

## Articles

# Blastocyst culture: facts and fiction



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### Abstract

The use of sequential media has made extended culture and transfer of blastocysts feasible for human IVF. Embryo transfer on day 5 has been claimed to result in higher implantation rates than transfer on day 3, on the basis of retrospective comparative studies. This is not supported convincingly, however, in randomized controlled trials published to date. Blastocyst culture imposes additional requirements in terms of personnel, equipment, education and cost and is associated with a greater incidence of monozygotic twinning and cycle cancellation rate than in the case of day 3 culture. In order for day 5 transfer to replace day 3 transfer, a convincing comparison between the two methods should therefore demonstrate the superiority of blastocyst transfer. There is still a need for properly designed randomized controlled trials to compare day 3 with day 5 transfer which will also address the effectiveness of a single blastocyst replacement in reducing the incidence of multiple pregnancies as well as the value of blastocyst cryopreservation.

**Keywords:** blastocyst, extended culture, monozygotic twinning

### Introduction

Activation of the embryonic genome occurs after the 8-cell stage is reached (Braude *et al.*, 1988). Otherwise the embryo will not survive further. Such an embryo can unfortunately be selected for transfer on day 3. In this case, implantation does not occur, while no pregnancy will ensue if a single embryo replacement is performed.

In terms of embryo quality, the improvement of human IVF outcome requires identification of those embryos that will progress beyond the 8-cell stage and therefore have an increased implantation potential. Lack of such identification necessitates the replacement of more than one embryo, so as to reduce the chance of a failed attempt. Replacement of more than one embryo can, of course, result in multiple pregnancies.

Blastocyst culture allows the transfer of embryos that clearly have an activated embryonic genome. In this way, it may be feasible not only to increase the implantation rate, by transferring embryos that have progressed beyond day 3, but even more importantly, to avoid the incidence of multiple pregnancies by reducing the number of embryos replaced. However, this requires that the elimination of embryos in

extended culture from day 3 to days 5–6 should be dependent solely on their inherited survival potential and not be a consequence of an adverse effect exerted by the sequential media used for culture.

Where this is the case, it is likely that even though blastocyst transfer may select embryos with a greater implantation potential, this will be at the cost of losing embryos that could have implanted if transferred prior to the blastocyst stage. This will mean that a number of patients who might have become pregnant if embryos had been replaced on day 3 will now fail to become pregnant.

In patients with poor prognosis (those with no 8-cell embryos on day 3), blastocyst culture and transfer has been reported to result in 0% pregnancy rate, in contrast with day 3 transfer, which has led to 33.3% ongoing pregnancy rate (Racowsky *et al.*, 2000). This suggests that the uterine environment may 'rescue' some of the embryos that will not survive blastocyst culture (Racowsky *et al.*, 2000). In this study, a sequential culture system with IVF-500 culture medium to support embryo growth from days 1–3 and S2 in turn for later growth was used, since the S1/S2 combination was reported to produce blastocysts of lower quality. Moreover, the

implantation rate achieved in patients with no 8-cell embryos with day 3 transfer (33.3%) indicates that those embryos were not compromised in culture.

A reduction in viability of embryos with cleavage abnormalities has also been shown by Alikani *et al.* (2000), who suggested that until culture media are further refined, extended culture should be limited to those embryos with optimal development for the first 3 days in culture.

If the uterine environment is better for some embryos on day 3 than a laboratory environment, then an attempt to predict blastocyst development or quality on the basis of embryo assessment on day 3 might be inefficient.

## Can blastocyst development be predicted on the basis of day 3 embryo evaluation?

In answer to this question, Shapiro *et al.* (2000) showed that 24% of the fastest growing embryos on day 3 did not develop into blastocysts. On the other hand, many of the embryos growing at a significantly slower rate did form blastocysts.

Graham *et al.* (2000) showed that only 48% of embryos selected on day 3 for embryo transfer or cryopreservation were selected again on days 5 and 6. Similar results were obtained by Rijnders *et al.* (1998), who noted that only 51% of the embryos transferred on day 5 had been previously selected for transfer on day 3.

Balaban *et al.* (2000) showed that although there was a significant difference in the blastocyst formation rate between grade 1 and 2 embryos on the one hand and grades 3 and 4 on the other (59.1 versus 25.9% respectively), ~40% of high quality embryos did not form blastocysts. Moreover, the quality of the cleavage-stage embryo was not a determinant of blastocyst quality.

Langley *et al.* (2001) showed that a significant number of embryos with initial retarded development were able to progress to the blastocyst stage (36% of the 4-cell, day 3 embryos reached the blastocyst stage).

Fisch *et al.* (2001) developed a graduated embryo score (GES) system comprising two interval evaluations of early developmental milestones along with a weighted assessment of conventional morphological characteristics on day 3. They suggested that high GES scoring embryos on day 3 could predict high quality blastocyst conversion better than cleavage rate and morphology. Sixty-four percent of high scoring (90–100) embryos resulted in high quality blastocyst formation. However, even with this scoring system, some embryos with low scores (30–65) will still form high-quality blastocysts (11%).

In summary, it appears that the ability of the embryo assessment systems currently used to predict blastocyst development is limited. The validity of such an attempt, however, is questionable as embryos with high implantation potential can also be identified on the basis of sophisticated criteria (Gerris *et al.*, 1999; Lunqvist *et al.*, 2001).

It is interesting to note that by analysing patients <34 years of age undergoing their first IVF trial, a pregnancy rate of 74.1% and an implantation rate of 48.1% were recorded following transfer of two top-quality embryos on day 3 (Gerris *et al.*, 1999). When, however, an uninterrupted sequence of patients was analysed by the same authors (Gerris *et al.* 2001), the same method (transfer of two top-quality day 3 embryos) yielded good but less impressive results (ongoing pregnancy rate: 49.6%, ongoing implantation rate: 39.5%). The importance of the population selected should therefore not be underestimated when evaluating the beneficial effect of day 3 or day 5 transfers.

It should be also clear that prior to embarking on blastocyst development prediction, it should be shown that transfer at the blastocyst stage results in a better overall outcome than does day 3 transfer.

## Is blastocyst transfer better than day 3 transfer?

### Evidence from retrospective studies

Milki *et al.* (2000) compared day 5 versus day 3 transfers and showed a significantly higher viable pregnancy rate (68 versus 46% respectively) and implantation rate (47 versus 20% respectively) in a small number of patients who had more than three 8-cell embryos on day 3 of culture. More embryos were transferred on day 3 than on day 5 (4.6 versus 2.4 respectively). Multiple pregnancy rates did not differ significantly between the two methods, although the rate was higher in the day 3 group than in the day 5 group (65 versus 44% respectively).

From a limited number of patients with multiple IVF failures and with at least three embryos available in the previous cycle, Cruz *et al.* (1999) concluded that blastocyst transfer leads to a higher clinical pregnancy rate (40%) than does day 3 transfer (9.1%). Moreover, the implantation rate was also higher in the day 5 group than in the day 3 group (11.3 versus 3.4% respectively), while more embryos were transferred on day 3 than on day 5 (5.4 versus 3.1 respectively). No multiple pregnancies were observed in the blastocyst group, although the difference with day 3 transfer was not significant.

Racowsky *et al.* (2000) reported that for patients with at least 8 zygotes after fertilization, similar pregnancy rates were observed between day 5 (43.2%) and day 3 (43.9%) transfers. The implantation rate was higher when embryos were replaced on day 5 (31.2 versus 19.5%). Again, more embryos were transferred on day 3 than day 5. No difference in multiple pregnancy rates was observed between the two groups.

Analysing day 5 versus day 3 transfer in patients with at least five oocytes fertilized, Abdelmassih *et al.* (2001) observed a higher ongoing pregnancy rate (41.0 versus 29.4% respectively) and implantation rate (18.5 versus 11.5% respectively) in the day 5 group. More embryos were transferred in the day 3 group than in the day 5 group (4.6 versus 3 respectively). A higher multiple pregnancy rate was present in the day 3 versus day 5 group (47.1 versus 28.5% respectively).

Balaban *et al.* (2001a) observed that blastocyst transfer results in a higher implantation rate than does day 3 transfer (15 versus 5.9% respectively) in patients with only grade 3 and 4 embryos available for transfer on day 3. More embryos were replaced on day 3 than on day 5 (5.2 versus 2.4 respectively). No difference was observed in pregnancy rates (27.2 versus 33.5% respectively) or multiple pregnancy rates between the two groups (13.6 versus 9.4% respectively).

Performing blastocyst transfer for all their patients, Marek *et al.* (1999) demonstrated a higher ongoing pregnancy rate and implantation rate than for patients in whom day 3 transfer was performed prior to extended culture introduction (pregnancy rates: 46.9 versus 36.9% respectively; implantation rates: 32.4 versus 23.3% respectively). Again, more embryos were transferred on day 3 (3 versus 2.5). The multiple pregnancy rates were similar in the day 3 and day 5 groups (49 and 42% respectively).

Wilson *et al.* (2002) showed a higher ongoing pregnancy rate (52 versus 42%) and implantation rate (43 versus 27%) when comparing day 5 ( $n = 570$ ) with day 3 ( $n = 419$ ) transfers. More embryos were replaced on day 3 (2.7) than on day 5 (2.0). Moreover, no difference was observed in multiple pregnancy rates between the two groups compared (day 3: 36% versus day 5: 34%).

In oocyte recipients, Schoolcraft and Gardner (2000) reported that blastocyst transfer compared with day 3 transfer results in a higher clinical pregnancy rate (87.6 versus 75.0% respectively) and implantation rate (65.0 versus 41.6% respectively). More embryos were replaced on day 3 than on day 5 (3.2 versus 2.1 respectively). Moreover, no difference was observed in multiple pregnancy rates when embryos were transferred on either day 3 (40.5%) or day 5 (44.2%).

To sum up, it appears that retrospective studies consistently support the view that blastocyst transfer results in a higher implantation rate than does day 3 transfer. Results are not always so clear regarding pregnancy rates, which in some studies did not differ between the two methods (Racowsky *et al.*, 2000; Balaban *et al.*, 2001a). Interestingly, multiple pregnancy rates were similar in both day 3 and day 5 transfers.

It should also be noted that any retrospective study must be regarded sceptically and several issues need specific attention.

Consistently more embryos were transferred on day 3 than on day 5 in all of the above studies. However, as the number of embryos replaced increases, a 'dilution effect' takes place.

If a comparison is made between the implantation rates achieved in two exactly similar groups of patients, in whom, however, unequal numbers of embryos are transferred on the same day of culture (e.g. day 3), the only case that there will not be no difference in implantation rates is if all the embryos are of similar quality. If, however, embryo quality is not the same within embryos, as in human IVF, then the chance to transfer embryos of inferior quality increases in parallel with the number of embryos transferred (assuming that the best embryos are transferred). As a consequence, the implantation rate will decrease in the group of patients in which more

embryos are transferred. This kind of bias is present in all of the retrospective studies mentioned.

As a consequence, arguing that the higher implantation rate observed with extended culture is proof that blastocyst transfer is superior than day 3 culture is not justified. Moreover, such a conclusion may be a source of further questionable arguments.

*'Blastocyst culture provides more physiological synchronization of the embryo with the endometrium, improving implantation rates.'*

This notion is frequently used in the literature to explain why blastocyst transfer leads to better outcome compared than does day 3 transfer. However, it is probably justified only when embryo replacement on day 5 is attempted during a natural cycle. Vlasisavljevic *et al.* (2001) compared day 2 to day 5 transfers in unstimulated ICSI cycles and obtained similar pregnancy rates per oocyte retrieval (12.2 versus 14.3% respectively) as well as per day 2 embryo available (23.8 versus 23.2% respectively).

In an important study, Gardner *et al.* (1996) determined the levels of metabolites surrounding the human oocyte and embryo *in vivo*. For that purpose, oviduct and uterine fluids were collected throughout the menstrual cycle from naturally cycling patients and it was shown that the human embryo is exposed to different metabolite concentrations as it passes along the genital tract. Therefore, a uterine transfer of a day 3 embryo in a natural cycle probably results in asynchrony as the day 3 embryo is exposed to uterine secretions 2 days earlier than in nature. If this embryo is cultured to day 5 *in vitro*, a subsequent transfer at the blastocyst stage will probably result in synchrony and more physiological exposure of the blastocyst to the normal uterine secretions.

However, in referring to IVF, it must be clear that during ovarian stimulation, together with the major deviations from normal function that the human ovary undergoes to produce multiple oocytes, the endometrium, under the influence of supraphysiological levels of oestrogen and progesterone, is also deviating strongly from normality (Ubaldi *et al.*, 1997; Nikas *et al.*, 1999; Kolibianakis *et al.*, 2002).

The combination of agonists and gonadotrophins, used in the majority of the retrospective studies mentioned, is known to result in endometrial advancement at oocyte retrieval in 100% of cases (Ubaldi *et al.*, 1997). The same is true for antagonist cycles (Kolibianakis *et al.*, 2002). An embryo transfer on day 3 or day 5 is therefore performed in an endometrium that was already abnormal at oocyte retrieval, 3 or 5 days earlier.

Moreover, by studying endometrial biopsies from donor cycles, Nikas *et al.* (1999) showed that the cycle days when pinopodes form are on average 1–2 days earlier in ovarian stimulation than in natural cycles. These changes in pinopode expression may reflect shifts in the window of receptivity, resulting in ovo–endometrial asynchrony and limiting implantation success in IVF.

Consequently, the basis on which to assume better embryo–endometrium synchronization in stimulated cycles

with blastocyst transfer rather than with day 3 transfer is not clear. The extrapolation from events occurring in a natural cycle between the embryo and the endometrium (synchrony with day 5 transfer) to those taking place in a hyperstimulated cycle is probably not justified.

Uterine contractility has been shown to be lower on day HCG+7 than on day HCG+4 and the day of HCG administration in assisted reproductive technology cycles where embryos were transferred on day 2 (Fanchin *et al.*, 2001). Although these results require further investigation, there is at present no study comparing uterine contractility between day 3 transfer and day 5 transfer.

### *'Blastocyst culture eliminates aneuploid embryos leading to a better implantation rate.'*

Sandalinas *et al.* (2001) reported that 19% of the aneuploid embryos and 21% of the polyploid embryos on day 3 developed to the blastocyst stage, suggesting that extended culture to day 5 or 6 cannot be used as a reliable tool to select against chromosome abnormalities.

Magli *et al.* (2000) analysed 143 6–9-cell embryos from patients who had preimplantation genetic diagnosis and showed that 51% of them were aneuploid by FISH on day 3. These embryos were then cultured to day 5. Although aneuploidic embryos on day 3 had a lower capacity to develop to morulae and blastocysts than did euploid ones (59 versus 34% respectively), 40% of the generated blastocysts were abnormal. Clearly an aberrant genome is not incompatible with blastocyst development.

Seeking why blastocyst transfer results in a better implantation rate than does day 3 transfer should follow the persuasive demonstration of such a statement. This can be achieved only by properly designed prospective randomized studies (Bennet, 2001).

### Evidence from randomized controlled trials

Eight randomized studies have been published so far (Scholtes and Zeilmaker, 1996; Gardner *et al.*, 1998; Coskun *et al.*, 2000; Huisman *et al.*, 2000; Karaki *et al.*, 2002; Levron *et al.*, 2002; Rienzi *et al.*, 2002; Utsunomiya *et al.*, 2002) (see **Table 1**) comparing day 3 with day 5 embryo transfers. The randomization procedures in the above studies were not always adequate. Scholtes and Zeilmaker (1996) and Huisman *et al.* (2000) randomized their patients using the day of the week. Pseudo-randomization was also employed by Gardner *et al.* (1998), Rienzi *et al.* (2002) and Utsunomiya *et al.* (2002). The type of randomization was not mentioned in the study by Levron *et al.* (2002). On the other hand, although adequate randomization was used in the studies by Coskun *et al.* (2000) and Karaki *et al.* (2002), this was carried out at the time of fertilization. The studies by Gardner *et al.* (1998), Coskun *et al.* (2000), Karaki *et al.* (2002), Levron *et al.* (2002) and Rienzi *et al.* (2002) analysed a selected population. Moreover, from the studies using sequential media, similar numbers of embryos were transferred between day 3 and day 5 groups only in the studies by Coskun *et al.* (2000), Rienzi *et al.*

(2002), and Utsunomiya *et al.* (2002). Sequential media were not used in the studies by Huisman *et al.* (2000) or Scholtes and Zeilmaker (1996).

Recognizing the above limitations, it can be observed that in none of the eight randomized controlled trials is pregnancy rate significantly better with blastocyst transfer as compared with day 3 transfer. In the study by Levron *et al.* (2002), blastocyst transfer resulted in a poorer pregnancy rate than day 3 transfer. Moreover, in only two out of eight randomized controlled trials was implantation rate better with blastocyst transfer, while in the study by Levron *et al.* (2002) it was worse than day 3 transfer.

It therefore appears that the published prospective randomized trials do not support the view that day 5 transfer results in a better outcome than day 3 transfer, even if only studies in which sequential media were used are considered.

Higher implantation rates for blastocyst transfer were reported in the studies by Gardner *et al.* (1998) and Karaki *et al.* (2002). However, at the same time, more embryos were transferred on day 3 than on day 5. The validity of comparing implantation rates is therefore questionable, since the denominator is different across the groups compared. If more embryos are transferred in day 3 patients, then a 'dilution effect', as mentioned above, is to be expected. Where the numbers of embryos replaced were similar between the two groups, no difference was observed in implantation rates.

Interestingly from the above prospective trials, the study by Gardner *et al.* (1998) is commonly used in the literature to support the notion that 'the patients who stand to benefit most from blastocyst technology are those who are at greater risk for high order multiple gestations'. However, without moving to extended culture but by limiting the analysis to better prognosis patients, in a way similar to that of Gardner *et al.* (1998), the halo surrounding these results starts to appear also in day 3 transfers. In patients <34 years of age, in their first cycle of IVF with at least two top quality embryos on day 3, Gerris *et al.* (1999) reported a 48.1% implantation rate and 74% ongoing pregnancy rate by transferring two embryos. The importance of patient selection is also suggested by the results of Gardner *et al.* (1998), since an equally high pregnancy rate after day 3 transfer was achieved in their study (66%).

A properly designed randomized trial comparing day 3 transfer with day 5 transfer should cover several points. Randomization should be performed prior to initiation of treatment. In this way, information about the superiority or inferiority of a procedure is applicable to all patients and can assist the informed choice the couple has to make. Analysis should be performed on an intention-to-treat basis, so as to avoid attrition bias. Delivery rate per randomized patient should be the primary outcome measure.

The numbers of embryos transferred should be equal among groups, so that valid conclusion can be drawn for both implantation rates and multiple pregnancy rates. Moreover, in order to develop a complete picture of the methods being compared, the combined success of both fresh and frozen embryo transfer must be considered between cleavage and

more advanced embryos. Such an analysis was performed in the study by Rienzi *et al.* (2002), although in a limited number of cycles.

Most importantly, non-inferiority as compared with day 3 transfer cannot be the outcome of a comparison between day 3 and day 5 transfer. In order to recommend switching to day 5 transfer, it should be proven convincingly that day 5 transfer has clear advantages over day 3. This is due to the fact that blastocyst culture is associated with an increased chance of having no transfer in a proportion of patients and that it requires additional equipment, extra personnel, more space and a higher cost than day 3 transfer.

A cost effectiveness analysis regarding blastocyst culture versus day 3 culture has not yet been performed. This is due to the fact that the denominator of the cost-effectiveness ratio,

which is the effect of the intervention (blastocyst culture and transfer), is still in debate. Such an analysis will have to take into consideration all costs involved in blastocyst culture, from the trivial cost of the medium to more substantial costs, such as that from the extra incubation space needed and that pertaining to the labour intensive nature of day 5 culture (Patton *et al.*, 1999) as well as a possible decrease in cost due to fewer embryos being frozen when blastocyst culture is performed. Interestingly, amongst embryologists the greater disadvantage of blastocyst culture appears to be the workload, cost and resources involved (Hartshorne and Lilford, 2002). Nevertheless, although superiority of day 5 transfer versus day 3 transfer has not been shown yet, it appears that its adoption is on the increase.

Two randomized controlled trials have so far compared day 2 transfer with day 5 transfer (Plachot *et al.*, 2000; Van Der

**Table 1.** Randomized trials comparing day 3 versus day 5 transfer.

Authors; RT <sup>a</sup> ; no. embryos transferred	Selection criteria	Culture media	Mean no. embryos transferred		Pregnancy rate (%)		Implantation rate (%)		Multiple pregnancy rate (%)	
			Day 3	Day 5	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5
Scholtes and Zeilmaker (1996); RT1; day 3 – 233, day 5 – 410	All IVF patients	Single medium	2.4	2.1	26	25	13	12	15	20
Gardner <i>et al.</i> (1998); RT2; day 3 – 47, day 5 – 45	Patients with >10 follicles of >12 mm on day 8 of the cycle	Sequential media	3.7	2.2	66	71	30.1 <sup>c</sup>	50.5	–	–
Huisman <i>et al.</i> (2000); RT1; day 3 – 590, day 5 – 709	All IVF patients	Single medium	1.9	1.9	21.7	22.1	14.4	15.5	26	32
Coskun <i>et al.</i> (2000); RT3; day 3 – 101, day 5 – 100	All patients who had 4 fertilized oocytes	Sequential media	2.3	2.2	39	39	21.3	23.8	33	38
Karaki <i>et al.</i> (2002); RT3; day 3 – 82, day 5 – 80	Patients with at least five 2PN embryos available	Sequential media	3.5	2.0	26	29	12.7 <sup>c</sup>	26.1	48	39
Levron <i>et al.</i> (2002); RT4; day 3 – 44, day 5 – 46	Patients with >5 zygotes on day 1	Sequential media	3.1	2.3	45.5 <sup>c</sup>	18.6	38.7 <sup>c</sup>	20.2	40	50
Utsunomiya <i>et al.</i> (2002); RT5; day 3 – 184, day 5 – 180	All patients	Sequential media	2.9	3.0	26.3	24.8	11.7	9.2	–	–
Rienzi <i>et al.</i> (2002) <sup>b</sup> ; RT2; day 3 – 48, day 5 – 50	<38 years, with at least eight 2PN zygotes on the day following ICSI	Sequential media	2.0	2.0	56	58	35	38	–	–

2PN = two-pronucleated.

<sup>a</sup>RT = randomization type code – RT1 = by weekday of retrieval; RT2 = by computer-generated list; RT3 = at fertilization by sealed envelopes; RT4 = on day 1, method not stated; RT5 = depending on the sequence of ovum retrieval.

<sup>b</sup>Cumulative delivery rates including both fresh and frozen cycles per oocyte retrieval – day 3 – 77% day5 – 52%,  $P < 0.01$ .

<sup>c</sup>Significantly different from corresponding day 5 rate ( $P < 0.05$ ).

Auwers *et al.*, 2002). The method of randomization is not stated in the study by Plachot *et al.* (2000). Moreover, a selected patient population was analysed (patients who had more embryos than the number required for transfer on day 2), while day 2 patients tended to have more embryos transferred than day 5 patients. Cryopreservation was not always performed on the day of transfer in the day 2 group. In the study by Van Der Auwers *et al.* (2002), an adequate randomization procedure was followed (sealed envelopes), patients were randomized before initiation of treatment and similar numbers of embryos were transferred between the two groups of patients. Follow-up included frozen–thawed cycles performed until 1 year after the completion of the study, but supernumerary embryos on day 2 were cultured until day 5 before cryopreservation. Implantation rates were similar between day 2 and day 5 in the study by Plachot *et al.* (18.9 versus 24.1%) in contrast to that by Van Der Auwers *et al.* (29 versus 46% respectively). Delivery rates per oocyte retrieval did not differ significantly in the two studies between day 2 and day 5 transfers (Plachot *et al.*: 41.7 versus 38.0% respectively; Van Der Auwers *et al.*: 27 versus 36% respectively). The cancellation rate was significantly higher in the blastocyst group than in the day 2 group in the study by Van Der Auwers *et al.* (27 versus 10% respectively).

## Points of attention for blastocyst transfers

Whilst waiting for properly designed randomized trials to justify or not the move from day 3 to day 5 culture, several aspects of a blastocyst culture programme require attention.

## Blastocyst transfer and monozygotic twinning

Peramo *et al.* (1999) described the first two cases of monozygotic twin pregnancies following blastocyst transfer, while 4.3% monozygotic twinning (MZT) has been reported by Racowsky *et al.* (2000). Moreover, da Costa *et al.* (2001) observed a higher incidence of MZT in blastocyst transfers (3.9%) than in 4–8 cell embryo transfers (0.7%) in ICSI pregnancies.

Tarlatzis *et al.* (2002) reported a higher incidence of MZT after ICSI than after IVF (5.9 versus 0% respectively). However, an increased incidence of MZT (5%) has also been reported after IVF and blastocyst transfer (Behr *et al.*, 2000).

The increased incidence of MZT associated with blastocyst transfer might be related to changes in the zona pellucida or in the hatching process caused by the artificial opening induced by ICSI.

It should be noted that an increased incidence of monozygotic twinning has also been reported without the use of extended culture (Sills *et al.*, 2000; Schachter *et al.*, 2001). In order to draw reliable conclusions on the potential effect of extended culture on the incidence of MZT, an accurate reporting of MZT after either short or blastocyst culture is therefore essential.

## Sex ratio after blastocyst transfer

Sex ratios favouring males have been reported after embryo transfer at the blastocyst stage (Quintas *et al.*, 1998; Menezo *et al.*, 1999). This might be associated with the faster rate at which male embryos cleave in culture and therefore with their increased chance of being selected for transfer (Menezo *et al.*, 1999). However, the association between blastocyst transfer and skewed male–female birth ratio was not confirmed in further studies (Kausche *et al.*, 2001; Wilson *et al.*, 2002).

## How successful is a blastocyst cryopreservation programme?

Behr *et al.* (2002) suggested that blastocyst cryopreservation results in a 16% implantation rate for embryos frozen either on day 5 or day 6 of culture. Moreover, implantation rates of 21.9% (Yokota *et al.*, 2001) and 23.7% (Choi *et al.*, 2000) have been reported following vitrification and subsequent thawing of human blastocysts.

A higher implantation rate was also reported by Langley *et al.* (2001) in a retrospective analysis comparing day 5 with day 3 transfers (21.9 versus 10.1% respectively). More frozen–thawed embryos were transferred in the day 3 group (3.1 versus 2.3).

It has also been shown that it is possible to refreeze supernumerary blastocysts produced by thawing of day 3 embryos and culture to day 5 for subsequent transfer (Farhat *et al.*, 2001). However, additional data relating to fetal outcome after such a procedure are needed before it is recommended routinely.

The importance of including cryopreservation in the evaluation of a blastocyst transfer versus day 3 transfer programmes lies in the fact that fewer blastocysts are available for cryopreservation than day 3 embryos. To compensate for this discrepancy, frozen–thawed blastocyst cycles should result in a better implantation rate than day 3 frozen–thawed cycles. How much better this rate should be depends on the blastocyst formation rate.

## What is the rate of blastocyst formation?

Blastocyst formation rates using sequential media appear to vary considerably (Table 2). The same holds true for cycle cancellation rates, which also vary according not only to the blastocyst formation rate but also, mainly, on the population studied, ranging from 4.5% (Gardner *et al.*, 1998: high responders) to 11% (Karaki *et al.*, 2002: patients with at least 5 2PN embryos available).

Differences in the media used for extended culture, which can be sequential or involve a one-step system (Biggers and Racowsky, 2002), might affect blastocyst development but other factors have also been considered as a source of variation.

## Blastocyst culture and ICSI/IVF

Gardner *et al.* (1998) reported no difference in blastocyst formation between intracytoplasmic sperm injection (ICSI)

**Table 2.** Blastocyst formation rate using sequential media.

Authors	Blastocyst formation rate (%)
Gardner <i>et al.</i> (1998)	46
Coskun <i>et al.</i> (2000)	28
Karaki <i>et al.</i> (2002)	33
Racowsky <i>et al.</i> (2000)	21–38
Balaban <i>et al.</i> (2001a)	21–47
Schoolcraft and Gardner (2000)	58
Vlaisavljevic <i>et al.</i> (2001)	55
Behr <i>et al.</i> (1999)	53
Cruz <i>et al.</i> (1999)	51
Utsunomiya <i>et al.</i> (2002)	21.6
Rienzi <i>et al.</i> (2002)	44.8

and IVF (45 versus 47% respectively). Similarly, no significant effect for the artificial reproductive technology method used was observed by Karaki *et al.* (2002).

On the other hand, ICSI was associated with a lower blastocyst development rate than IVF (44.2 versus 51.0% respectively) in the study by Schoolcraft *et al.* (1999). Similarly, Miller and Smith (2001) reported that ICSI for severe male-factor is associated with a lower blastocyst formation rate compared than is IVF.

Although controversial, an ICSI-associated adverse effect on blastocyst formation rate is probably due to the severity of the male factor for which ICSI is performed. Balaban *et al.* (2001b) compared the blastocyst formation rate after ICSI in relation to the origin of spermatozoa used (ejaculated, epididymal and testicular from obstructive and non-obstructive azoospermic patients). These authors showed that increasing severity of spermatogenetic defect is associated with a lower rate of blastocyst formation. A higher rate of blastocyst formation has also been reported (Coskun *et al.*, 2000) in tubal as compared with male-factor infertility patients (41 versus 26% respectively).

## Age and blastocyst development

The effect of age on blastocyst transfer is also controversial. Gardner *et al.* (1998) did not observe an adverse effect of maternal age on blastocyst development, whereas Pantos *et al.* (1999) reported a lower blastocyst formation rate in women >40 years of age (22.2%) as compared with women <40 years of age (40.5%).

In summary, the number of day 3 embryos progressing to the blastocyst stage during extended culture may be affected by several factors. It appears, however, that blastocysts produced may have different implantation potential.

## Do differences among blastocysts provide additional indications of their viability?

Gardner *et al.* (2000) suggested that the quality of individual human blastocysts can be quantified by using a three-part scoring system. Transfer of at least one grade 1 or grade 2 blastocyst or one hatching blastocyst was associated with very high implantation and pregnancy rates, as opposed to grade 3 blastocysts (Balaban *et al.*, 2000).

It has been proposed that quantitative measurements of the inner cell mass (ICM) are highly indicative of blastocyst implantation potential. Blastocysts with relatively large and/or slightly oval ICMs are more likely to implant (Richter *et al.*, 2001).

It also appears that the transfer of blastocysts is more successful than that of non-cavitating embryos (Scholtes and Zeilmaker, 1996). Shapiro *et al.* (2001) reported that embryos that develop to the expanded blastocyst stage and are transferred on day 5 after retrieval are approximately twice as likely to implant compared with those for which expansion and transfer are delayed until day 6.

Blastocyst quality therefore seems to be related to the implantation and pregnancy rates achieved. Contrary to animal studies, blastocyst viability following transfer does not seem to be associated with glucose metabolism in humans (Jones *et al.*, 2001).

## Conclusions

Blastocyst culture and transfer is feasible with the use of sequential media. Considering the additional requirements with which blastocyst transfer is associated, it must be proved to be superior to day 3 transfer in order to replace it in artificial reproductive technologies.

Randomized controlled trials published to date do not show convincingly that blastocyst results in a better outcome than does day 3 transfer. There is still a need for properly designed randomized controlled trials to compare day 3 and day 5 transfers, which will also address the effectiveness of a single blastocyst transfer in reducing the incidence of multiple pregnancies and will assess the value of blastocyst cryopreservation.

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