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ARTICLE

Preimplantation factor negates embryo toxicity and promotes embryo development in culture

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Abstract Preimplantation factor (PIF) is secreted by viable mammalian embryos and promotes implantation and trophoblast invasion. Whether PIF also has a direct protective or promoting effect on the developing embryo in culture is unknown. This study examined the protective effects of synthetic PIF (sPIF) on embryos cultured with embryo toxic serum (ETS) from recurrent pregnancy loss patients ($n = 14$), by morphological criteria at 72 h of culture, and determined sPIF-promoting effect on singly cultured bovine IVF embryo development. sPIF negated the ETS-induced effect by increasing mouse blastocyst rate versus other embryonic stages (odds ratio (OR) 2.01, 95% confidence intervals (CI) 1.14–3.55, chi-squared = 12.74, $P = 0.002$), increased blastocyst rate (39.0% versus 23.7% ETS alone) and lowered embryo demise rate (11.0% versus 28.8%, OR 0.24, 95% CI 0.11–0.54), which was not replicated by scrambled PIF or the control. sPIF added to bovine embryos for 3 days promoted development at day 7 of culture (11% versus 0%, chi-squared = 4.0, $P = 0.045$). In conclusion, sPIF prevented embryo demise caused by exposure to ETS and promoted development of singly cultured bovine IVF embryos following short-term exposure. sPIF-based therapy for reducing recurrent pregnancy loss and improving lagging cultured IVF embryo development should be explored.

KEYWORDS: embryo development/survival, embryo toxic serum, preimplantation factor, recurrent pregnancy loss, trophic effect

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Introduction

The viable mammalian embryo (semi-allograft) controls its own destiny. It has been postulated that the embryo emits specific 'pro-pregnancy' signals to the mother/host throughout viable pregnancy (Barnea and Coulam, 1997). *In situ*, within the uterus, these signals support implantation (Aplin and Kimber, 2004; Paidas et al., 2010) and systemically, at the periphery, they induce and/or maintain immune tolerance without causing deleterious immune suppression (Chaouat et al., 2010). Given pregnancy's unique immune milieu, it is expected that specific embryo-derived compounds play a crucial modulating role leading to maternal recognition and tolerance of pregnancy. To orchestrate such a 'cross-talk', a viable embryo must be present and will be accepted by the mother. Whereas, a non-viable conceptus will be rejected and maternal acceptance and tolerance for the embryo will not develop (Barnea, 2004).

In patients with recurrent pregnancy loss (RPL), many embryos are aneuploid (non-viable) and self-eliminated (most fail to implant) since maternal recognition of pregnancy does not initiate (Allison and Schust, 2009; Suzumori and Sugiura-Ogasawara, 2010). Impaired maternal receptivity and failure to develop tolerance toward the embryo are also important causes of RPL. In at least ~25% of cases, the serum of RPL patients is toxic when added directly to the medium of cultured embryos. Assaying embryo toxicity in the embryo toxic serum (ETS) is therefore used as a screening diagnostic tool for these patients (Fein et al., 1998; Kaider et al., 1999; Roussev et al., 1995). However, since specific factors that are present in ETS have remained poorly defined to date, effective clinical countermeasures are yet to be identified (Chávez and McIntyre, 1984; Haimovici et al., 1998; Klein, 1997; Sargent and Dokras, 1996; Ito et al., 1996; Ornoy, 2007; Roussev et al., 1995; Thomason et al., 1995). Among several other factors, certain auto-antibodies directly target the embryo and impair its development (Kaider et al., 1999).

The Barnea group reported that viable mouse embryos secrete preimplantation factor (PIF), a peptide that is present in maternal circulation and expressed by the placenta (Barnea, 2004; Barnea et al., 1999; Roussev et al., 1996 (Barnea et al., 2007; Barnea, 2007a,b, Barnea and Sharma, 2006; Than et al., 2007). Synthetic PIF analogue (sPIF), replicating native PIF action, modulates peripheral immune cells to create tolerance without immune suppression and so is effective in controlling autoimmunity (Barnea, 2007b; Weiss et al., 2011). sPIF at a concentration range present in pregnant women (~0.16–0.20 μg/ml) displays essential multitargeted effects, regulating immunity, promoting embryo-decidual adhesion and regulating apoptosis in cultured human decidual cells (Paidas et al., 2010). sPIF also promotes human trophoblastic cell invasion, reflecting its autotrophic effect on the developing placenta (Duzyj et al., 2010). Recently it was reported that, using anti-PIF-based monoclonal antibody enzyme-linked immunosorbent assay, PIF concentrations in cultured mouse and bovine preimplantation embryos correlate with their progress to the blastocyst stage. Further, endogenous PIF is also required for optimal embryo development, likely through an autotrophic effect (Stamatkin et al., 2011).

Because endogenous PIF plays a critical role in embryo development, exogenous (added) sPIF also could be effective in protecting embryos against various environmental insults prior to implantation. To examine such possibility, two complementary in-vitro models in two different species were studied.

First whether sPIF can reduce toxicity was tested by adding it to mouse embryos cultured with toxic serum derived from several RPL patients having diverse aetiologies. This mimics the maternal environment to which the embryo is exposed prior to implantation, at the most vulnerable stage of pregnancy. Such data could help in designing a targeted therapy that would reduce chromosomally competent RPL.

A second culture model used a bovine model, where IVF embryos develop poorly mostly when cultured alone, since no optimal culture methods have been thus far developed. In contrast to humans, the currently available culture methods in multiple species do not sustain a good embryo development potential. Herein, this study tested whether sPIF added in the short term can improve long-term embryo development in culture, which is also relevant for human IVF as a possible treatment for lagging but viable embryos.

Materials and methods

Peptide synthesis

sPIF (MVRIKPGSANKPSDD) and scrambled PIF (GRVDPSNKSMPKDIA) were obtained by solid-phase peptide synthesis (Peptide Synthesizer, Applied Biosystems, Foster City, CA, USA) using 9-fluorenylmethoxycarbonyl chemistry. Final purification was conducted by reverse-phase HPLC and identity was verified by matrix-assisted laser desorption/ionization time-of-flight spectrometry and amino acid analysis (Biosynthesis, Lewisville, TX, USA).

sPIF effect on mouse embryos cultured with ETS

The study was approved by the CARI Research Institute, Chicago, IL, USA. A total of 30 archived frozen serum samples from patients with RPL of various aetiologies were studied. Fourteen serum samples which were previously found to be toxic for cultured mouse embryos (ETS) and 16 sera which were non-toxic, were studied using established criteria (Kaider et al., 1999; Roussev et al., 1995). Briefly, 2-cell-stage embryos were collected from superovulated mated CB6F1/J mice. Removed oviducts were dissected under microscope and embryos were removed into modified human tubal fluid culture medium (cat. 2001 InVitroCare, Frederick, MD, USA). Embryos were cultured in Nunc in 500 μl culture medium under mineral oil by incubating at 37°C with 5% CO_2 for 3 days maintaining pH 7.2 throughout the experiment. Embryos were cultured with 0–15% ETS or non-toxic-serum sPIF (0.043–3.12 μg/ml) adjusting the total culture volume with 2% bovine serum albumin in all cases to 500 μl. Results were compared with scrambled PIF (the same composition as PIF but with the amino acids in a random order) and tested in parallel or medium alone, both serving as controls. At the end of the culture period

at 72 h, embryos' developmental stages were recorded using established morphological criteria by microscopy (Stamatkin et al., 2011). Embryos were categorized as blastocysts, pre-blastocysts, morula, number of cells, 6–8, 2–4 or atretic embryos, and evaluated by two different observers (CWS, RGR).

IVF procedure to retrieve bovine oocytes

At the Louisiana State University Embryo Biotechnology Laboratory, IVF was performed on bovine oocytes obtained from a commercial source (Ovitra, TX, USA). Oocytes arrived in a climate-controlled container via Fedex approximately 24 h following their collection. A standard bovine IVF laboratory procedure was performed on groups of 10 oocytes in 40 μl droplets of fertilization medium (IVF-Tyrode's albumin lactate pyruvate). Briefly, 2 μl heparin (2 μg/ml), 2 μl penicillamine, hypotaurine and epinephrine and 2 μl spermatozoa were added to each fertilization droplet with the meiosis-II oocytes. This made the total medium volume 46 μl. Frozen–thawed spermatozoa from a fertile Holstein bull was used in these experiments (Purpera et al., 2009) The IVF interval was 18 h, incubated in a humidified atmosphere of 5% CO_2 in air at 39°C. Following fertilization, the presumptive zygotes were removed from the fertilization droplets and treated with hyaluronidase (1 mg/ml) to remove the cumulus cells. The embryos were then washed in HEPES-Tyrode's albumin lactate pyruvate medium and transferred to CR1aa culture medium (Rosenkrans and First, 1994). A single embryo from the group of IVF-derived embryos was then placed into a fresh 40 μl droplet of CR1aa at 39°C in a humidified atmosphere of 5% CO_2 in air. On day 3 of culture, individual embryos were transferred to a new 40 μl droplet of CR1aa and incubated under the same conditions until day 7 of culture. At the end of the experiment embryos were assigned a quality grade (1 = good, 3 = poor) and evaluated for morphological development (2-cell to blastocyst).

Addition of sPIF to IVF-derived bovine embryos during in-vitro culture

There are no effective culture methods to achieve a high rate of blastocyst formation in the bovine species following IVF. To examine whether PIF could improve that rate, IVF was performed on bovine meiosis-II oocytes ($n = 100$). The study used 0.16 μg/ml sPIF since this is the concentration of the peptide previously found effective to modulate decidual function and trophoblast invasion (Duzyj et al., 2010; Paidas et al., 2010). Following the IVF procedure, sperm-exposed oocytes ($n = 74$) were randomly allotted to two treatment groups. Treatment A included culturing zygotes in 40 μl droplets of CR1aa with 100 nM (0.16 μg/ml) exogenous sPIF ($n = 37$) added immediately prior to culture. Treatment B included culturing zygotes without the addition of sPIF ($n = 37$) and served as control. On day 3 of culture, developing embryos from both groups were individually transferred to fresh 40 μl droplets of CR1aa and cultured to day 7. Morphological development of the embryos was compared between the two groups at culture days 3 and 7.

Statistical analysis

Data were analysed using a multigroup chi-squared analysis determining the odds ratio (OR) and 95% confidence interval for ETS experiments. In other studies a multigroup chi-squared analysis was carried out using Analyse-it for Microsoft Excel. $P < 0.05$ was considered to be significantly different in these studies.

Results

Serum of certain patients with RPL were toxic for embryos. Initially the toxicity of previously frozen archived ETS samples was confirmed (**Table 1**). It demonstrated that at 10% concentration, practically all embryos became atretic, whereas at lower concentrations, 5% ETS and below, some embryos did survive following exposure. This documented that the ETS effect was dose-dependent and that 5% ETS was suitable to test the sPIF-protective effect.

sPIF protected against ETS derived from patients with RPL. This study tested whether sPIF could block ETS-induced adverse effects on embryos when added into the culture medium. Consequently, a checkerboard-type analysis was carried out to compare the effect of different ETS concentrations added to cultured mouse embryos with and without different sPIF concentrations (**Table 2**). As expected, the high-concentration (15%) ETS led to the demise of all embryos. At 5% ETS, the effect was still significant, although not all embryos became atretic ($P < 0.0001$). Addition of sPIF (0.78 μg/ml) to this culture medium was effective in negating toxicity, as compared with 5% ETS alone (chi-squared $P = 0.02$). The higher sPIF concentrations exposed to 5% ETS were less effective. The data also demonstrated that 3.12–0.78 μg/ml sPIF tested alone was not toxic and that the number of blastocysts that developed was similar to the medium-only control.

Lower sPIF concentrations were less effective in negating ETS. Lower sPIF doses were also tested to confirm that the 0.78 μg/ml dose was the most effective. **Table 3** shows an independent experiment, where the 0.124 μg/ml dose was tested using ETS from 10% to 1%. Data showed that the sPIF effect was borderline (not statistically significant) at 10% ETS. However, it was effective in negating toxicity at 5% ETS (chi-squared $P = 0.03$). When the protective effect of sPIF was compared against 5% ETS, obtained at 0.124 μg/ml,

Table 1 Embryo toxic serum is toxic for embryos.

Embryo	*Embryo toxic serum (%)*				
	10	*5*	*2.50*	*1*	*0*
Blastocyst	0	0	5	4	23
Pre-blastocyst	0	0	2	3	5
Morula	0	0	3	2	2
6–8 Cells	0	0	0	1	0
2–4 Cells	0	1	0	0	0
Atretic	10	8	0	0	0

Values are *n*. Mouse embryos ($n = 9–30$/group) were cultured in presence of various concentrations of embryo toxic serum for 72 h.

Table 2 Checkerboard analysis: serum of a patient with recurrent pregnancy loss is toxic to mouse embryos and synthetic preimplantation factor acts as a rescue factor.

Embryo	*Synthetic preimplantation factor*			
	3.12 μg/ml	*1.56 μg/ml*	*0.78 μg/ml*	*Control*
15% ETS				
Blastocyst	0	0	0	0
Pre-blastocyst	0	0	0	0
Morula	0	0	0	0
6–8 Cells	0	0	0	0
2–4 Cells	2	1	1	2
Atretic	8	9	9	8
5% ETS				
Blastocyst	1	2	6[a]	1
Pre-blastocyst	2	1	0	0
Morula	3	4	3	4
6–8 Cells	4	3	1	1
2–4 Cells	0	0	0	0
Atretic	0	0	0	0
1% ETS				
Blastocyst	8	9	9	10
Pre-blastocyst	2	1	1	0
Morula	0	0	0	0
6–8 Cells	0	0	0	0
2–4 Cells	0	0	0	0
Atretic	0	0	0	0
Control				
Blastocyst	8	10	8	10
Pre-blastocyst	2	0	2	0
Morula	0	0	0	0
6–8 Cells	0	0	0	0
2–4 Cells	0	0	0	0
Atretic	0	0	0	0

Various concentrations of embryo toxic serum (ETS) were added to mouse embryo cultures ($n = 10$/group) in presence of synthetic preimplantation factor (sPIF) at different concentrations. 5% ETS and 0.78 μg/ml sPIF were chosen to test the peptide's protective effects.
[a]Chi-squared = 5.5, $P < 0.02$.

Table 3 Lower synthetic preimplantation factor (sPIF) concentrations are less effective in negating embryo toxic serum (ETS) toxicity.

Embryo	*Control*	*0.124 μg sPIF*	*P-value*
10% ETS			
Blastocyst	0	0	NS
Pre-blastocyst	0	1	–
Morula	0	2	–
6–8 Cells	0	0	–
2–4 Cells	0	0	–
Atretic	10	8	–
5% ETS			
Blastocyst	0	1	0.03[a]
Pre-blastocyst	0	1	–
Morula	0	2	–
6–8 Cells	0	0	–
2–4 Cells	1	2	–
Atretic	8	4	–
2.5% ETS			
Blastocyst	5	6	NS
Pre-blastocyst	2	2	–
Morula	3	2	–
6–8 Cells	0	0	–
2–4 Cells	0	0	–
Atretic	0	0	–
1% ETS			
Blastocyst	4	7	NS
Pre-blastocyst	3	2	–
Morula	2	1	–
6–8 Cells	1	0	–
2–4 Cells	0	0	–
Atretic	0	0	–

Various concentrations of ETS were added to mouse embryo cultures ($n = 9$–10/group). In the presence of 0.124 μg/ml sPIF, the 5% ETS-induced toxicity was negated. However, the degree of protection was lower than observed with the higher sPIF concentration. NS = not statistically significant.
[a]Chi-squared test.

with even lower doses which were tested in parallel (0.024 μg/ml and 0.0043 μg/ml, the lowest dose tested), no protection against embryo toxicity was observed and only 0/10 and 1/10 embryos survived, respectively. Thus the sPIF effect was bell-shaped and was effective at a narrow range of concentrations. Although the lower sPIF dose (0.124 μg/ml) was effective, it was not as pronounced as with the 0.78 μg/ml dose. Therefore, the protective effects of sPIF were further examined using a fixed maximally effective dose of the peptide combined with fixed 5% ETS serum derived from different patients.

sPIF rescued embryos cultured in ETS serum derived from different RPL patients. To further validate the initially observed sPIF-induced embryo protective effects against toxicity ETS sera, six independent studies each testing a different patient serum were carried out to confirm the observations in a larger number of embryos. Addition of sPIF (0.78 μg/ml) to cultured embryos ($n = 113$/group) negated 5% ETS-induced toxicity by acting as a 'rescue factor'. Specifically, sPIF increased the blastocyst rate (39.0%) as compared with ETS alone (23.7%) while reducing the atretic embryo rate (11.0% versus 28.8%, control, respectively; **Fig. 1**). **Table 4** shows the number of blastocysts and atretic embryos following treatment with sPIF in the combined experiments. It demonstrates that sPIF negated the ETS effect by increasing mouse blastocyst percentage as compared with all other developmental stages including atretic embryos (chi-squared = 12.74, $P = 0.002$). **Table 5** shows the significant OR using the different models of analysis. Thus sPIF exposure doubled the odds of an embryo developing to the blastocyst stage compared with ETS alone. On the other hand, sPIF reduced three-fold the likelihood of an embryo becoming atretic as compared with ETS alone. Therefore sPIF protection against a maternally induced toxic environment was dual, increasing blastocysts while decreasing atretic embryos.

The protective effect of sPIF was specific and was not replicated by the control peptide. In contrast to sPIF,

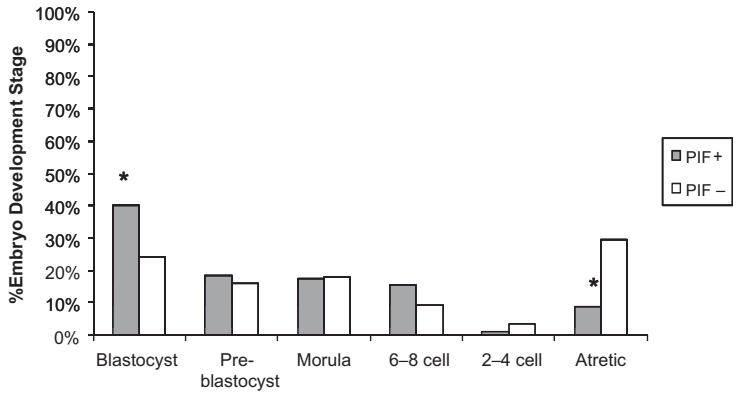

Fig. 1 Synthetic preimplantation factor (sPIF) protects against toxicity of embryo toxic serum (ETS). Mouse embryos were cultured with 5% ETS and 0.78 μg/ml sPIF, which increased blastocyst formation rate while reducing significantly atretic embryos as compared with ETS-only treated embryos (chi-squared = 12.74, $P < 0.002$). **Table 1** details the statistical analysis of the six independent experiments.

Table 4 Synthetic preimplantation factor (sPIF) protects against embryo toxic serum-induced toxicity.

Treatment	*Blastocyst*	*Other*	*Atretic*	*Total*
sPIF	45	55	13	113
No sPIF	28	52	33	113
Total	73	107	46	226

Values are n. Other = pre-blastocyst, morula, 6—8 cells and 2—4 cells.
Chi-squared = 12.74, $P = 0.002$.

scrambled PIF (3.12—0.78 μg/ml) added to embryos ($n = 14$—40/group) did not protect against ETS-induced toxicity, demonstrating that the sPIF effect was specific (data not shown).

High-dose sPIF did not affect embryos cultured under optimal conditions. The initial study (**Table 1**) showed that when sPIF alone was tested at a high 3.12 μg/ml concentration, cultured embryo development was similar to medium control. The study was repeated again (**Fig. 2**) and showed that the effects of sPIF and media-alone treatment were similar (84% versus 77% blastocyst rate). Thus high-dose sPIF appeared to be safe as it did not interfere with normal embryo development.

sPIF promoted cultured IVF-derived bovine embryo development. To demonstrate sPIF's ability to enable embryos to overcome the frequently encountered developmental arrest when cultured in single cultures, a second model was used. Single bovine IVF-derived zygotes were cultured with 0.16 μg/ml sPIF or medium alone for 3 days ($n = 37$/group), followed by change of culture media without added sPIF and observation until day 7 of culture. After 3 days of culture, no differences between sPIF-treated zygotes versus untreated controls were found (83% versus 75% cleavage rate). **Table 6** shows that after 7 days 17% of sPIF-treated zygotes progressed and reached >16 cells versus none reaching such an advanced stage of development in the control (degrees of freedom = 4, chi-squared = 4.0, $P = 0.045$). Thus short-term sPIF administration improved embryo development and the peptide's effect persisted even post exposure.

Discussion

Preimplantation factor (PIF) secreted by viable embryos reaches maternal circulation where it plays a critical role to create maternal tolerance and promote embryo implantation, starting post fertilization (Duzyj et al., 2010; Paidas et al., 2010). Endogenous PIF is further required for embryo development acting through possible autocrine mechanisms having a self-sustaining role (Stamatkin et al., 2011). Herein this study reports that sPIF added to mouse embryo cultures acts as a rescue factor negating ETS-induced toxicity by increasing blastocysts and reducing atretic embryos. Thus sPIF-based therapy may have a role in reducing recurrent

Table 5 Odds ratio (OR) analysis of synthetic preimplantation factor (sPIF) effect on embryo morphology.

OR ratio calculation	*Model 1: blastocyst versus all other embryos*[a]	*Model 2: atretic versus all other embryos*[a]	*Model 3: blastocyst versus atretic embryos*[b]
sPIF	2.01 (1.14—3.55)	0.32 (0.15—0.64)	0.24 (0.11—0.54)
No sPIF	Reference	Reference	Reference

Values are OR (95% confidence interval).
[a] $n = 226$ embryos.
[b] Subset of $n = 119$ embryos with either blastocyst or atretic outcome.

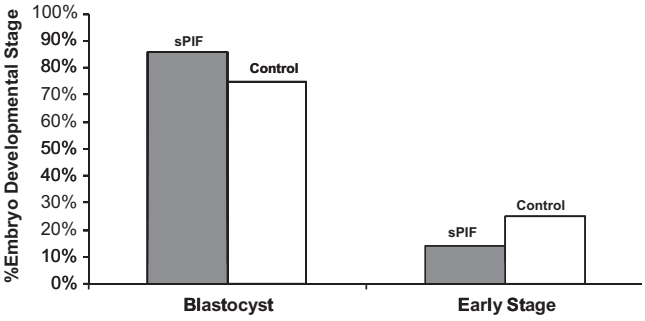

Fig. 2 High-dose exogenous synthetic preimplantation factor (sPIF) does not affect embryos cultured under optimal conditions. Embryos were cultured with 3.12 μg/ml sPIF up to the blastocyst stage. Blastocyst rate of formation was slightly better than controls. Results are composite of two independent experiments.

Table 6 Short-term synthetic preimplantation factor (sPIF) addition promotes singly cultured bovine embryos development in the long term.

Embryo	*No sPIF*	*sPIF*
Day 3		
NC	9 (24)	6 (16)
2 Cells	4 (11)	8 (22)
3 Cells	3 (8)	3 (8)
4 Cells	9 (24)	9 (24)
5 Cells	0 (0)	1 (3)
6 Cells	6 (16)	3 (8)
8 Cells	6 (16)	6 (16)
16 Cells	0 (0)	1 (3)
% Cleavage	76	84
Day 7		
Degenerate	16 (57)	17 (57)
ND	9 (32)	8 (27)
5 Cells	1 (3)	0 (0)
8 Cells	1 (3)	1 (3)
16 Cells	1 (3)	0 (0)
32 Cells	0 (0)	1 (3)
Morula	0 (0)	2 (7)
Blastocyst	0 (0)	1 (3)
% Cleavage	11	17

Singly cultured bovine embryos were exposed to 0.16 μg/ml sPIF for 3 days. Subsequently the media was changed and embryos were cultured for an additional 4 days. After 3 days there was already a slight increase in the cleavage rate. However, at day 7 of culture none of the control embryos progressed beyond the 16-cell stage as compared with the sPIF-treated embryos, chi-squared = 4.0 $P = 0.045$.

miscarriage rates in patients with ETS. sPIF also promotes suboptimally cultured bovine embryo development and may aid in lagging embryos' progress to blastocyst stage.

RPL remains a serious condition with few established effective targeted therapies. Beyond embryonic aneuploidy which causes up to 60% of losses, the rest may be amenable for therapy. Despite intense efforts, no uniform and specific therapies have been developed to treat RPL. The aim is to improve maternal environment, including the immune milieu, and make it receptive for the embryo. Studies have demonstrated that PIF has such an effect on the decidua, as well as promoting trophoblast invasiveness (Duzyj et al., 2010; Paidas et al., 2010). There is evidence that circulating toxins and antibodies are present in the serum of some RPL patients which may cause the demise of exposed embryos. However, targeted interventions to negate such toxicity to improve patient outcome have not been addressed previously. Using sPIF as a protective agent in this embryo culture model was intended to specifically examine a possible therapeutic approach. Using total ETS provided a suitable model to determine an overall adverse maternal environment that the embryo might encounter post fertilization and prior to implantation, thereby mimicking the in-vivo scenario.

The embryo at the peri-implantation phase is most vulnerable to exposure to an adverse (toxic) maternal environment (Kaider et al., 1999). sPIF added to mouse embryos cultured in groups was successful in negating ETS-induced toxicity derived from non-pregnant RPL patients. Such data on PIF-induced embryo protection create a strong link between embryo self-preservation and its ability to negate maternal hostility successfully. sPIF both increased blastocyst formation and reduced atretic embryo rates when exposed to 5% ETS. The protective effect was confirmed by testing several patients expected to have diverse RPL aetiologies because of various toxins and antibodies (Kaider et al., 1999). As sPIF protected both ends of the spectrum of embryo development, it could counteract the embryo-toxic agents that cause a delay in development or lead to demise. As far as is known, this is the first report that exogenous sPIF negates ETS-induced effects and acts as an embryo rescue factor.

Such embryo-protective effects were not obtained by testing other known factors including transforming growth factor α, gonadotrophin-releasing hormone I analogue, insulin growth factors, acrogranin, epidermal growth factor, embryotrophic factor 3 and granulocyte-macrophage colony-stimulating factor (Block and Hansen, 2007; Brinson, 2000; Diaz-Cueto et al., 2000; Jousan et al., 2008; Kawamura et al., 2005, 2007; Sjoblom et al., 2002; Wei et al., 2001; Xu et al., 2004). The specificity of the protective action of sPIF was documented by using scrambled PIF (the same amino acids as sPIF but in a random order) as a control, which had no discernible protective effect. This protection is in line with data demonstrating that fluorescein isothiocyanate (FITC) PIF is taken up by the bovine blastocyst thus aiding in its self-preservation. In contrast, FITC scrambled PIF failed to bind demonstrating specificity (Stamatkin et al., 2011). The minimal protective effect of sPIF at high concentrations might be due to down-regulation of putative embryo binding site(s). As sPIF reversed ETS effects from several patients, very likely of different aetiologies, the current study suggests that sPIF may be a targeted therapy for treating RPL patients, especially those with ETS activity. Evidencing PIF's potential safety, this study demonstrates that high-dose sPIF added to mouse embryos cultured under optimal conditions is safe, reflecting exogenous sPIF effectiveness directly, acting only according to need.

To demonstrate the sPIF-promoting effect on cultured IVF embryos, this study used a single bovine-embryo model as it compares well with the single human IVF embryo model. sPIF improved bovine IVF embryo development and thereby could aid in improving lagging embryo progress to blastocysts. The bovine embryo model is the closest to human embryos because of similar expression of *OCT4* (Berg et al., 2011).

In singly cultured bovine IVF embryos, in contrast to human, lack of supportive cross-talk greatly impairs embryo development. The poor progress is also caused by the ill-defined culture conditions available for bovine embryos. In reality, in this study, only a few of several hundred embryos cultured reached the blastocyst stage without treatment at day 7 of culture. Presence of detectable PIF concentrations in the medium of individually cultured bovine embryos was associated with progress up to the blastocyst stage (Stamatkin et al., 2011). A low sPIF concentration added to these embryos in the short term improved their long-term development. Possibly, sPIF-induced zygote activation triggered an endogenous PIF production as well, leading to the continued autocrine-trophic effect which also lasted after removing sPIF from the culture medium. These trophic effects of sPIF are particularly relevant in a hot climate such as Louisiana where the heat-stressed bovine oocytes were collected for the current study (Flamenbaum and Galon, 2010). Fertilizing all bovine oocytes using the same sperm donor assured uniformity of the fertilization process and avoided a possible embryo selection bias. Culturing bovine and other species' embryos in groups improves development, suggesting the presence of embryo cross-talk (Contramaestre et al., 2008; Gopichandran and Leese, 2006; Stokes et al., 2005; Teruel et al., 2005; Guérin and Ménézo, 2010). sPIF added to single bovine IVF embryos produced a similar promoting effect. Hence, sPIF could optimize IVF embryos outcome by promoting those that were viable but delayed because of suboptimal culture conditions. In human IVF, sPIF could also improve the development of lagging embryos from poor responders in a safe and targeted manner.

This study has certain limitations. Although implied, the direct translation of sPIF-based therapy for treating RPL patients has to be confirmed in a clinical setting. sPIF has unique immune-modulatory properties and is effective in preclinical models (Barnea, 2007a,b; Weiss et al., 2011). Clinical trials for treating non-pregnant patients with immune disorders are planned shortly, following ongoing FDA-directed toxicology studies (Reuven Or, Clinical Trials.gov). Also, observations made on bovine embryos may not be directly translatable to the human IVF setting because of species differences.

The strength of the study is the checkerboard testing of sPIF and ETS combinations, defining best testing conditions, and multiple independent experiments from several patients analysed by rigorous statistical analysis. The use of scrambled PIF as a negative control demonstrated sPIF's specificity. The use of single-embryo IVF cultures to test sPIF under harsh conditions documented its promoting properties, especially since sPIF had no effect under optimal culture conditions.

sPIF protected against embryo toxicity induced by serum derived from RPL patients, increasing blastocysts and reducing atretic embryos irrespective of the putative offending agent(s) present in ETS. Further, sPIF promoted bovine embryo development by overcoming the developmental arrest present in this species. The sPIF effect was direct and defined on the embryo. Therefore, sPIF therapy may benefit patients experiencing RPL and sPIF added to culture could promote suboptimal embryo development.

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Declaration: PIF is a patented compound owned by BioIncept, of which ERB is (uncompensated) Chief Scientist and CBC is a minority shareholder. CWS and RGR received funding from BioIncept. PIF is in preparation for multicentre clinical trials for the treatment of immune disorders.

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