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Introduction

The success of the in-vitro fertilization (IVF) and embryo transfer procedure is limited by the low proportion of embryos which undergo implantation. Factors which govern embryo viability are poorly understood. One theory proposes that embryos require autocrine growth factors for normal development (Paria and Dey, 1990) and that their production is disrupted by IVF procedures (O’Neill, 1997). Platelet-activating factor (PAF) may be one of several possible embryonic autocrine growth factors. In mice, embryos resulting from IVF release seven times less PAF than is released by equivalent embryos fertilized in the reproductive tract (O’Neill, 1997), while human IVF embryos release low but highly variable amounts of PAF (O’Neill et al., 1987; Vereecken et al., 1990). Culture of embryos in vitro may also compromise PAF production (Ryan et al., 1989), while addition of PAF to culture media stimulates embryo metabolism (Ryan et al., 1990a) and development (O’Neill et al., 1989, 1997; Roberts et al., 1993), and results in significantly higher pregnancy rates following transfer (mouse: Ryan et al., 1990b; human: O’Neill et al., 1989).

PAF (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine) is an ether phospholipid with an acetate group at its C2 glycerol carbon. Upon removal of this acetate, PAF is converted to its inactive form, lyso-PAF (Blank et al., 1981). An enzyme known as PAF:acetylhydrolase (PAF:AH; EC 3.1.1.47) (Farr et al., 1980) catalyses this reaction, and appears to be essentially ubiquitous in body fluids and cells. The presence of this enzyme ensures that PAF is very rapidly degraded and can thus have only local short-term actions. This lability limits the potential for the addition of PAF to culture media when complex protein sources such as serum are present. Serum is a rich source of PAF:AH; however, it has been shown that it may be inactivated at low pH (Farr et al., 1980). In a previous trial (O’Neill et al., 1989) we tested the effects on the outcome of human IVF of supplementing media with PAF following treatment of serum with acidification/neutralization to reduce PAF:AH activity. In the current study we investigated the efficacy of the method for PAF:AH deactivation.

Materials and methods

Serum preparation and acid treatment

Serum was obtained from the female partner (or the male partner in the presence of female anti-sperm antibodies) of couples undergoing...
IVF at the Human Reproduction Unit, Royal North Shore Hospital of Sydney, Australia. For a control group, serum was also prepared from healthy (both male and female) volunteers. Blood (10 ml) was collected by venepuncture using a 21-gauge needle (Terumo Medical Corporation, Elkton, MD, USA). Following transfer of the blood to a 10 ml tissue culture tube (Falcon 2001; Becton-Dickinson, Franklin Lakes, NJ, USA), the tube was immediately placed on ice during clot formation. After 1 h, the blood was centrifuged (2500 g for 10 min at 4°C) and the supernatant transferred to another 10 ml tissue culture tube. The tube was incubated at 37°C until a fibrin clot was fully formed (~1 h). After removing the fibrin clot, the resulting serum was then heat-inactivated (20 min at 56°C).

The method of PAF:AH deactivation used in the previously reported PAF supplementation trial (O’Neill et al., 1989) involved acidification of serum by adding 1 M HCl (BDH, Poole, Dorset, UK) dropwise until the pH was 3.0. The pH was measured using a PHM61 laboratory pH meter with a GK2401C combined electrode (Radiometer A/S, Copenhagen, Denmark). The acidified serum was incubated for 5 min at room temperature before returning the pH to 7.4 by the dropwise addition of 1 M NaOH (BDH). The acid/alkali treatment dissolved in Tyrode’s buffer and prepared as 1 mM and 100 µM respectively. Dose–response curves for PLA₂ were obtained by adding 50 µl of PLA₂ vehicle to the PAF:AH assay. Results were expressed as pmol acetate released/min. PLA₂ catalyses the same reaction as PAF:AH. Concentrations of PLA₂ were chosen that had equivalent activity to that in the serum pool before and after acid treatment. The effect of PLA₂ inhibitors on PLA₂ and pooled sera were examined by adding inhibitors (in 430 µl of Tyrode’s buffer) to the PAF:AH assay.

**SDS-PAGE serum profiles**

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the PhastSystem (Pharmacia, Uppsala, Sweden). Sera were diluted 1:20 (v/v) with reducing SDS sample buffer, boiled for 1.5 min, then centrifuged (5 min at 3000 g) to clarify samples. The reducing SDS sample buffer was 62.5 mM Tris-HCl, pH 6.8 (Sigma); 2% (w/v) SDS (Bio-Rad); 10% (v/v) glycerol (BDH); 0.001% (w/v) bromophenol blue (Bio-Rad); 5% (v/v) 2-mercaptoethanol (ICN Biomedicals). Samples (~2–3 µg) were applied to PhastGel gradient 10–15% (molecular weight range: 10–250 kDa) and detected with combined Coomassie Brilliant Blue/silver stain (Pharmacia).

Interpretation of serum profiles and molecular weight estimations were performed by scanning PhastGels with the PhastImage Gel Analyzer (Pharmacia) to measure the optical density at 603 nm, then comparing the distance of the protein peaks from the cathode with a molecular weight standard calibration graph, using the Pharmacia Analysis Programme (Version 1.0; Pharmacia).

**IVF**

Human oocytes were collected by a transvaginal ultrasound-guided approach following ovarian stimulation. On collection, ova were washed extensively and placed individually into 5 ml tissue culture tubes containing 1 ml of human tubal fluid medium (HTFM) with 10% (v/v) acid-treated serum. Insemination was performed 5 h later with 80–120×10⁵ motile spermatozoa. After incubation at 37°C for 18 h, in 5% CO₂ in air, the fertilization status of the oocytes was checked and the resulting embryos transferred to fresh medium for 24 h. During this incubation period, embryos were cultured in the presence of PAF. After 24 h, most embryos had reached the 4-cell stage. A maximum of two embryos were transferred to the uterus and the remaining embryos were cryopreserved.

If menstruation had not occurred 17 days after embryo transfer, urine samples were collected for detection of β-human chorionic gonadotrophin by the Tandem Icon II HCG ImmunoConcentration Assay (Hybritech Incorporated, San Diego, CA, USA).
Optimization of PAF supplementation in IVF culture medium

PAF (L-$\alpha$-phosphatidylcholine, $\beta$-acetyl-$\gamma$-O-alkyl; Sigma) was aseptically prepared as 1 mg PAF/ml in chloroform (BDH). A 80 mg PAF/ml stock solution was prepared by placing an aliquot into a siliconized glass tube (Vacutainer; Becton-Dickinson), evaporating the solvent with N$_2$ and suspending in HTFM with 3 mg human serum albumin/ml. Sterile aliquots (100 µl) of this stock solution were stored at −20°C. A final concentration of 800 ng PAF/ml was prepared by adding 10 µl of the 80 µg PAF/ml stock solution to 1 ml of HTFM with 10% (v/v) serum, in 5 ml tissue culture tubes (Falcon 2003; Becton-Dickinson).

Statistical analysis

Comparison of means of two populations was made using the Student’s $t$-test (Minitab Statistical Package, Version 10; State College, PA, USA). Data presented as percentages were analysed by $\chi^2$ test (Minitab). Correlations between two variables were performed using linear regression analysis (Statview 512+; BrainPower Incorporated, Calabas, CA, USA). Analyses were summarized as the regression equation, correlation coefficient ($r$) and the level of significance ($P$).

Results

Deactivation of PAF:AH in IVF culture medium

Sera from 18 females undergoing IVF treatment were used in a preliminary investigation of the effectiveness of PAF:AH deactivation by acid treatment (pH 3.0) at room temperature for 5 min. Parallel tubes were prepared containing 10% (v/v) serum which had been either acid-treated or left untreated. Each tube was supplemented with 800 ng PAF/ml. To simulate IVF culture conditions, these tubes were incubated for 24 h (at 37°C in 5% CO$_2$ in air) and then extracted and partially purified by thin-layer chromatography, and bioassayed for PAF.

In media with serum that was not acid-treated, the amount of PAF remaining was 0.016 ± 0.016% (mean ± SEM) of that originally added. For acid-treated serum, 45.836 ± 9.175% (mean ± SEM) remained. It was noted, however, that differences in the effectiveness of the acid-treatment procedure occurred (Figure 1). Of the 18 serum samples examined, five had 90–100% PAF remaining after 24 h (showing that the acid treatment was effective), eight had 10–90% of the PAF remaining after 24 h, and five samples only had 0–10%.

PAF:AH specific activity in sera before and after acid treatment

The PAF:AH specific activity was measured in the 18 serum samples before and after acid treatment. In untreated samples, the average (mean ± SEM) PAF:AH specific activity was 381.2 ± 32.4 pmol acetate released/min/mg protein (Figure 2A). Following acid treatment for 5 min at room temperature, the average PAF:AH specific activity was 12.1 ± 5.6 pmol acetate released/min/mg protein. Some sera were resistant to acid treatment, with several samples still displaying marked PAF:AH activity. There was, however, no correlation between the PAF:AH specific activity ($r = 0.023$) and the protein concentration ($r = 0.005$) present in serum before acid treat-
There was also no association with age, cause of infertility, or type of ovarian stimulation regimen of the subjects; nor was there correlation between the preovulatory plasma oestradiol peak concentration (7389.2 ± 1407.6 pmol oestradiol/l, mean ± SEM) for these subjects and PAF:AH specific activity, either before (r = 0.042) or after acid treatment (r = 0.026).

**Acceptable amounts of PAF:AH for use with PAF supplementation**

The regression equation between the amount (%) of PAF remaining after incubation for 24 h and the PAF:AH specific activity in serum after acid treatment was $y = 88.2 - 17.9x$, $r = 0.81$, $P = 0.0004$ (after excluding serum samples where no PAF remained) (Figure 2B, insert). This relationship predicts that the PAF:AH specific activity in serum must be <0.5 pmol acetate released/min/mg of protein for the PAF to remain largely undegraded. On this basis, it was concluded that acid treatment of sera in 12 of the 18 subjects (67%) was not effective.

**Comparison of PAF:AH specific activity in the IVF and a control group**

The PAF:AH specific activity (mean ± SEM) in sera from healthy volunteers ($n = 20$) before and after acid treatment was 390.4 ± 23.7 and 2.7 ± 0.7 pmol acetate released/min/mg protein respectively. There was no significant difference between the serum PAF:AH specific activity after acid treatment for the control group compared to that collected from IVF subjects ($P = 0.11$). A similar proportion of sera from the control group was not effectively acid-treated [13/20 (65%) had PAF:AH specific activities >0.5 pmol acetate released/min/mg protein]. It was concluded that this control group was suitable for further development of the PAF:AH deactivation technique, and a pool of this serum was prepared.

**Phospholipase A$_2$ and inhibitors**

To exclude the possibility that the persistent PAF:AH-like activity was due to long-acyl-chain PLA$_2$ activity rather than residual PAF:AH, pharmacological inhibitors of PLA$_2$ ($p$-bromophenacyl bromide and mepacrine) were used to inhibit the activity of PLA$_2$. Dose–response curves for PLA$_2$ were established, and a range of concentrations was chosen to give equivalent activity to the serum pool before (1–50 IU/ml) and after acid treatment (0.1–1.0 IU/ml). Mepacrine and $p$-bromophenacyl bromide caused significant inhibition of PLA$_2$ activity, but had no effect on the enzyme activity in sera before (Figure 3A and B) or after acid treatment (Figure 4A and B), indicating that the enzyme in the acid-treated serum which caused some degradation of PAF was not PLA$_2$. It was therefore assumed that this residual activity was PAF:AH, which was less susceptible to acid deactivation.

**Development of a new acid treatment protocol**

The serum pool was acidified to pH 3.0 with 1 M HCl and maintained for a period of 5, 10, 20, 40 and 80 min at either room temperature or 37°C, prior to neutralization with 1 M NaOH. Acid treatment was more effective when performed at 37°C, and at this temperature PAF:AH inactivation was complete by 20 min (Figure 5). A new acid-treatment protocol of 20 min at 37°C may be more effective than the earlier protocol of 5 min at room temperature.

**Effectiveness of new acid-treatment protocol**

The effectiveness of the new acid-treatment protocol was examined in a screen of 53 couples undergoing IVF. Serum was prepared and split into two fractions and then acid-treated by the two protocols. After 5 min acid treatment at room temperature, 32 out of 53 sera (60%) had PAF:AH specific activities >0.5 pmol acetate released/min/mg protein. By comparison, after acid treatment for 20 min at 37°C, all sera had PAF:AH specific activities <0.5 pmol acetate released/min/mg protein, showing this protocol to be an effective treatment for the removal of PAF:AH from serum.

**SDS-PAGE serum profiles**

Figure 6 is a schematic representation of PhastImage analysis of serum from one IVF subject. There were only minor changes in the protein profile of the serum following acid treatment.
Optimization of PAF supplementation in IVF

Figure 4. Amount of acetate released (pmol/min) by (A) phospholipase A₂ (PLA₂; ○) and (B) pooled sera after acid treatment for 5 min at room temperature (●), and the effect of the PLA₂ inhibitors p-bromophenacyl bromide (1 mM; □) and mepacrine (100 μM; ■). Data shown are representative results.

Figure 5. Effect of acid treatment incubation at room temperature (○) or 37°C (●) and incubation time on the PAF:AH specific activity (pmol acetate released/min/mg protein) of pooled sera.

Figure 6. Schematic representation of the effect of acid treatment on serum. Serum samples from a woman undergoing in-vitro fertilization were (A) left untreated, (B) acid-treated for 5 min at room temperature, or (C) acid-treated for 20 min at 37°C. Reduced sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed using a gradient 10–15 PhastGel and a combined Coomassie/silver stain. Interpretation of serum profiles and molecular weight estimations were performed by scanning PhastGels with the PhastImage Gel Analyzer to measure the optical density (O.D.) at 603 nm (y-axis), then comparing the distance of the protein peaks from the cathode (x-axis) to a molecular weight standard calibration graph.

for 5 min at room temperature. Increasing the acid treatment to 20 min at 37°C did not induce further changes in the profile. Specifically, acid treatment induced a loss of a protein band at ~190 kDa and a marked reduction in the intensity of a band at ~170 kDa, while there was the appearance of a band at ~105 kDa, possibly due to the formation of subunits after breakdown of the larger acid-labile proteins.

PAF supplementation trial with new acid-treatment protocol

A trial was performed to assess the effect of the new acid-treatment protocol on IVF outcome, following PAF supplementation of IVF culture medium. Women undergoing IVF were alternately allocated to one of two groups (5 min at room temperature versus 20 min at 37°C). A total of 164 IVF treatment cycles were examined in the trial. The oocyte recovery, fertilization and embryo development rates were equivalent for both groups (Table I). Also, approximately equal numbers of embryos were transferred or cryopreserved. The pregnancy rates were not significantly different (13/89 = 14.6% versus 15/75 = 20.0%) for the two treatments, although there was a trend towards a higher pregnancy rate with the new acid-treatment protocol. The results show that this new
procedure for acid treatment of serum in combination with PAF supplementation does not have detrimental effects on embryos and their pregnancy outcome and is therefore suitable for use in IVF. The PAF concentration in media after embryo culture was not measured in this study.

**Discussion**

PAF is released by embryos soon after fertilization and throughout the preimplantation phase. While it may impose several effects on maternal physiology during early pregnancy (see Stein and O’Neill, 1994 and references therein), an important function may be its actions as an autocrine embryotrophic factor. While some studies have failed to detect the release of PAF from embryos (human: Amiel et al., 1989; mouse: Smal et al., 1990), this was likely to be due to the use of unconventional extraction procedures for PAF assay (Ammit and O’Neill, 1997). PAF stimulates embryo metabolism (Ryan et al., 1990a), cell-cycle progression (Roberts et al., 1993) and viability (O’Neill et al., 1989; Ryan et al., 1990b). A common observation has been that the amount of PAF released by embryos is highly variable. A recent study (O’Neill, 1997) showed that mouse embryos produced by IVF produced seven times less PAF in vitro than did corresponding embryos produced by fertilization in the reproductive tract. Furthermore, the mouse embryos produced by IVF had significantly worse development rates to the blastocyst stage. Supplementation of culture media with PAF resulted in improved development rates of these embryos. A trial of supplementation of human IVF culture media with PAF also resulted in a significant improvement in embryo viability as assessed by implantation rates (O’Neill et al., 1989) and live-born babies (O’Neill et al., 1992).

It is noteworthy that embryo-derived PAF is detected in IVF culture media, despite the presence of serum which would contain PAF:AH (O’Neill et al., 1987). Recent studies (Ammit and O’Neill, 1997) indicate that PAF released from embryos occurs in association with a carrier which protects the PAF from the actions of PAF:AH. Exogenous PAF added to culture media does not appear to be able to access this carrier and is susceptible to rapid hydrolysis. Thus, the supplementation of IVF culture media with PAF can be problematic if complex protein sources such as serum or follicular fluid are used, since each contains PAF:AH. To achieve effective concentrations of PAF in culture media, efficient strategies for overcoming the effects of PAF:AH are required. At high concentrations PAF is cytotoxic; hence a strategy of adding excess initial concentrations is undesirable. Repeated application is a possibility, but in a busy clinical setting is likely to be impractical, while the repeated interventions would make the achievement of stable culture conditions difficult.

The results of this study show that, when using acid treatment for 5 min at room temperature, sufficient PAF:AH activity persisted so that ~28% of cases had <10% of the original PAF remaining after 24 h, while in another 28% almost all PAF added remained bioactive. Thus, the addition of PAF under these circumstances would result in a wide range of effective bioactive PAF concentrations being available to the embryo. It was confirmed that the enzyme activity remaining after treatment was not due to long-acyl chain PLA₂, nor was it related either to the initial PAF:AH activity of the serum, or to any obvious treatment parameters of the subject. It was shown that extension of the treatment time at pH 3.0 enhanced the degradation of PAF:AH activity and this was further facilitated by undertaking the acid treatment at 37°C. A standard protocol of acid treatment for 20 min at 37°C was chosen. This treatment caused all sera tested to have PAF:AH deactivated to a satisfactory level.

The role of complex protein supplements in promoting embryo development (if any) is unknown. Recently the use of defined media with simple protein supplementation, such as albumin, has gained some favour. If such protein supplements are homogeneous, they are unlikely to contain PAF:AH activity and may be compatible with PAF supplementation without the need for acid treatment. The possibility remains that acid treatment may cause serum to be an unsuitable media supplement. One consequence is the increase in osmotic strength caused by the addition of HCl and NaOH. This was solved by dilution of serum with water and measurement of its osmotic strength prior to its use in media. The analysis of protein size with SDS-PAGE before and after acid treatment showed that at this gross level there were only relatively modest changes in the serum protein profile. In a trial to examine the effect of PAF supplementation of culture media on treatment outcome, it was found that extending the acid treatment had no adverse effects on embryo development in vitro or on pregnancy following transfer. There was a non-significant trend for a higher pregnancy rate after use of the harsher acid-treatment protocol. The trial size was, however, too small to draw conclusions from this outcome. It is conceivable that acid treatment may have other adverse effects on serum that are not detected by PAGE. A further control of acid-treated serum which was not supplemented with PAF compared to non-treated serum may help address this. An alternative strategy would be to use serum-free media supplemented with albumin. PAF supplementation of media with albumin as the only macromolecular supplement was found to increase the viability and pregnancy rates of mouse embryos following embryo transfer (Ryan et al., 1990b).

The results of this study show that an optimized method of acid treatment of serum for the degradation of PAF:AH has

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<tr>
<th>Acid treatment protocol</th>
<th>5 min at room temperature</th>
<th>20 min at 37°C</th>
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<tbody>
<tr>
<td>No. of patients receiving embryos</td>
<td>89</td>
<td>75</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>13</td>
<td>15</td>
</tr>
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<td>Pregnancy rate (%)</td>
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<td>20.0</td>
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<tr>
<td>No. of oocytes retrieved</td>
<td>7.51 ± 0.44</td>
<td>7.73 ± 0.51</td>
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<tr>
<td>No. of embryos fertilized</td>
<td>5.40 ± 0.38</td>
<td>5.48 ± 0.38</td>
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<tr>
<td>No. of normally developed embryos</td>
<td>4.87 ± 0.36</td>
<td>4.91 ± 0.35</td>
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<tr>
<td>No. of embryos transferred</td>
<td>2.02 ± 0.06</td>
<td>2.11 ± 0.08</td>
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<tr>
<td>No. of embryos frozen</td>
<td>2.97 ± 0.36</td>
<td>2.80 ± 0.33</td>
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References


been developed. With the use of this method, the results show that PAF added to embryo culture media containing serum will remain largely undegraded. This provides a basis for carrying out controlled dose–response studies of PAF supplementation. This method allows for future studies to examine the relationship between total PAF concentration in media (supplement plus embryo-derived PAF) and the subsequent viability of embryos after transfer. It is noted that, despite this optimized methodology and the addition of PAF, the pregnancy rate was still below a clinical ideal. It has recently been shown in the mouse (O’Neill, 1997) that while addition of PAF to media in which embryos were cultured enhanced embryo development, it only partially compensated for the adverse effects of IVF and subsequent culture. That study showed that insulin-like growth factor (IGF)-I and IGF-II could also partially compensate for the adverse effects of IVF and culture in vitro. It is likely that assisted reproductive technology causes multiple aberrations in the growth stimuli of the early embryo. Future studies may allow these to be alleviated by understanding the nature of the aberrations and the appropriate conditions for media supplementation.