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CLINICAL ASSISTED REPRODUCTION

An Analysis of Spontaneous Hatching in a Human Endometrial Epithelial Coculture System: Is Assisted Hatching Justified?

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Purpose: *To evaluate spontaneous embryo hatching in an endometrial epithelial coculture system, and compare it with cases where coculture was performed because of maternal age, previous repeated implantation failures, or both. To clarify in which cases assisted hatching would be appropriate.*
Methods: *Individual human embryos were cocultured on an endometrial epithelial cell monolayer until Day 6.*
Results: *Blastocyst hatching rate at Day 6, depending on maternal age, was 9.1% (age <37 years) and 3.4% (age ≥ 37 years). However, blastocyst hatching rates depending on number of previous IVF failures were similar.*
Conclusions: *Maternal age and previous implantation failures are factors affecting the ability of human embryos to reach the blastocyst stage in coculture. However, assisted hatching is not justified in these populations because of the absence of hatching rate differences between blastocysts obtained from these two groups and the control group.*

KEY WORDS: Assisted hatching; blastocyst; coculture; implantation.

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INTRODUCTION

Before the blastocyst attaches to the endometrial epithelium it must escape from the zona pellucida. This step is referred to as embryo hatching and is a necessary step in blastocyst implantation.

Failure of hatching may be due to the hardening of the zona pellucida, intrinsic factors such as genetic problems, as well as poor quality embryos resulting from suboptimal in vitro culture conditions. It has been reported that a low proportion (5–8%) of embryos develop to the in vitro hatching stage (1–4).

To enhance in vitro hatching, the zona pellucida can be manipulated with a technique named assisted hatching. However, it is not clear if this would be of benefit to all patients undergoing embryo transfer. While some studies have shown that assisted hatching improves pregnancy and implantation rates in unselected patients (5–8), in older patients (9–12), in patients with repeated IVF failures (13,14), or in poor prognosis patients (15–18), other studies demonstrated that this technique does not increase pregnancy and implantation rates in the same groups: unselected patients (19–21) or older patients (22,23).

The coculture system is another technique employed for improving preimplantation embryo development. Generally this technique is used for patients with repeated implantation failure, patients in whom multiple pregnancies were to be avoided, and patients where embryo development or chromosomal analysis (preimplantation diagnosis) needed to be assessed.

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Furthermore, a combination of both the techniques used to enhance human embryonic development and increase implantation rate has been analyzed, and the results are equally controversial. While Cohen (24) suggested that this combination had a significantly detrimental effect on the results of assisted hatching, other groups show acceptable pregnancy and implantation rates in older patients with or without multiple failed IVF (25) and in poor prognosis patients, with multiple failed IVF, high basal FSH, and ovulatory disorders (26).

During coculture, some embryos hatch spontaneously at Days 6–7. Thus, we have learnt about the hatching ability of embryos derived from different clinical situations. Therefore, this study was retrospectively designed to evaluate the influence of maternal age and number of implantation failures on the rate of in vitro blastocyst development, so as to determine if assisted hatching in the IVF laboratory would be of benefit to these patients.

MATERIALS AND METHODS

Patients

A total of 202 patients participating in the Instituto Valenciano de Infertilidad coculture programme, from May 1996 to December 1998, were included in this study. A total of 1,372 embryos were studied.

Patients were divided as follows: 74 patients from the ovum donation programme were considered as controls because oocytes were obtained from younger donors (mean $= 25.7 \pm 0.3$ years). Thus, the actual performance of the technique and embryo development up to the blastocyst stage—in our laboratory—can be evaluated by analyzing this group of women in which no male factor was registered.

One hundred and twenty eight patients were analyzed for maternal age and previous implantation failures. Patients were divided into two subgroups according to age: <37 years ($n = 97$) and ≤ 37 years ($n = 31$). Mean maternal age was 32.6 ± 0.2 years and 38.7 ± 0.4 years, respectively. The mean number of implantation failures in these subgroups was similar (3.2 ± 0.2 and 3.4 ± 0.5, respectively). Then, the same patients ($n = 128$) were analyzed based on previous implantation failures and were divided into two subgroups: ≤ 2 ($n = 49$) and ≥ 3 ($n = 79$) previous failures. The mean number of previous failures was 1.4 ± 0.1 and 4.3 ± 0.2, respectively. Mean maternal age was similar in both groups (33.4 ± 0.5 years and 34.6 ± 0.4 years, respectively).

Endometrial Culture

Primary culture of epithelial endometrial cells for coculture embryos were employed. Endometrial biopsies were minced into small pieces of less than 1 mm, and subjected to mild collagenase digestion with 0.1% collagenase type IA (Sigma, Madrid, Spain). Epithelial and stromal endometrial cells were isolated as previously described (30). Stromal cells were discarded and epithelial cells were cultured to confluence over 4–6 days in 75% Dulbecco's Modified Eagle's Medium (Sigma, Madrid, Spain) and 25% MCDB-105 (Sigma, Madrid, Spain), medium supplemented with 10% fetal bovine serum (Gibco, Madrid, Spain), 5μg/ml Insulin (Sigma, Madrid, Spain), and antibiotics.

Embryo Coculture

Individual human embryos were cocultured on an endometrial epithelial cell monolayer. Embryos were incubated at 37°C, in an atmosphere of 5% CO_2 in air. Day 0 was defined as the day of oocyte retrieval. On Day 2, after insemination, when embryos were at the 2–4-cell stage, they were cocultured in 1 mL of IVF: S2 medium (1:1) (Scandinavian IVF Science AB, Gothenburg, Sweden). On Days 3, 4, 5, and 6, until transfer, the embryos were cocultured in S2 medium. Media were removed every 24 h. Embryos achieving the blastocyst stage were transferred back to the mother with a Frydman catheter. The percentage of hatching embryos transferred was 17.2% in the control group. In the subgroups studied according to the age, the percentages were 25% and 7.9%, respectively. Depending on the number of previous failures the percentages were 23.1% and 20.1%, respectively.

Statistical Analysis

Chi-square tests were used to analyze statistical differences between groups. A p value of $<.05$ was considered statistically significant.

RESULTS

A total of 1,372 embryos were analyzed. In the control group, 275 out of 447 embryos developed to blastocyst stage, but only 38 reached the hatching stage at Day 6. Thus, the actual performance of normal embryos in our system in terms of hatching is 8.5%. The results comparing different maternal ages in our

Table I. Embryo Development Until Blastocyst Hatching at Day 6 Depending on Maternal Age

Group	Patients (*n*)	MII oocytes (*n*)	Fertilisation (%)	Embryos (*n*)	Blastocyst (%)	Hatching BI (% per embryo)	Hatching BI (% per blastocyst)	Implantation (%)
Control	74	629	85.9	447	275 (61.5)	38 (8.5)	38 (13.8)	21.3
<37 years	97	1,302	83.4	748	433 (57.9)	68 (9.1)	68 (15.7)	14.3
≥37 years	31	326	78.7	177	83 (46.9)*	6 (3.4)**	6 (7.2)	9.4

Note. Data analysed by Chi-square.
* $p < .01$, Significantly different from control; ** $p < .05$, Significantly different from control.

IVF population are shown in Table I, in which it can be observed that hatching was significantly ($p < .05$) lower in the group of older patients as compared with the younger and control groups because significantly less blastocysts were obtained in the group of older patients. No significant difference was obtained between the control and the <37 years group. However, when we compared blastocyst hatching per blastocyst obtained in these groups, no significant differences were obtained. Table II shows the results divided according to the number of previous IVF failures. It shows that the number of previous failures significantly ($p < .05$) affected the ability of the embryos to reach the blastocyst stage. However, as far as hatching was concerned, there was no difference among groups.

DISCUSSION

Assisted hatching has been developed to promote embryo implantation in in vitro human embryo culture. To improve the hatching of blastocysts, artificial alteration of the zona pellucida has been carried out in many laboratories, using several methods. However, micromanipulation may have serious consequences for embryonic development. Therefore, the effectiveness of this technique has varied, depending on its individual application.

The impact of assisted hatching on monozygotic twinning is also discussed. This technique may enhance not only pregnancy rate but also multiple gestation rate (26–30). The transfer of blastocysts (in human IVF) should result in a decrease in multiple gestations by reducing the number of embryos transferred to the mother, but so long as the twinning rate is not increased. Recently, Meldrum *et al.* (8) analyzed the safety of assisted hatching with respect to the incidence of multiple gestations. Their results illustrated that this technique did not increase monozygotic twinning.

The objective of this report was to evaluate the rate of spontaneous embryo hatching in a coculture system in optimal conditions. In addition, to compare this observation with cases in which coculture was performed because of maternal age or repeated IVF failure, or both, so as to determine whether or not the embryos derived from these two situations would benefit from the micromanipulation methods described by Cohen *et al.* (24). We selected as a control population the embryos created by insemination of donated oocytes with normal sperm, since oocytes employed in our programme are provided by healthy women (mean age of 25.7 years). Based on our experience, the rate of in vitro embryo hatching is 8.5%, which compares favourably with other authors who obtained rates between 4.3% and 8% (1–4).

Another objective of the present study was to clarify whether assisted hatching would be necessary in cases in which the literature reports the need for it, such as older women or those with repeated IVF failures, or both (9,14,16,18,22,25). We hypothesised that if unassisted in vitro hatching occurs in these two situations at the same rate as controls, assisted hatching

Table II. Hatching of Human Blastocysts at Day 6 Depending on the Number of Previous IVF Failures

Group	Patients (*n*)	MII oocytes (*n*)	Fertilisation (%)	Embryos (*n*)	Blastocyst (%)	Hatching BI (% per embryo)	Hatching BI (% per blastocyst)	Implantation (%)
Control	74	629	85.9	447	275 (61.5)	38 (8.5)	38 (13.8)	21.3
≤2 Failures	49	639	81.3	360	221 (61.4)	31 (8.6)	31 (14.0)	12.7
≥3 Failures	79	989	82.6	558	300 (53.8)*	43 (7.7)	43 (14.3)	14.1

Data analysed by Chi-square.
* $p < .05$, Significantly different from control.

may not be required. Our data have clearly shown that maternal age significantly decreases the chances of an embryo to develop until the blastocyst stage, but when embryos achieve this stage hatching rate is not impaired.

Implantation has been found to be consistently higher than hatching, demonstrating that in vitro hatching is not a prerequisite, since some embryos will hatch after replacement. Moreover, it is not possible to ascertain whether the embryos that finally implant are those that hatched in vitro or not.

We were unable to find differences in the rate of hatching when repeated IVF failure was analyzed. However, we did find a difference in the percentage of embryos developing to the blastocyst stage, suggesting that repeated failure may be caused, at least in part, by decreased embryo quality.

In keeping with this concept, we have recently analyzed our experience with coculture of human embryos in IVF and oocyte donation patients (31). We have observed that coculture improves pregnancy rates in oocyte donation but not in IVF. These data reinforce the concept that the quality of the endometrium is crucial for implantation. In IVF cases in which the endometrium is affected by the endocrine milieu generated by simultaneous ovarian stimulation, the transfer of embryos cultured to the blastocyst stage has no positive effect on outcome. Moreover, in ovum donation in which external steroid replacement provides a more physiological environment, blastocyst replacement makes a difference. All these data together suggest that coculture is a useful system in which natural embryo selection can be obtained. In this sense, a lower rate of in vitro development can be expected in women with repeated failure. But, an optimal endometrium is as crucial as a good embryo to achieve successful implantation.

In conclusion, the results of this study indicate that spontaneous hatching in humans may occur, in our in vitro coculture system. Repeated IVF failure and maternal age are associated with lower embryo quality, but the ability of the embryos to hatch is not impaired.

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