesis of recurrent spontaneous abortion (Rote *et al*., 1992). PAF promotes local production of early pregnancy factor, an immunosuppressive glycoprotein (van der Welden *et al*., 1991). Conceivably, antibodies to surface phospholipids and to this glycoprotein could reduce the efficiency of implantation and promote autoimmune rejection of the conceptus.

It has been postulated that in situations of local or systemic tissue damage, cellular surface phospholipids convert from a bilaminar configuration to a hexagonal phase II structure. In this isomeric form phospholipids combine with lipoproteins to become antigenic and lead to APA produc-

Table I. Mean age, mean number of embryos transferred per in-vitro fertilization (IVF)/embryo transfer attempt and antiphosoholipid antibody (APA) serology status for 429 women undergoing IVF/embryo transfer: a comparison between women with and without organic pelvic pathology

	Group I Organic pelvic pathology	Group 2 No organic pelvic pathology[a]
No. of IVF/embryo transfer cycles	365	64
No. of women	365	64
Age (mean ± SE)	35 ± 1	34 ± I
No. of embryos transferred per IVF/embryo transfer attempt	4.0 ± 1	3.0 ± I
APA +		
No. of IVF/embryo transfer cycles	194	9
No. of women	194	9
Incidence/woman (%)	53	14[b]
APA -		
No. of IVF/embryo transfer cycles	171	55
No. of women	171	55
Incidence/woman (%)	47	86

[a]Male factor and unexplained infertility.
[b]$p < 0$ .001.

Table II. Group I comprises 365 IVF/embryo transfer cycles (365 women) stratified by organic pelvic aetiology in relation to antiphospholipid antibody (APA) serology status, treatment regimen and pregnancy rates per cycle

	Ongoing clinical pregnancies and births per IVF/embryo transfer cycle		
	APA + (*n* = 194)[a]		APA - (*n* = 171)[a]
	H/A regimen (*n*= 169)	No H/A regimen (*n*= 25)	No H/A regimen
Group 1A: endometriosis	24/47 (51%)	0/5	5/27 (19%)
Group 1B: PID	36/74 (49%)	2/11 (18%)	31/102 (30%)
Group 1C: iatrogenic adhesions	22/48 (46%)	2/9 (22%)	11/42 (26%)
Pregnancy rates per IVF/embryo transfer cycle	82/169 (49%)	4/25 (16%)	47/171 (27%)[c]

H/A = heparin + aspirin; PID = pelvic inflammatory disease.
[a]*n* = no. of women and IVF/embryo transfer cycles.
[b]Significant difference (P < 0.05) between H/A and no H/A.
[C]Significant difference (P < 0.001) between H/A APA+ and APA- groups.

tion (Rauch and Janoff, 1990). These antibodies have been identified in a number of autoimmune disorders (including but not limited to systemic lupus erythematosus, scleroderma and Hashimoto's thyroiditis) that are known to be associated with a high incidence of pregnancy wastage (Lockshin *et al*., 1985). As demonstrated by this study, infertility associated with pelvic inflammatory disease, endometriosis and post-surgical pelvic adhesions is likewise associated with a high prevalence of APA seropositivity. This phenomenon could explain the reduced implantation rate per embryo as compared with implantation rates in women without these pathologies and following the transfer of embryos to a third party (IVF/ovum donation and/or IVF/surrogacy) who does not have pelvic pathology (Asch, 1993).

APA have been shown to be transiently produced during ovarian stimulation and/or as a consequence of oocyte retrieval, with subsequent disappearance within several weeks (Fisch *et al*., 1991). This might explain the reduced implantation rate per embryo that occurs following embryo transfer in cases of ovarian stimulation, as well as explain the increased miscarriage rate that occurs in spontaneous pregnancies that immediately follow failed cycles of IVF/ embryo transfer (Schwartz and Jewelewicz, 1991; Scialli, 1986).

When present, APA bind with surface phospholipids on the trophoblast and result in direct cellular injury, inhibit syncytia formation and cause indirect damage through intravascular thrombosis (Rote *et al*., 1992). Heparin, whether endogenous (manufactured by trophoblast) or exogenously administered, inhibits binding of APA with phospholipids, protecting the trophoblast from injury (McIntyre *et al*., 1993). Aspirin on the other hand, exerts an anti-thromboxane effect and inhibits platelet aggregation (Harris *et al*., 1985). It is postulated that H/A therapy facilitates and promotes implantation through these mechanisms.

While it is reasonable to link recurrent reproductive failure in APA seropositive women (in whom pregnancy has already been diagnosed) to failed implantation, it is difficult to attribute a failure to conceive following IVF/embryo transfer (where pregnancy has not yet been diagnosed) to a similar mechanism.

This study indicates that there is a high prevalence of APA seropositivity in women with organic pelvic disease, that such women experience poor IVF/embryo transfer success rates and that, as with recurrent abortion in association with APA seropositivity, H/A therapy has significant therapeutic value. Accordingly, it is reasonable to postulate that a similar pathogenesis may be applicable to both situations.

The rationale for prescribing aspirin in cases of recurrent reproductive failure associated with APA seropositivity is that aspirin may counter APA-mediated hypercoagulability in the choriodecidual space, a situation which if left unaddressed would traumatize the trophoblast and compromise feto-maternal exchange. However, a haemo-chorial relationship is only established with placentation. (i.e. after establishment of a biochemical pregnancy) and accordingly, it is unlikely that aspirin therapy would influence early implantation. Rather, the possible benefit of aspirin therapy could lie in an ability to protect the trophoblast from damage after placentation has been established. Heparin on the other hand, through preventing APA from interfering with syncytialization of the early cytotrophoblast and by countering APA interference with phospholipid-induced decidual reactions that are vital to early implantation, might potentially promote both early implantation and subsequent placentation.

The prevalence of APA seropositivity in the general population ranges from 5 to 17%, while in patients who experience recurrent spontaneous abortion it is as high as 59% (Matzner *et al*., 1994). Our findings indicate that the prevalence of APA seropositivity in patients undergoing IVF/ embryo transfer due to organic pelvic disease is similar to that seen in women who suffer from recurrent spontaneous abortion. In contrast, patients undergoing IVF/ embryo transfer cycles in the absence of female pelvic pathology demonstrate similar APA seropositive rates to the general population (14%). Damage to pelvic organs from endometriosis, infection or iatrogenic trauma may induce the production of APA, and these antibodies may contribute to a woman's inability to conceive naturally or via IVF.

The observation that H/A-treated APA seropositive patients achieved a higher clinical pregnancy rate than their untreated APA seronegative controls raises the possibility that IVF/embryo transfer patients in general may benefit from this adjunctive therapy. Fisch *et al*. (1991) have shown that there is a transient rise in APA titres in women undergoing ovarian stimulation. Thus, it is possible that some IVF/ embryo transfer failures in the pre-cycle APA seronegative women are indeed due to antibody induction and/or transient rises in titres previously below detection. We are currently investigating the potential benefit of treating APA seronegative women undergoing IVF/ embryo transfer with H/A. Further studies many elucidate whether the levels of APA or specific epitopes are more important in affecting IVF/ embryo transfer outcome.

The results of this study support the contention that immunological factors play a pivotal role in regulating normal embryo implantation and placentation. Accordingly, it is likely that autoimmune antibodies predispose women to poor pregnancy outcomes. Given our findings, it is indeed possible that the long-awaited breakthrough in achieving significantly improved success rates with IVF/embryo transfer might well have arrived and that a 50% birth rate per embryo transfer could become routine in many [VF centres of excellence. We conclude that all women undergo-

ing IVF/embryo transfer due to female pelvic organic disease should be tested for APA prior to initiating ovarian stimulation, and that APA seropositivity is an indication for H/A treatment.

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