Human Reproduction Vol.17, No.5 pp. 1306–1310, 2002

Embryonic platelet-activating factor: an indicator of embryo viability*

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BACKGROUND: A definitive need exists to identify a biomarker of embryonic viability. Platelet-activating factor (PAF) production by human embryos is related to pregnancy potential. METHODS: Conditioned embryo culture media were obtained following conventional IVF on day 3, with PAF levels and pregnancy outcomes correlated. RESULTS: Overall pregnancy rate was 68% (17/25) with a mean of 84.1 (± 8.5) pmol/l/embryo PAF level. PAF levels ranged from a 216.4 pmol/l/embryo (pregnant) to a 3.7 pmol/l/embryo (not pregnant). There was a significant difference ($P < 0.05$) in PAF content between pregnant (92.1 ± 9.5 pmol/l/embryo) and non-pregnant groups (52.5 ± 16.6 pmol/l/embryo). Patients were categorized into three groups based upon PAF levels: low (≤5 pmol/l/embryo); medium (51–100 pmol/l/embryo) and high (>100 pmol/l/embryo). The low (60%) group had a significantly ($P < 0.05$) lower pregnancy rate than either the medium (85%) or high (89%) groups. A receiver–operator characteristic curve predicted a cut-off limit of 45 pmol/l/embryo for PAF content in human embryo conditioned culture media. CONCLUSIONS: The data demonstrate a correlation between PAF levels in human embryo conditioned culture media and pregnancy outcome. Additionally, as embryonic PAF levels increase so does the corresponding pregnancy rate. Therefore, PAF may be used as an indicator of embryo viability and for predicting pregnancy outcome.

Key words: embryo culture/embryo development/platelet-activating factor/pregnancy/viability

Introduction

Preimplantation embryos produced through IVF are generated for the primary purpose of assisted procreation. Generally, a number of these embryos are produced, yet only a few (two or three) are routinely transferred into the patient. A definitive need exists to identify a biomarker of embryonic viability. A method for selecting the best quality embryo has been a quest of the laboratory embryologist and the infertility physician. While such methods for selection exist they are typically based upon morphological assessments, i.e. embryo grading (Cummins *et al.*, 1986; Claman *et al.*, 1987; Puissant *et al.*, 1987; Steer *et al.*, 1992; Gardner *et al.*, 2000). For example, scoring an embryo grade is primarily dependent upon cell stage, presence of irregular blastomeres and degree of fragmentation. Selecting an embryo based upon its grading score has its limitations, e.g. qualitative grading does not necessarily indicate embryo viability (Dokras *et al.*, 1993; Rijnders and Jansen, 1998; Nakagawa *et al.*, 1999; Desai *et al.*, 2000; Graham *et al.*, 2000; Balaban *et al.*, 2001). As the development of improved culture conditions for the human embryo and the need for selection of a single best embryo for transfer progress (day 3 or blastocyst) there is a need for an indicator of viability (Saith *et al.*, 1996). A number of endogenous factors have been attributed to the regulation of preimplantation embryo development and implantation, for example insulin-like growth factors (Liu *et al.*, 1987) and platelet-activating factor (PAF) (O'Neill *et al.*, 1987). PAF is a unique and novel signalling phospholipid that has pleiotrophic biological properties in addition to platelet activation and is produced by a variety of cell types (Snyder, 1987). These embryonic 'regulators' may be used as indicators of embryo viability (Liu *et al.*, 1987).

PAF production by human embryos is related to their pregnancy potential (O'Neill *et al.*, 1987; Roudebush *et al.*, 2001a,b). Since its discovery in the early 1970s (Benveniste *et al.*, 1972) this novel compound has been implicated in a wide variety of reproductive functions (Harper, 1989). The exact mechanism is uncertain, yet its importance in normal fertility is significant.

PAF plays a significant role in mammalian reproduction. Exogenous PAF will stimulate early embryo metabolism and cell division (Ryan *et al.*, 1992; Roudebush *et al.*, 1996; O'Neill, 1997). Additionally, exogenous PAF will enhance

*Presented, in part, at the Annual meeting of the European Society of Human Reproduction and Embryology (ESHRE 2001), Palais de Beaulieu Congress and Exhibition Centre, Lausanne, Switzerland, July 1–4, 2001

implantation rates of exposed mouse embryos (Nishi *et al.*, 1995).

Whereas embryo-derived PAF levels have been measured in conditioned human embryo culture media (O'Neill *et al.*, 1987), there is limited information available on its content with regard to embryo viability (as determined by pregnancy outcomes). Therefore, the study objective was to determine if the level of PAF production by human embryos correlates with pregnancy outcome following assisted reproduction.

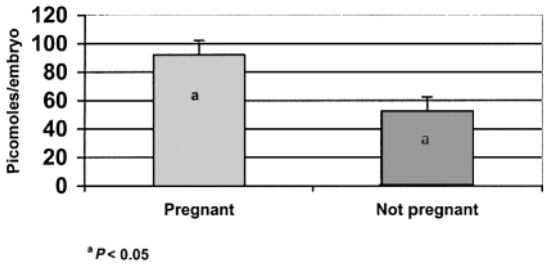

Figure 1. Mean PAF content of HECM between pregnant and non-pregnant patients.

Study design

PAF levels in human embryo conditioned culture media (HECM) were determined by a PAF-specific radioimmunoassay and correlated with pregnancy outcomes following assisted reproduction via conventional IVF and day 3 embryo transfer.

Study population

Inclusion criteria were as follows: basal FSH level <15 mIU/ml, evidence of a normal uterine cavity, and no contraindication to pregnancy. Infertility diagnoses included ovulation dysfunction, endometriosis, tubal blockage, and male factor. Controlled ovarian stimulation was initiated with leuprolide acetate (Lupron; TAP Pharmaceuticals, North Chicago, IL, USA) administered either from the midluteal phase at 0.5 mg s.c. daily until gonadotrophin injections began, at which time the Lupron dose was lowered to 0.25 mg/day until the morning of HCG injection, or as a flare protocol, in which Lupron 1.0 mg/day was were begun on cycle day 1 and continued until day 5, when the dose was lowered to 0.25 mg/day until the morning of HCG injection. Recombinant FSH (Gonal-F; Serono Laboratories Inc., Norwell, MA, USA; or Follistim; Organon Inc., West Orange, NJ, USA) was begun after pituitary down-regulation and continued until at least two follicles reached a mean diameter of 18 mm. Oocyte retrieval was scheduled 36 h after HCG injection.

Embryo culture and transfer

All culture media and protein supplements used in the study were commercially available and purchased from Irvine Scientific, Santa Ana, CA, USA. Media P1 was supplemented with 10% v/v Synthetic Serum Substitute and equilibrated in 5.0% carbon dioxide at 37°C before use. Sperm were prepared with a 90/45 discontinuous gradient wash, and the final pellet was resuspended in 500 µl P1 medium. Conventional insemination (study day 0) was performed by adding 100 000 sperm/ml to the oocytes, which were in groups of 3–5 in 75 µl droplets of P1 media under sterile mineral oil. Fertilization checks were carried out 16–18 h after insemination. Embryos, in cohorts (1–5) with two pronuclei, were transferred to a new 50 µl drop of P1 medium for culture (study day 1). All embryo transfers (study day 3) were performed with a Wallace catheter (Marlow Technologies, Willoughby, OH, USA) in P1 medium. Methylprednisolone (16 mg/day) and doxycycline (100 mg twice daily) were administered for 5 days beginning on the day of oocyte retrieval. The luteal phase was supplemented with 50 mg progesterone in oil i.m. or 8% vaginal progesterone (Crinone; Serono Laboratories).

PAF content determination

Conditioned human embryo culture media were collected following conventional IVF and embryo transfer on day 3 (day 1 = pronuclear stage). An equal volume (50 µl) of 20% glacial acetic acid was added to the HECM to inactivate PAF-acetylhydrolase which is typically present (Wells and O'Neill, 1994). PAF content in HECM was determined by a PAF specific radioimmunoassay [^{125}I] according to the manufacturer's instructions (Perkin Elmer Life Sciences, Inc., Boston, MA, USA). Briefly, primary antibodies were added to tubes containing the HECM samples, mixed and incubated for 15 min at room temperature. Secondary antibodies and tracer were added, mixed and incubated for 24 h+ at room temperature. Following centrifugation (2000 *g*; 30 min), supernatants were decanted and the tubes blotted and counted. The standard curve was calculated by regression analysis (logit value of normalized percentage bound versus log of ng PAF assayed). Content of PAF in the HECM is expressed as pmol/l/embryo (i.e. amount of PAF measured in the HECM/number of embryos cultured). PAF levels in the HECM were compared with pregnancy outcomes. Patients were further categorized into three groups based upon PAF levels: low (≤50 pmol/l/embryo); medium (51–100 pmol/l/embryo); and high (>100 pmol/l/embryo). Clinical pregnancy was defined as the presence of a fetal heartbeat on ultrasonography.

PAF radioimmunoassay performance was as follows: sensitivity (detection limit), 0.19 pmol/l; intra-assay coefficient of variation, 8.51% (determined by five replicates of samples measured in a single assay), 10.06 ± 0.29 pmol/l; inter-assay coefficient of variation 7.76% (determined by assaying same replicates in four separate assays), 10.73 ± 0.76 pmol/l.

Statistical analysis

Data, screened for normality, were analysed by Student's *t*-test and Mann–Whitney rank sum test. Statistical calculations were performed with SigmaStat for Windows, version 2.03 (Jandel Scientific Corporation, San Rafael, CA, USA). Receiver–operator characteristic (ROC) curve analyses were performed using StatTools (Chang, 2000) using PAF levels in HECM to pregnancy outcomes.

Results

A total of 46 HECM samples from 25 conventional IVF cycles were assayed and quantified for PAF content as described. A total of 66 embryos was transferred (2.6 embryos per patient), there was no significant difference in the number of embryos transferred between patients who became pregnant (2.7 embryos per patient) versus those who did not become pregnant (2.6 embryos per patient). The overall pregnancy rate for this study was 68.0% (17/25) with a mean (± SEM) PAF level of 84.1 (± 8.5) pmol/l/embryo. PAF levels ranged from a high of 216.4 pmol/l/embryo (this patient became pregnant) to a low of 3.7 pmol/l/embryo (this patient did not become pregnant). The mean PAF level in the HECM media samples for patients who became pregnant versus those who did not become pregnant are presented in Figure 1. Patients who became pregnant [92.1 (± 9.5) pmol/l/embryo] had significantly ($P < 0.05$) higher PAF levels in their HECM than

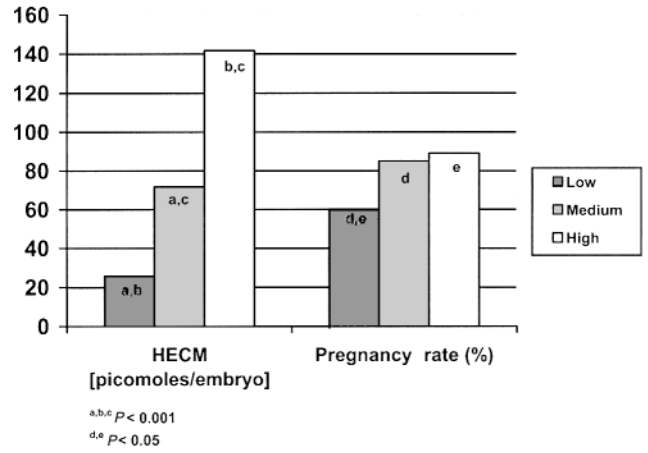

Figure 2. Mean PAF production in HECM and respective pregnancy rates. For statistical comparisons between like letters, $^{a,b,c}P < 0.001$, $^{d,e}P < 0.05$.

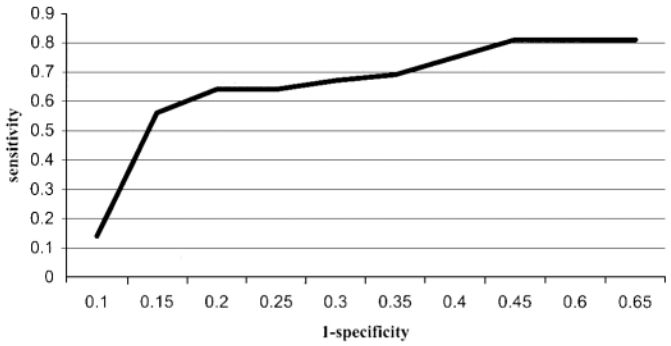

Figure 3. Receiver operator curve (ROC) for embryonic-PAF on pregnancy outcome.

patients that did not become pregnant [52.5 (± 16.6) pmol/l/embryo]. The overall implantation rate was 1.5. There was no significant relationship between PAF and implantation rate. The total number of multiple gestations was 7 (28%).

The mean PAF levels in the HECM samples and their respective pregnancy rates for each PAF group (low, medium or high) are presented in Figure 2. The low PAF group had a significantly ($P < 0.05$) lower pregnancy rate (60%) than either the medium (85%) or high (89%) PAF groups. There was no significant difference in pregnancy rates between the medium or high PAF groups.

A ROC curve was drawn to determine which cut-off level of PAF content in HECM best predicted a positive pregnancy outcome. The sensitivity and 1-specificity for HECM PAF levels measurements (range 3.7–216.4 pmol/l/embryo) were calculated for different cut-off limits to predict pregnancy outcome as illustrated by the ROC curve presented in Figure 3. The ROC curve and the calculation of the probability that a positive pregnancy outcome will be predicted indicated a cut-off limit of 45 pmol/l/embryo for PAF content in HECM. The sensitivity and specificity values for this cut-off are 0.81 and 0.60 respectively. The diagnostic accuracy is 0.76, the positive predictive value is 0.88 and the negative predictive value is 0.46.

Discussion

As the development of improved culture conditions for the human embryo progresses and the need for selection of a single best embryo for transfer increases there is a need for an indicator of embryo viability. A number of endogenous factors have been attributed to regulate preimplantation embryo development and implantation. These embryonic 'regulators' may be used as an indicator of embryo viability. One such 'regulator' is PAF (O'Neill *et al.*, 1987). It was reported that embryonic PAF (determined by a platelet count bioassay) was related to ovulation induction regimen, follicular size and estradiol production, time of PAF measurement and embryo morphology. Whereas, the authors stated a relationship between PAF and pregnancy outcome, the results were qualitative in nature. In the present study, we have quantitatively (by radioimmunoassay) demonstrated that PAF levels in HECM and pregnancy outcomes have a positive and significant relationship. As embryonic PAF levels increase so does the corresponding pregnancy rate. Patients who became pregnant had more PAF in their respective conditioned culture medium than patients who did not become pregnant. The ROC curve predicted a cut-off limit of 45 pmol/l/embryo for PAF content in HECM. Therefore, embryo-derived PAF may be used as an indicator of embryo viability and for predicting pregnancy outcome.

PAF is present in uterine fluid, produced by both endometrium and preimplantation embryos. PAF uterine levels in the rabbit increase from day 3–5 of pregnancy (Angle *et al.*, 1988). Embryonic-PAF production in the rabbit and mouse also increases during the preimplantation phase, with maximum levels at the expanded blastocyst stage (Minhas *et al.*, 1993; Ripps *et al.*, 1993). Embryonic-PAF can act as an autocrine stimulator of embryo development (Ryan *et al.*, 1992). The action(s) of this embryo-derived PAF can be blocked by PAF-antibodies or PAF antagonists (Nishi *et al.*, 1995; Roudebush *et al.*, 1995). PAF production by human embryos has been correlated with pregnancy potential, i.e. the ability for the embryos to implant successfully (O'Neill *et al.*, 1987; Nakatsuka *et al.*, 1992; Roudebush *et al.*, 2001a). Addition of PAF to the culture media promotes 2-cell development to the blastocyst stage in the mouse by stimulation of embryonic metabolism (Ryan *et al.*, 1992; Nishi *et al.*, 1995; Roudebush *et al.*, 1996; O'Neill, 1997). The effect of PAF on mouse embryo development *in vitro* does, however, appear to be strain specific (Radonjic-Lazovic and Roudebush, 1995). Improved rabbit blastocyst development after IVF with PAF-treated sperm has also been reported (Roudebush *et al.*, 1993).

In other cell types, the action of PAF is receptor-mediated, and this may also be true in the embryo since different PAF antagonists and PAF antibodies competitively inhibit its action (Nishi *et al.*, 1995; Roudebush *et al.*, 1995). Additionally, PAF antagonists inhibit implantation (Spinks and O'Neill, 1988; Andu *et al.*, 1990). These data provide further evidence on the presence and requirement of embryo-derived PAF during the pre-, peri- and implantation periods. The PAF receptor has been characterized in the mouse (Ishii *et al.*, 1996; Roudebush *et al.*, 1997). Earlier results in our laboratory suggest that

temporal embryonic expression of PAF and the PAF-receptor, are related (Roudebush *et al.*, 1998). The embryo-derived PAF production rate peaks about the time that the mouse preimplantation embryo normally enters the uterus. The highest production level for embryonic-PAF is at the expanded blastocyst stage. Preimplantation stage embryos in a variety of species (human, mouse, sheep, rabbit and pig) produce and release PAF (O'Neill, 1987; Collier *et al.*, 1988; Battye *et al.*, 1991; Mook *et al.*, 1998). Furthermore, mouse preimplantation embryos cultured in the presence of PAF have enhanced developmental rates (Roberts *et al.*, 1993) and higher implantation rates upon transfer to synchronized recipients (Ryan *et al.*, 1987). This may be due to embryo-derived PAF stimulation of embryonic metabolism (Ryan *et al.*, 1989). PAF directly influences the oxidative metabolism of glucose and lactate in the mouse preimplantation embryo (Ryan *et al.*, 1990). Cholinephosphotransferase and acetyltransferase (the enzymes that catalyse the final step in the biosynthetic pathways for PAF production) are present in mouse preimplantation stage embryos (Wells and O'Neill, 1994).

Enhanced embryo development has also been reported in rabbit oocytes fertilized *in vitro* with PAF-treated sperm (Roudebush *et al.*, 1993). Preliminary results in our laboratory suggest that PAF-receptor expression is not uniform throughout the preimplantation period. Eight-cell stage embryos have a lower degree of PAF-receptor expression. This, taken with the significant increase in PAF production, suggests that PAF may regulate expression of its own receptor. In most cells, PAF binds to surface receptors inducing the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) resulting in an increase of intracellular calcium (Lapetina, 1982). Exogenous PAF affects intracellular calcium levels in mouse preimplantation embryos (Roudebush *et al.*, 1997; Emerson *et al.*, 2000). Therefore, PAF appears to bind to cell surface receptors on preimplantation embryos, initiating the formation of IP_3 and DAG, and increasing intracellular calcium. As a secondary messenger, calcium can regulate preimplantation embryonic development by modulating the activity of molecules that transduce intracellular signals, which in turn influence embryonic growth and development. PAF may effect preimplantation embryo development and implantation via a receptor-mediated control of intracellular calcium. Additional studies are needed to elucidate the reproductive significance of PAF activity in preimplantation embryos and PAF's role in the establishment of pregnancy.

In summary, pregnancy outcomes may be predicted by measuring PAF levels in HECM. Additional studies are required for further development of a quick, high-throughput system to adequately determine embryonic PAF production levels by individual embryos prior to this becoming clinically routine. We are currently developing an ELISA system that will be used in a clinical trial to determine the efficacy of measuring PAF production by singleton embryos so as to select the best embryo(s) for transfer. This may allow the laboratory embryologist and/or infertility physician an opportunity to determine the embryo's viability, thus improving pregnancy potential in addition to minimizing multiple gestations.

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Submitted on August 1, 2001; resubmitted on October 19, 2001; accepted on January 10, 2002