FERTILITY AND STERILITY
Vol. 45, No. 1, January 1986
Printed in U.S.A.

Failure of implantation in human in vitro fertilization and embryo transfer patients: the effects of altered progesterone/estrogen ratios in humans and mice*

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Daily blood samples were taken for progesterone (P) and estradiol (E_2) measurements from women who showed a platelet response consistent with the presence of viable embryos after in vitro fertilization and embryo transfer procedures. A comparison of steroid levels between those women who became pregnant and those who did not revealed the following: at and after the time of transfer, women who failed to become pregnant had significantly higher E_2 levels and a lower ratio of P/E_2 than women who became pregnant. The P/E_2 ratio was a better predictor of implantation failure than was the absolute level of either hormone. Experiments were done in mice to test the hypothesis that P could protect implantation of the embryo against the inhibitory effects of high E_2. In mice, implantation was inhibited by relatively high levels of E_2. This effect was overcome by concomitant administration of P. There was a significant dose-response-related interaction of P with the E_2.
Fertil Steril 45:69, 1986

Early embryonic mortality in man and animals is considerable. In a review of this topic, Short[1] supported the idea that this may be because of "genetic causes arising de novo during gametogenesis." The high incidence of human embryonic loss before implantation[2-4] has become particularly apparent with the development of in vitro fertilization and embryo transfer (IVF-ET) procedures.[5, 6] This is supported by Jones et al.,[7] who quoted a maximum pregnancy rate of 25% of patients undergoing ET. The recent work of O'Neill[8] and O'Neill et al.[9] has provided a means of monitoring the presence and viability of the embryo in utero before implantation. The results of this work with human transfer patients suggest that up to 50% of apparently normal embryos may not be viable. These results support the idea expounded by Short[1] but still leave 20% to 30% of apparently viable, transferred embryos failing to result in pregnancy.

It has long been known that alterations in estrogen and progesterone (P) levels affect repro-

Received July 1, 1985; revised and accepted September 10, 1985.
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ductive outcome and that the two hormones do not act independently.[10] Alterations in amounts of estrogen and P affect various parts of the reproductive process[10]; among these is the inhibition of implantation in animals and humans by high levels of estrogens.[10, 11] The use of exogenous P to overcome the effects of estrogen[10] and to support the luteal phase[12] has been widely used. The inability to monitor embryo survival before implantation has prevented supporting evidence for the efficacy of this treatment.

This study monitored in utero embryo viability and measured daily 17β-estradiol (E_2), P, and human chorionic gonadotropin (hCG) levels in women undergoing IVF-ET. The aim was to discover any differences in the levels and ratio of these hormones that might have affected the pregnancy outcome of viable embryos. The second part of the study was concerned with testing, in experimental animals, the efficacy of P in protecting the process of implantation against inhibitory effects of high estrogen levels.

MATERIALS AND METHODS

HUMAN DATA

The data reported here were collected from women participating in the IVF-ET program at Royal North Shore Hospital and Hunters Hill Private Hospital in Sydney, New South Wales, Australia. Details of ovulation induction, ovum collection, IVF, embryo culture and transfer, blood collection, and counting of blood platelets were the same as previously described by O'Neill et al.[9]

Blood samples were taken from patients immediately before ET and at the same time daily for the following 2 weeks. Embryo viability was determined with the use of changes in the blood platelet levels as described by O'Neill[8] and O'Neill et al.[9] Concentrations of P, E_2, and hCG were measured for all patients. The concentrations of E_2 were also measured daily during the latter part of the follicular phase for all women.

Duplicate samples were used for the hormone assays at each time interval, and all samples from an individual were measured in the same assay at the conclusion of the collection period. E_2 was measured with the use of a direct ^{125}I E_2 competitive radioimmunoassay (RIA) (Endocrine Sciences, Tarzana, CA). P was measured with the use of a double-antibody ^{125}I P competitive RIA (Farmos Diagnostica, Turku, Finland). The hCG was measured with the use of an ^{125}I hCG competitive RIA (Serono Diagnostics, London, UK).

The endocrine data were analyzed by analysis of variance, with the time interval between samples as the controlled variable and the hormone levels as the dependent variable. The treatment groups comparison was between confirmed pregnant and confirmed nonpregnant women.

EFFECTS OF INJECTING PROGESTERONE AND/OR 17β-ESTRADIOL BEFORE IMPLANTATION IN MICE

Mice

We used Quackenbush strain virgin white mice aged 8 to 10 weeks and weighing 30 to 35 gm.

Dose of Steroids

We chose the doses of steroids used in the experiment because they included and exceeded the range required to initiate implantation in replacement experiments with the use of these mice.[13, 14] Steroids were injected separately in 0.1 ml of sesame seed oil. Control mice received oil alone and experimental animals received P + oil, E_2 + oil, or P + E_2. The doses of P were 1 or 10 mg/mouse, and the doses of E_2 were 0.1, 1.0, or 10.0 μg/mouse.

Experimental Design

After random allocation to treatment groups, eight mice per group were injected at 9:00 A.M. on day 4 of pregnancy with the various treatments and doses. (Day 1 of pregnancy was the day on which the copulatory plug was detected.) The treatments were carried out on day 4 because the embryos are in the uterus and are unattached at that time. Injection of steroids before this time can affect tubal transport of embryos[15] and would therefore confuse any effect of the altered P/E_2 ratio on implantation. Only mice that mated on the third day after being placed with male mice were used in this experiment. This was done because it has been shown that a significantly different number of eggs are shed on different days after contact with male mice.[16] The mice were killed and an autopsy performed on day 7 of pregnancy. The number of implants, implant weight, and body weights were recorded for all mice.

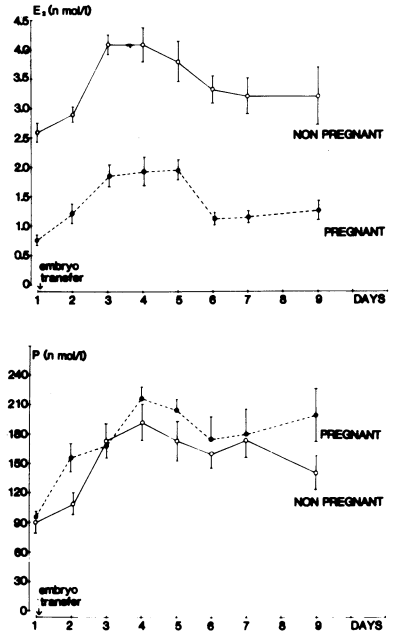

Figure 1
E_2 (*top*) and P (*bottom*) values (means and standard deviations) for women who became or failed to become pregnant after ET.

Analysis of Data

An analysis of variance for number of implants per mouse per treatment was carried out. To obviate the problem of including mice with no implants in the data, the data were transformed with the use of the square root of implant number + 0.5 before the analysis. This allowed the inclusion of all mice from all the treatment groups. Specific comparisons between treatments and doses were made by partitioning the analysis of variance with the use of polynomial coefficients.

RESULTS

HUMAN DATA

All the women in this comparison showed a significant decrease in platelet number after ET. This response indicates the presence of a viable fertilized embryo and did not occur in women undergoing the same procedures but with unfertilized ova or culture medium transferred.[8, 9] We compared levels of P and E_2 and the P/E_2 ratio between five women who showed a positive hCG result and a confirmed physical pregnancy with eight women who showed a negative hCG result and a confirmed lack of pregnancy.

The data in Figure 1 show the means and standard deviations for P levels during the 9 days after ET in the pregnant and nonpregnant groups of women. There was no significant difference in the P levels between the two groups. It also shows the means and standard deviations for E_2 levels during the 9 days after ET in the pregnant and nonpregnant groups of women. There was a significant difference ($P < 0.01$) in the E_2 levels between the two groups, with the women who failed to become pregnant showing much higher levels.

The means and standard deviations for the P/E_2 ratio between the pregnant and nonpregnant women are shown in Figure 2. The P/E_2 ratio was calculated simply by dividing the P value in nanomoles per liter by the E_2 value in nanomoles per liter measured in the same sample. There was a significant difference ($P < 0.01$) in the P/E_2 ratio between the pregnant and nonpregnant groups of women. The ratio of P/E_2 was higher (i.e., P was dominant) in the women who became pregnant.

The results in Figure 3 are the individual P and E_2 values and P/E_2 ratio for 13 days after transfer in two women who showed prolonged depression of platelet counts after ET. Both women were injected with 3000 IU hCG (Organon, Oss, The Netherlands) on day 9 after ET. Woman 1 showed no steroid response to the hCG and failed to establish a pregnancy. Woman 2 showed a dramatic increase in steroid levels after the hCG injection and went on to have a normal, healthy infant. The striking difference between the steroid endocrine profiles of these patients was the relatively high E_2 and low P of patient 1, compared with patient 2. That is, at the time of ET and in the

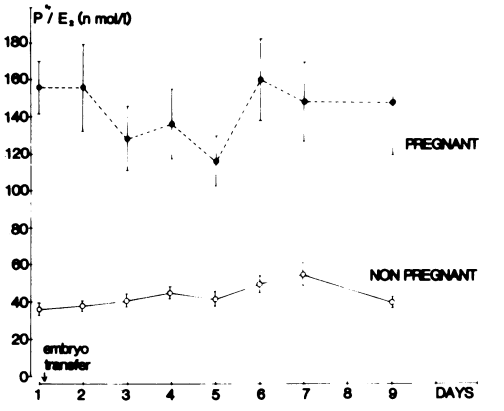

Figure 2
The P/E_2 ratios (mean and standard deviations) for women who became or failed to become pregnant after ET.

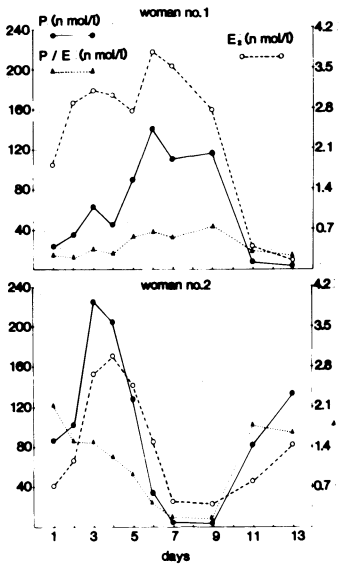

Figure 3
Individual P and E_2 values, and P/E_2 ratios for two women who showed a delayed return to normal platelet levels after ET. Woman 1 failed to become pregnant, and woman 2 did become pregnant.

days immediately thereafter, the P/E_2 ratio strongly favored P in the woman who became pregnant.

Table 1 shows the mean E_2 concentrations for the pregnant and nonpregnant women on the day of and the 4 days before the hCG injection used to induce ovulation. An analysis of variance of the data showed no significant differences between the two groups up to the time of hCG injection.

MOUSE DATA

Table 2 shows the number of mice pregnant and the number of implants per mouse after the various steroid treatments. The results convincingly show that increasing doses of E_2 reduce the number of embryos implanted per mouse and the number of mice that remain pregnant. Concomitant administration of P protects against this effect. The protective effect of P shows a dose-dependent interaction with doses of E_2. We conclude from the results that absolute levels of E_2 are not critical for implantation, but the ratio of P/E_2 is critical.

The analysis of variance for the data summarized in Table 2 reveals a significant difference between the treatments ($P < 0.001$). Partitioning the analysis of variance revealed the following significant effects. There was a significant effect of E_2 ($P < 0.001$) and a significant difference between the doses of E_2 ($P < 0.01$). There was also a significant effect of P ($P < 0.001$) and a significant difference between the doses of P ($P < 0.01$). The interaction between P and E_2 was significant ($P < 0.001$).

DISCUSSION

Until recently, it has been difficult to investigate the causes of pregnancy failure after IVF-ET procedures. With the use of changes in circulating maternal platelet levels as an indicator of embryo viability, we investigated the importance of maternal P and estrogen levels as a cause of pregnancy failure. Previous work in the mouse[8] and human[9] showed a significant thrombocytopenia associated with the presence of viable embryos and a return to normal platelet numbers occurring around the time of implantation.

In this study, women who showed this platelet response after routine IVF-ET were divided into two groups, those who had a confirmed pregnancy and those with a confirmed failure of pregnancy. We compared the groups regarding P and E_2 levels from the day of ET until day 9 after ET. There was no significant difference in P levels between the two groups over this time, but there was a significant difference in E_2 levels and ratios of P/E_2. The women who failed to have implanted embryos had significantly higher E_2 levels and lower ratios of P/E_2. There was no significant difference in the number of eggs collected from the women in the two groups. This suggests that the high level of E_2 in the nonpregnant group was because of ovarian hyperstimulation rather than superovulation. The range of E_2 levels from individuals within the two groups did overlap, whereas the ratios of P/E_2 did not. At the time of and during the few days after ET, it would appear that the ratio of P/E_2, rather than absolute levels of either steroid, is a critical indicator for successful implantation.

To test this hypothesis, we did experimental work in mice. Humans and mice have a similar

Table 1. Mean E_2 Concentrations in Millimoles per Liter for Pregnant and Nonpregnant Women on the Day of and 4 Days Before hCG Injection

Days before hCG	Day 4	Day 3	Day 2	Day 1	Day of hCG
Pregnant women	1.68	2.60	3.66	3.98	5.49
Nonpregnant women	1.56	2.83	3.83	5.33	6.37

Table 2. *Effect of E_2 and P on Implantation in Mice*

Group	Treatment at 9:00 A.M., day 4 of pregnancy	No. of mice pregnant out of 8/group	% Mice pregnant	Mean no. of implants/mouse
1	0.1 ml oil + 0.1 ml oil	8	100	16.4
2	0.1 ml oil + 1 mg P	7	87.5	17.6
3	0.1 ml oil + 10 mg P	8	100	18.1
4	0.1 ml oil + 0.1 μg E_2	8	100	13.5
5	0.1 ml oil + 1.0 μg E_2	6	75	9.7
6	0.1 ml oil + 10.0 μg E_2	1	12.5	3.0
7	1 mg P + 0.1 μg E_2	7	87.5	16.1
8	1 mg P + 1.0 μg E_2	8	100	14.3
9	1 mg P + 10.0 μg E_2	4	50	6.5
10	10 mg P + 0.1 μg E_2	8	100	17.0
11	10 mg P + 1.0 μg E_2	7	87.5	15.7
12	10 mg P + 10.0 μg E_2	8	100	14.4

hemochorial placentation and similar steroid endocrinology associated with implantation. After ovulation, both species have rising P levels and a secondary rise in E_2 before implantation. The secondary rise in E_2 is an absolute requirement for implantation in mice, and this may be the case in human beings. In mice, experimental alteration of the P/E_2 ratio to favor estrogen at the time of the secondary E_2 rise causes reovulation and a failure of implantation of the original fertilized eggs.[17] A major difference between the species is the endocrine support for the corpus luteum (CL). Mice do not have an active CL unless copulation occurs. Regardless of whether the mating is sterile or fertile, the CL is activated and supported by the secretion of pituitary prolactin. This pituitary support lasts for 11 days of the 20-day gestation period, after which placental prolactin takes over this function.[14] Implantation occurs late on day 5 after copulation in mice; thus there is no requirement for embryonic hormones to support the CL at this time. In human beings, embryonic hCG is required to support the CL at the time of implantation and for its maintenance during early pregnancy.[18]

Mice, therefore, are useful experimental animals in which to test the effects on implantation of altering ratios of P/E_2, without the complication of an embryonic gonadotropin affecting the results. The results clearly show that the P/E_2 ratio is important and that P can protect the process of implantation against the inhibitory effect of high E_2 levels. This may explain the comparatively higher success rate of Jones et al.,[7] who routinely gave P to patients after ET.

The individual results shown for the two women in Figure 3 raise some particular and interesting points. These two women were injected with hCG on day 9 after ET. It is important that hormone levels were measured retrospectively and that the decision to inject these women was based solely on the failure of their platelet levels to return to normal at the expected time. This suggested that viable embryos were still present but had failed to implant. The retrospective measurement showed no hCG in either woman on day 9 and was consistent with this idea. Woman 1 failed to respond to hCG, showed declining steroid levels after the injections, and did not become pregnant. Woman 2 responded to the single injection of hCG with rising steroids, implantation, and a successful pregnancy. A comparison of the steroid profiles for these two women shows that the E_2 levels were high and the P/E_2 ratio low in woman 1 at the time of and during the few days after the ET. The opposite was true of woman 2, however; her steroid levels fell dramatically around the expected time of implantation. This was presumably due to failure of luteotropic support caused by lack of embryonic hCG.

The failure of exogenous hCG to stimulate the CL in woman 1 suggests that even if embryonic hCG had been produced, the CL would not have responded. This could have been because of an inherent lack of receptors of hCG in the CL or, more likely, a direct or indirect effect on the receptors of the relatively high E_2 levels.[19] Thus the inhibitory effects of relatively high E_2 levels may prevent implantation by affecting the uterus and/or embryo and/or CL. Apart from the correct steroid environment for successful implantation, there may be a concomitant extra steroidal requirement for hCG. Hearn et al.[20] showed that marmoset embryos grown in culture will not attach to fibroblast layers in the absence of chorionic gonadotropin (CG). Embryos that do not at-

tach do not continue to grow and develop. In this study, CG appeared to be an absolute prerequisite for attachment to occur and attachment could be blocked by the addition of antibody against CG.

In summary, the practical implications from this work suggest that we should endeavor to mimic the normal endocrinology as closely as possible for improved success in IVF-ET. Ideally, the stimulation of follicular growth before ovulation should not stimulate excessive estrogen. If this does occur, then P and/or hCG could be administered to counteract its effect. To mimic the normal physiologic situation, P treatment should be given for a few days, followed by hCG treatment. It would probably be beneficial to counteract the effect of excess estrogen as early as possible. We suggest that P treatment should start after egg collection and before transfer of embryos.

Acknowledgments. We wish to thank Dr. Ian Martin for help and advice with the statistical analyses and Mrs. Patricia Mote, Mr. Michael Bonofacio, Mr. Anthony Zakker, and Mr. Peter Sloss for technical assistance.

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