

Evidence that the adverse effect of controlled ovarian hyperstimulation on successful pregnancy outcome following embryo transfer may be related to premature trophoblast invasion

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Summary

Purpose: To determine if premature trophoblast invasion may be a contributing factor to lower fecundity associated with controlled ovarian hyperstimulation (COH) and in vitro fertilization (IVF).

Methods: Blood samples were obtained three-five days after ET to measure expression by lymphocytes of a 34 kDa protein known as the progesterone-induced blocking factor (PIBF) using an immunocytochemistry technique. Clinical and viable pregnancy rates were determined according to whether PIBF was detected or not.

Results: Progesterone-induced blocking factor was positive in 14 of 67 (21%). Clinical pregnancy rates following fresh ET were 7.1% for those positive for PIBF versus 43.4% for those negative for PIBF.

Conclusions: Progesterone-induced blocking factor production requires allogeneic induction of progesterone receptors in gamma/delta T-cells. This suggests early detection of PIBF may be related to premature trophoblast invasion possibly into an endometrium not yet prepared for the trophoblast, thus possibly leading to early immune rejection of the fetus.

Key words: Hostile uterus; Immunomodulatory protein; Controlled ovarian hyperstimulation.

Introduction

There are some data supporting the concept that controlled ovarian hyperstimulation (COH) may adversely affect implantation [1]. One of the first studies matched patients having in vitro fertilization (IVF) to oocyte recipients using donor oocytes with regards to age and previous conception [2]. Despite the transfer of similar numbers of embryos and the finding of no difference in embryo morphology between standard IVF patients and recipients, the clinical and ongoing pregnancy rates (PRs) and implantation rates were significantly higher in recipients [2].

In another study using a shared oocyte program for donors and recipients, the PRs following fresh embryo transfer (ET) were twice as high in the older recipients than the younger donors [3]. However, no significant differences were seen in the PRs between donors and recipients for frozen ETs (though there was a small trend for higher PRs in recipients following frozen ET) [3]. This trend, even with frozen ET, was subsequently found to be related to a lower implantation rate because of hydrosalpinges [4]. In this subsequent study, which also corroborated higher implantation rates in recipients vs donors in fresh ET cycles, the trend for higher PRs in recipients, even with frozen ET, was abrogated once the policy to perform salpingectomies for hydrosalpinges was invoked [4]. A very vivid example of the potential adverse affect of the COH regimen in certain individuals was provided by a case

report of a 38-year-old woman with ten years of infertility related to polycystic ovarian syndrome who failed to conceive after six years of ovulation induction and even ten cycles of IVF-ET where 92 embryos had been transferred; however, she conceived on her first frozen ET on an estrogen-progesterone therapy cycle after transfer of only five embryos [5].

The assumption has been made that the COH regimen somehow inhibits implantation. However, another possibility is that implantation is not impaired but there is premature trophoblast invasion.

The study presented herein was an attempt to determine if premature trophoblast invasion could be an explanation for poor fecundity in some women following COH and IVF-ET by evaluating whether the appearance of some early pregnancy factor, possibly detected at an earlier time than normal, and which would normally require trophoblast invasion, might correlate with poor pregnancy outcome. The factor measured for this study was progesterone-induced blocking factor (PIBF), a protein whose production by gamma/delta T-cells requires the induction of progesterone (P) receptors which is initiated by the allogeneic stimulus of the invading trophoblast [6-10]. The earliest that PIBF has been previously detected is in the late luteal phase, and its presence has been associated with a positive pregnancy outcome [11]. For this study, the attempt was made to measure PIBF at the peri-implantation time to see if detection might be associated with a negative pregnancy outcome.

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Materials and Methods

A prospective study was conducted. Patients age 40 or younger undergoing ET either following oocyte retrieval or the thawing of cryopreserved embryos were enrolled in the study. Oocyte recipients were excluded. Patients consented to have an additional vial of serum drawn three-five days post-ET to measure expression of PIBF by lymphocytes.

The measurement of PIBF expression was determined by an immunocytochemistry method using a PIBF-specific polyclonal antibody. Mononuclear cells were removed using Isoprep (Robbins Scientific, Sunnyvale, CA) and cold centrifugation and were adjusted to a concentration of $2 \times 10^6/\text{ml}$; 100 μl aliquots of cell suspension were added to sample chambers and air dried then fixed in cold acetone. The cells were first incubated with a protein blocking agent and then incubated overnight with anti-PIBF. The cells were washed in phosphate buffered saline (PBS) (Gibco, Grand Islands, NY) and then covered with anti-rabbit peroxidase. Following a second PBS wash, fresh chromogen solution was added and the cells incubated; the reaction was then stopped with distilled water and the cells counterstained with hematoxylin and the slides were read under oil immersion (100 x objective). A positive reaction was indicated by a reddish precipitate at sites of specific cellular antigen localization; 300 cells were counted. The percent of the cells positive was then determined. A test was considered positive if there were at least four lymphocytes of the 300 counted demonstrating the reddish precipitate. This cut-off level of $>1\%$ was chosen based on previous unpublished data using this immunocytochemistry technique in which the large majority of non-pregnant women showed $\leq 1\%$ of the lymphocytes expressing PIBF.

Oocyte retrieval followed stimulation using either the luteal phase leuprolide acetate/gonadotropin protocol [12] or the follicular phase leuprolide acetate/gonadotropin protocol [13]. Embryo transfer was performed three days following retrieval. Assisted hatching and intracytoplasmic sperm injection were used as needed. The transfer of thawed cryopreserved embryos was performed in cycles in which patients were given graduated doses of oral estradiol for two weeks beginning at 2 mg and ending at 6 mg followed by P vaginal suppositories 200 mg 2x/day and IM P (100 mg/day). Down regulation with leuprolide acetate 0.5 to 1 mg daily SC was added if indicated.

The outcome measures evaluated were PIBF expression, positive pregnancy test, and clinical pregnancy (ultrasound evidence of viable pregnancy at eight weeks). Chi-square analysis was used to evaluate the association between outcome of ET and PIBF expression. To check for possible confounding variables, patient and stimulation characteristics were compared by PIBF expression and outcome of ET. Analysis of variance or chi-square analysis was used as appropriate. A p value of .05 was used to determine significance.

This study was approved by the ethics committee of the Cooper Institute for Reproductive Endocrinology. It was not necessary to submit to the Institutional Review Board of Cooper Hospital because treatment rendered to patients was unaltered whether PIBF was positive or not and the blood tests for PIBF were obtained at the same time that other hormonal studies were obtained so no extra venipunctures were necessary. Patients were not charged for these tests, but were made aware that they were taken. All patients who became patients in the reproductive endocrinology division of Cooper Hospital University Medical Center were requested to sign general release forms allowing some of their blood samples to be used for research purposes. Only patients having already signed their releases were included.

Results

Progesterone-induced blocking factor was positive in 20.8% (14 of 67) of women receiving COH and in 11.4% (8 of 70) women undergoing frozen ET ($p < .05$).

Clinical PRs following COH and subsequent ET were 7.1% (1 of 14) for those positive for PIBF compared to 43.4% (23 of 53) for those negative for PIBF ($p < .05$). With sample sizes of 14 and 53, and pregnancy rates of 7.1% and 43.4%, the study had 81% power to detect a difference in the pregnancy rates at the 5% significance level.

Following frozen ET, clinical PRs were 50.0% (4 of 8) for those positive for PIBF and 45.2% (28 of 62) for those negative for PIBF ($p = \text{NS}$).

The mean \pm SD for the serum P levels on the day of human chorionic gonadotropin (hCG) was $1.8 \pm .7$ ng/ml for those patients receiving COH who had positive PIBF expression vs $1.4 \pm .7$ for those with negative PIBF expression and receiving COH ($p = .10$). It is interesting to note that 50% of patients with positive PIBF had serum P > 2 ng/ml on the day of hCG vs 21% for those negative for PIBF.

A distribution of the etiology by PIBF results and IVF outcome can be seen in Table 1. There did not appear to be an inadvertent selection of any given etiology that could explain the data rather than the presence or absence of PIBF. A comparison of possible confounding variables can be seen in Table 2. There was a significant difference in the mean serum estradiol at mid-luteal phase between those with a positive PIBF versus those negative but not

Table 1. — Distribution of etiology for infertility by PIBF results and IVF outcome

	Negative PIBF		Positive PIBF	
	Not Pregnant (n=30)	Pregnant (n=23)	Not Pregnant (n=13)	Pregnant (n=1)
Tubal only	9 (30.0%)	7 (30.4%)	5 (38.5%)	
Male factor only	8 (26.7%)	8 (34.8%)	2 (15.4%)	
Male and female factor	5 (16.7%)	4 (17.4%)	2 (15.4%)	
Female only (non-tubal)	6 (20.0%)	4 (17.4%)	4 (30.8%)	1 (100.0%)
Not available	2 (6.7%)			

Table 2. — Comparison of possible confounding variables

	Positive PIBF		Negative PIBF	
	Pregnant (n=1)	Not Pregnant (n=13)	Pregnant (n=23)	Not Pregnant (n=30)
Age	39	32.5 \pm 2.6	32.2 \pm 3.7	33.3 \pm 4.3
No. of oocytes retrieved	10	12.5 \pm 7.4	13.1 \pm 6.4	12.7 \pm 7.4
No. embryos transferred	4	3.1 \pm .9	3.4 \pm .7	3.1 \pm .9
Day of hCG				
E2	1424	2934.1 \pm 1428.3	2198.5 \pm 1031.7	2396.1 \pm 845.3
P	.9	1.9 \pm .6	1.5 \pm .8	1.6 \pm .6
Mid-luteal phase				
E2	N/A	2199.7 \pm 1281.3*	1543.8 \pm 107.4	1291.3 \pm 711.2*
P	N/A	111.8 \pm 49.1	107.4 \pm 41.7	92.5 \pm 42.7

* $p < .05$

on the day of hCG. There were too few patients with positive PIBF levels in frozen ET cycles to make valid statistical inferences on confounding variables.

Discussion

There are previous data that support the concept that in a pregnant woman exposure of the maternal vascular system to the allogeneic stimulus of the fetus, and thus trophoblast invasion, is required to allow PIBF expression by T lymphocytes [6-10]. Normal lymphocytes in non-pregnant women do not demonstrate P receptors [14]. However normal human lymphocytes express P receptors after in vitro allogeneic or mitogenic stimulation [8]. Furthermore, P receptors were also demonstrated in peripheral lymphocytes of liver transplanted and transfused patients [7]. In the peripheral blood of pregnant women there is an increased ratio of gamma/delta T-cell receptor positive lymphocytes and more than 90% of these cells are activated and express P receptors [10, 15]. The exact nature of the paternal antigen that stimulates the P receptor is not known but some data suggests that the antigens may be class I or class I-like molecules [16-18]. In the presence of P, P-receptor positive lymphocytes synthesize the immunomodulatory protein PIBF [9]. For these reasons it was considered that PIBF could be a marker for trophoblast invasion.

The data suggest that if earlier implantation than normal does occur, the event has negative effects on subsequent fecundity only when the endometrial environment has been exposed to COH. Nevertheless, it is possible that some alternative explanation other than premature trophoblast invasion could account for the earlier detection of PIBF. Whatever the mechanism, there appears to be a strong association of poor pregnancy outcome with earlier detection of PIBF.

These data need to be corroborated with another IVF center also measuring PIBF and subsequent outcome. Furthermore these data will hopefully interest some other IVF centers to evaluate other markers for earlier implantation, and to corroborate or refute the hypothesis that the adverse affect of COH may be related to not only increasing the chance of premature trophoblast invasion, but more importantly, creating an environment not conducive for survival of a prematurely invading trophoblast.

The 20% frequency of premature trophoblast invasion following COH and IVF-ET would not completely account for differences in pregnancy and implantation rates seen between donors and recipients in shared oocyte programs [3, 4]. The possibility exists that the PIBF assay is not sensitive enough to identify all cases of premature trophoblast invasion. However COH in some instances may also inhibit implantation. Experience from oocyte donation programs has demonstrated that embryo-endometrial synchronization within two days was necessary for blastocyst implantation [19-21]. Studies on endometrial pinopodes, which may be present for only 48 hours, have been suggested as indicators of endometrial receptivity [22, 23]. Some have suggested that premature recep-

tion of P in COH cycles despite the use of gonadotropin releasing hormone analogues can advance the implantation time and possibly the premature appearance of pinopodes and thus inhibit the embryos from attaching to the endometrium [24-27]. If some proteins are released by the multicell embryo/blastocyst while in the uterine cavity prior to implantation, they may provide the allogeneic stimulus for induction of P-receptors on gamma/delta T-cells. Using this model, the early production of PIBF may not be related as much to premature trophoblast invasion but to the earlier production of a critical P level. However, the data presented here did not demonstrate higher mean serum P levels in those positive vs negative for PIBF expression.

Even if it is eventually found that the poor PRs related to earlier detection of PIBF expression are not related to premature trophoblast invasion, the test itself could prove useful as a marker for whether IVF-ET cycles with COH should be continued or if ET should be deferred with all embryos cryopreserved for future transfer. Another option to be evaluated would be whether day five blastocyst transfers would overcome this abnormal state. These data need to be confirmed by a larger series and hopefully by other IVF centers.

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