Clinical Outcome of Day 2 Versus Day 3 Embryo Transfer Using Serum-Free Culture Media: A Prospective Randomized Study

G. ERTZEID,[1,2] P. O. DALE,[1] T. TANBO,[1] R. STORENG,[1] E. KJEKSHUS,[1] and T. ÅBYHOLM[1]

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Purpose: *The objective was to evaluate whether extending the embryo culture period from 2 to 3 days would yield a more optimal selection of viable embryos, thereby increasing the implantation and live birth rates.*
Methods: *Patients undergoing in vitro fertilization with at least one oocyte fertilized were prospectively randomized to 2 or 3 days of embryo culture in serum-free media. On the basis of their morphology and cleavage rate, a maximum of three embryos was selected for transfer.*
Results: *Embryos transferred on day 2 or day 3 were similar morphologically, however, a higher proportion of retarded embryos was observed on day 3. The implantation rate was 15.8 and 14.3% for day 2 and day 3 transfers, respectively. The increase in live birth rate from 18.5 to 22.6%, possibly suggesting a better embryo selection on day 3, was not statistically significant.*
Conclusions: *Extending the embryo culture period from 2 to 3 days had no effect on implantation and live birth rates.*

KEY WORDS: embryo transfer; extended culture; implantation rate; live birth rate; serum-free media.

INTRODUCTION

Even though there have been several major advances in assisted reprodution technology in recent years, the implantation rate per embryo remains low, as high numbers of transferred embryos fail to implant. Poor embryo quality and/or low endometrial receptivity may explain the high rate of embryo wastage. To optimize the likelihood of pregnancy, selection of human embryos with a high developmental competence for transfer is essensial. Short-term embryo culture with transfer of embryos on day 2 (day 0 being the day of oocyte retrieval) is usually utilized in the Nordic countries. Embryos are therefore transferred at least 2 days before they would normally have entered the uterus and prior to the activation of the embryonic genome at the four- to eight-cell stage (1). One of the major concerns of performing embryo transfer of day 2 cleavage-stage embryos is to predict effectively the developmental competence of early human embryos in the clinical in vitro fertilization (IVF) laboratory (2,3). Embryos selected for transfer are usually chosen on the basis of their morphology and rate of development. Extending the culture period to beyond the time of expected activation of the embryonic genome might therefore optimize the selection of viable embryos for transfer. With this the implantation rate per embryo might increase, thereby limiting the number of embryos replaced to obtain an acceptable pregnancy rate but, at the same time, avoiding the occurrence of high-order multiple pregnancies.

The development of the coculture technique is one approach for extended culture. Although coculture systems seem to promote the pattern and rate of embryo development, the method has disadvantages, as it is time-consuming and expensive and exposes human embryos to animal cells as well as to potentially infectious agents. Supplementation of culture media with serum for improving culture conditions may also contribute to contamination. Therefore, the desired goal for IVF is the culture of human embryos in a serum-free chemically defined medium. Media supplemented with synthetic serum substitutes are now available and have proved to be successful in sustaining embryo development (4–6) even beyond day 2 (7–11). In these retrospective as well as prospective studies, either extended culture of "spare" embryos after embryo transfer on day 2 were utilized for evaluating blastocyst development (8,9) or the pregnancy rate was assessed

[1] Department of Obstetrics and Gynecology, The National Hospital, University of Oslo, 0027 Oslo, Norway.
[2] To whom correspondence should be addressed.

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in selected patients who had several day 2 embryos (7,10,11). Hence, the high rate of embryo development to the blastocyst stage as well as the high implantation rate of blastocyst transfers reported in these studies is achieved in selected groups of good responders.

The present report, on the contrary, is a prospective randomized study with the objective of evaluating whether extending the embryo culture period from 2 to 3 days in an unselected group of patients would yield a more optimal selection of viable embryos, thereby increasing the implantation and live birth rates.

MATERIALS AND METHODS

Patients

The study group consisted of patients undergoing IVF following ovarian stimulation at the National Hospital, University of Oslo. Patients with an infertility diagnosis of tubal factor, unexplained infertility, endometriosis, and others, for instance, ovulatory disorders, were included. Male partners had normal semen parameters according to World Health Organization criteria. Patients with diploid fertilization of at least one oocyte were randomized by computer based block randomization to 2 or 3 days of embryo culture.

Clinical Procedures

Ovarian stimulation for all patients consisted of down regulation with intranasal buserelin (Suprefact; Hoechst, Frankfurt am Main, Germany), 100 μg six times daily starting in the midluteal phase, followed after 2 weeks by stimulation with human menopausal gonadotropin (Pergonal, Serono, Geneva, Switzerland), 225 IU i.m. daily. Cycles were monitored using a combination of vaginal ultrasound examination and serum estradiol concentrations. Ovulation was induced with human chorionic gonadotropin (Profasi, Serono) when at least three follicles with a mean diameter of at least 17 mm were visualized. Transvaginal ovum pickup (OPU) was performed 36 hr later.

In Vitro Culture

Semen preparation and insemination of oocytes have been described previously (12). During the first 2 days embryos were cultured in groups, each containing up to five or six embryos, in 1 ml of the same culture medium, Universal IVF Medium (Medi-Cult, Copenhagen, Denmark), in a 5% CO_2 humidified atmosphere at 37°C. This medium is a commercial serum free medium supplemented with synthetic serum replacement (SSR) and pyruvate, as well as human serum albumin (HSA) and insulin. For extended culture to day 3, embryos were transferred to the commercially available M3 medium (Medi-Cult), which is an enriched serum-free medium based on Ham's F10 and F12 media.

Assessment of Fertilization and Embryo Development

At approximately 16 hr after insemination, oocytes were scored for the presence of pronuclei. Only oocytes with diploid fertilization were cultured further. Cleavage stages were registered at 24 and 48 hr after observation of pronuclei and morphological assessment of embryos was performed as follows: grade 1—blastomeres are of equal size or unequal size, as a result of asynchronous cleavage, and anucleate fragments are absent; grade 2—not all blastomeres are of equal size, and anucleate fragments are present in less than 20% of the volume; grade 3—not all blastomeres are of equal size, and anucleate fragments are present in 20 to 50% of the volume; and grade 4—anucleate fragments are present in more than 50% of the volume or the embryo is totally fragmented. Embryos were selected for transfer on the basis of their morphology and cleavage rate. A maximum of three embryos (grades 1–4 and all cleavage stages), when available, was replaced into the uterus. Totally fragmented embryos, however, were not replaced. The implantation rate defines the number of gestational sacs by the total number of embryos transferred and the live birth rate by the number of live births per 100 embryo transfer cycles. Pregnancy was determined by serum β-human chorionic gonadotropin (hCG) measurement 14 days post-OPU, confirmed by ultrasound at 7 weeks post-OPU and followed to delivery.

Statistical Analyses

The number of patients included was calculated based on a mean number of embryos transferred in each patient being 2.5, an increase in implantation rate from 15 to 25%, an α level of 0.05, and a power of 90%. Assuming this, a minimum of 682 embryos had to be transferred. Results are presented as means ± SD. The data were analyzed by Student's t test and χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

Patients

The two groups of patients studied were similar in age and primary/secondary infertility as well as infertility diagnosis for IVF as shown in Table I.

Oocytes and Embryo Quality

There was no significant difference between day 2 and day 3 cultures regarding the number of oocytes recovered per oocyte retrieval (10.2 ± 7 versus 10.9 ± 7) or the number of fertilized oocytes (7.2 ± 5 versus 7.0 ± 4), but a small difference in the number of replaced embryos (2.19 ± 0.68 versus 2.39 ± 0.57). Embryo transfer was not performed in four cycles with a single, fertilized oocyte but no further embryo development. Hence, a total of 162 embryo replacements was performed on day 2, and 159 replacements on day 3. Embryos selected for transfer on day 2 or day 3 showed no significant difference in morphological assessment; the proportion of grade 1 to 4 embryos was 42, 38, 15, and 5% on day 2 versus 46, 37, 12, and 5% on day 3. The cleavage stage of embryos transferred day 2 or day 3 is shown in Table II. On day 2, the proportion of embryos transferred which had completed the second cleavage division and hence had reached the expected four cell-stage (≥4 blastomeres) when replaced was 66% (237/360). On day 3, however, only 45% (171/382) of the embryos transferred had proceeded through the third cleavage division to the expected eight-cell stage (≥8 blastomeres), a statistically significant difference ($P = 0.01$).

Pregnancy Outcome

The implantation rate per transferred embryo was the same for the two groups: 15.8% for day 2 and 14.3% for day 3. Details of pregnancy outcome are presented in Table III. There was no significant difference as to the number of miscarriages or ectopic pregnancies. A total of 30 live births following 162 embryo replacements on day 2 resulted in a live birth rate of 18.5%, compared to 22.6% as a result of 36 live births following 159 replacements on day 3, a difference not reaching statistical significance ($P = 0.3$) (Table III).

Table I. Characteristics of Patients Randomized for Embryo Transfer on Day 2 or Day 3

	Day 2	Day 3
Cycles	165	160
Age (yr)	33.6 ± 3.9	33.7 ± 3.9
Primary infertility(%)	84 (51)	86 (54)
Infertility diagnosis		
Tubal factor (%)	103 (63)	98 (61)
Unexplained (%)	28 (17)	30 (19)
Endometriosis (%)	14 (8)	11 (7)
Others (%)	20 (12)	21 (13)

Table II. Number of Blastomeres in Embryos Selected for Replacement on Day 2 or Day 3

Blastomeres (*n*)	Day 2 embryos		Day 3 embryos	
	n	(%)	*n*	(%)
2	75	(21)	7	(2)
3	48	(13)	10	(3)
4	174	(49)}	44	(12)
5	30	(8)}	40	(10)
6	26	(7)}(66)*	81	(21)
7	0		29	(8)
8	7	(2)}	121	(32)}
9	0		21	(5)}(45)*
10	0		20	(5)}
>10	0		9	(2)}

* The proportion of embryos transferred which had proceeded through the second cleavage division on day 2 and the third cleavage division on day 3 ($P=0.01$).

DISCUSSION

The aim of this study was to investigate whether an extended period of in vitro culture would result in a more optimal selection of viable embryos and hence an increased implantation rate and live birth rate. The

Table III. Outcome of Pregnancies After Embryo Transfer on Day 2 Versus Day 3

	Day 2	Day 3
Embryo transfers (*n*)	162	159
Pregnancies (*n*)	45	48
Clinical abortions		
<12 weeks (*n*)	12	8
>12 weeks (*n*)	1	3
Ectopic pregnancies (*n*)	2	1
Live birth		
Singletons (*n*)	20	29
Twins (*n*)	8	7
Triplets (*n*)	2	0
Implantation rate per embryo (%)	15.8	14.3
Birth rate (live births/ ET) (%)	18.5	22.6*

* $P = 0.3$ (NS).

implantation rate per transferred embryo on day 3, however, was not improved and the increase in live birth rate following embryo replacement on day 3 versus day 2, suggesting a better selection of viable embryos, was not statistically significant. No significant difference in pregnancy rate or live birth rate was also observed in a previous retrospective study after delaying transfer to day 3 (13). However, in this study, the implantation rate, as measured by the proportion of embryos developing to the fetal heart stage, was higher on day 3 (23%) than on day 2 (19%), most likely as a result of fewer embryos on average transferred on day 3 than on day 2. In our study, three embryos were replaced when available. Hence, if we, as well, had reduced the number of embryos replaced to a maximum of two, many low-quality embryos would not have been replaced, and consequently, the implantation rate per embryo on day 3 might have been increased as a result of a more optimal selection against poorer embryos.

Furthermore, the outcome might have been different if only patients with several embryos on day 2 were included, always rendering possible a selection of the most viable embryos for transfer after prolonged culture. Previous studies of embryo development and pregnancy rate after embryo culture beyond day 2, usually include only "good responders"; patients with many fertilized oocytes (14) or with several viable embryos on day 2 (7,10,11). The validity of an implantation rate per embryo of 23.5% on day 2, 25% on day 3 (14), or as high as 50% after blastocyst transfer on day 5 (11), may therefore be limited, as good responders always have a good chance of conception compared with the overall population. Consequently, in a large study of an unselected group of patients, the implantation rate per blastocyst was not higher than 23% (15). In the present study, all patients were included.

Most IVF centers use scoring systems mainly based on morphological criteria to select embryos for transfer. Embryos selected for transfer on day 2 and day 3 in our study showed no difference in morphological assessment. There appears, however, to exist a low correlation among morphology, viability, and implantation. Development to the blastocyst stage has been shown to be poorly associated with the grade of the embryo on day 2 (16). In another study, no difference in implantation and pregnancy rates was observed between transfer of day 2 and transfer of day 3 embryos, although significantly more embryos cultured for day 3 transfer were of moderate to poor quality (14). However, the morphological criterion of multinucleated blastomeres is important, as in transfers of embryos containing mulitinucleated blastomeres, the implantation rate is low, and the incidence of chromosomal abnormalities high, especially when multinucleation appears as early as 2 or 3 days after fertilization (17,18).

The rate of embryo development appears to be a more reliable indicator of embryo viability than morphology. Evaluation of embryo viability based on timing of the first cell division has been studied (19), and a higher frequency of clinical pregnancies resulting from early cleaved two-cell embryos was observed (20). Furthermore, on day 2, embryos at the four- to six-cell stage had the best chance of reaching the blastocyst stage (21), while two- to three-cell embryos never developed to the blastocyst stage (22). Moreover, the rate of development to the blastocyst stage is of importance; the faster the embryo reached the blastocyst stage, the higher was the chance of implantation (23). Hence, the increased success with blastocyst transfers on day 5, with an implantation rate (fetal heartbeat/embryo transferred) as high as 50% (11), may not be the result of the blastocyst transfer itself, but attributed to intrinsic characteristics of the embryo, laid down in the oocyte under maternal control before ovulation. In our study, with embryos selected for transfer on the basis of their morphology as well as cleavage rate, a significantly higher degree of retarded embryo development on day 3 was observed, as only 45% of the embryos transferred had proceeded through the third cleavage division, compared to 66% of day 2 embryos that had completed the second cleavage division. On the contrary, in a previous study with improved clinical outcome with delay of transfer from day 2 to day 3, after selection of embryos only on the basis of their cell number without regard of fragmentation, no retardation of embryo development by extended time in culture was observed, as 49% of embryos transferred on day 3 had eight or more blastomeres, while only 43% of embryos on day 2 had four or more cells (24). However, from the results, the day 2 group seems to be a negatively selected group, which might explain the improved outcome with day 3 transfer in this retrospective study.

The presence of a suitable environment that fulfills the growth requirements to support the development of the embryo is another factor necessary for successful outcome. The potential for embryo development varies under different culture conditions (10,11,25). Extension of the culture period beyond day 2 may itself impose new drawbacks and risks because the embryonic genome is activated at the four- to eight-cell stage.

Analysis on day 2 and day 3 of embryos cultured in different media did reveal a difference in the incidence of multinucleated blastomeres and was generally higher in day 3 than in day 2 embryos (26). Consequently, the effect of a better selection of viable embryos in extended culture may be compromised by the suboptimal embryo development in vitro compared to that in vivo.

In conclusion, extending the culture period in serum-free media from 2 to 3 days did not increase the implantation rate or pregnancy rate in this prospective, randomized study, in an unselected group of patients with a maximum of three embryos replaced when available. On the other hand, these data demonstrate that transfers can be safely scheduled for day 2 or day 3 at the convenience of the patient and the IVF center. To improve the implantation rate in the future, we have to learn more about parameters for embryo quality assessment and embryo culture as well as endometrial receptivity as suboptimal endometrial quality is another possible cause for low implantation rate. Clinical studies, as well as basic science investigating genetic and physiological factors in the embryo and uterus which influence successful implantation, are mandatory.

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