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The rate of development and time of transfer play different roles in influencing the viability of human blastocysts

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Improved embryo culture protocols now render more feasible the possibility of obtaining human blastocysts after in-vitro fertilization. In this study we present: (i) results of blastocyst development from supernumerary embryos after co-culture on green monkey kidney epithelial cells and (ii) pregnancy rates after transfer of frozen blastocysts. In addition, we have examined the influence of the day of blastocyst freezing and the day of transfer after the luteinizing hormone (LH) peak on pregnancy and implantation rates. Of 423 supernumerary embryos, 200 developed to the blastocyst stage (47.3%). By days 5 and 6, 67% of the blastocysts had reached the blastocyst stage, and were frozen, compared to 28.5% by day 7. When we compared the cases where only blastocysts frozen on days 5 and 6 were transferred compared to those frozen and transferred on or after day 7 the pregnancy rates were 7/18 (38.9%) and 1/16 (6.2%) respectively. In contrast, when we examined the influence of the day of transfer we found that pregnancies were established from day 5 up to day 9 post LH peak. Based on these results, we suggest that every attempt should be made to increase the development rate of supernumerary embryos to the blastocyst stage, as it appears that the quality of blastocysts transferred, as shown in this study by rate of development, plays a more crucial role than the timing of transfer.

Key words: blastocysts/human/in-vitro fertilization/transfer/viability

Introduction

The embryo enters the uterus during the later stages of preimplantation embryo development. It has therefore been argued for some time that it would be more appropriate to transfer in-vitro fertilized human embryos to the uterine cavity at the blastocyst stage (Olivennes *et al.*, 1994; Kaufmann *et al.*, 1995). This would provide a better synchrony between the uterine endometrium and the embryo. The other advantages of such a procedure include the possible selection of embryos with a higher implantation potential (Nakayama *et al.*, 1995), supporting the concept that some embryos with chromosomal or genetic abnormalities may cleave, but fail to reach the blastocyst stage (Edwards and Hollands, 1988). Moreover, the transfer of blastocysts may reduce the risk of multiple pregnancy if only one or two better-quality embryos can be selected. The use of blastocysts, however, has not been widely accepted for two reasons: (i) pregnancies can be established when embryos are transferred to the uterus at the 4- to 6-cell stage, even though they remain in the uterus for ~90–99 h prior to implantation, and (ii) human in-vitro fertilization (IVF) embryos can be cultured to the blastocyst stage with only limited success.

Recently, however, improved embryo culture protocols have rendered the possibility of obtaining blastocysts after in-vitro fertilization more feasible. Numerous culture systems, with (Weimer *et al.*, 1989a, b; Ménézo *et al.*, 1990; Bongso *et al.*, 1991; Birkenfeld and Navot, 1991; Plachot *et al.*, 1993; Quinn and Margalit, 1996) and without feeder cells (Hardy *et al.*, 1989; Dokras *et al.*, 1993; Muggleton-Harris *et al.*, 1995; Scholtes and Zeilmaker, 1996; Desai *et al.*, 1997), now exist which can support development of human embryos to the blastocyst stage. In our own studies, we have used a co-culture system with green monkey kidney epithelial (Vero) cells (Sakkas *et al.*, 1994). Recently, a number of studies have reported the successful culture of human embryos to the blastocyst stage and improved pregnancy rates after the transfer of fresh (Olivennes *et al.*, 1994; Scholtes and Zeilmaker, 1996) and frozen blastocysts (Kaufmann *et al.*, 1995). The question however remains as to whether the transfer of blastocysts conveys an advantage to the infertile couple. In addition, little is known as to the effect of the time of transfer and the quality of blastocysts transferred in the human. One study supporting the use of blastocysts was that of Buster *et al.* (1985) who recovered in-vivo developed human blastocysts by uterine lavage and transferred them to achieve a high implantation rate (3/5, 60%), well above that currently observed in most IVF cycles.

In this study, we present our results of blastocyst development from supernumerary embryos. In the first part of the study, we examined the influence of the day of blastocyst freezing and day of transfer after the luteinizing hormone (LH) peak on pregnancy and implantation rates. In the second part of the study, we investigated whether development to the blastocyst stage could be used as an indicator of the overall viability of a patient's embryos in the initial treatment cycle.

Materials and methods

The present study was performed on patients entering the IVF programme at the Clinic of Infertility and Gynaecologic Endo-

crinology, Department of Obstetrics and Gynaecology, University Hospital of Geneva, Geneva, Switzerland between April 1992 and March 1997. In all, 90 cycles were assessed where the patient underwent a routine IVF treatment with transfer and development of supernumerary embryos. All patients without supernumerary embryos were excluded. The stimulation protocol adopted by our group has been previously described (Sakkas *et al.*, 1994).

Collected oocytes (day 0) were fertilized using our standard culture medium, Whittingham's T6 (Quinn *et al.*, 1982) supplemented with 10% maternal serum. Serum was prepared from blood taken on the first day of stimulation. Oocyte retrieval took place between 0830 and 1030. Insemination was performed between 1530 and 1630 (~1600) in tubes. The following morning (day 1) the oocytes were removed from the tubes, washed and placed in 20 µl culture drops under oil (Light white mineral oil; Sigma Pharmaceuticals, Buchs, Switzerland) in Petri dishes and the presence of pronuclei assessed. Embryo transfer was routinely performed on day 2; however, when a patient had had three or more failures to achieve pregnancy after transfer, the embryos were placed in co-culture from the 2 PN stage and the transfer was performed on day 3. Routinely, a maximum of three embryos was transferred to the patient and the transfers were normally performed between 1000 and 1400.

On the day of transfer, all embryos were assessed for the number of cells per embryo, to ascertain their cleavage rate, and given a quality score based on the presence of fragments and clarity of the cytoplasm of the blastomeres, similar to that previously described by Cummins *et al.* (1986). The ratings given to embryos for cell number were 1 for 1-cell, 2 for 2-cells, 3 for 3-cells, 4 for 4-cells, 6 for between 4- and 8-cells and 8 for >8-cells. The ratings given to embryos for quality were the same as those used by Cummins *et al.* (1986) except that the values of 0 for poorest and 3 for best embryo were given. Peripheral levels of human chorionic gonadotrophin (HCG) were measured 14 days after transfer (total HCG: IMX, Abbot, Abbot Park, USA). Patients with three consecutive HCG values >5 mIU/ml and in which the fetus or fetuses displayed a heartbeat by ultrasound examination 4–5 weeks after transfer were considered to have achieved a clinical pregnancy.

Supernumerary embryos were co-cultured on Vero cells until the blastocyst stage using T6 medium supplemented with vitamins and amino acids as previously described (Sakkas *et al.*, 1994). The blastocysts were then frozen using the protocol described by Ménézo *et al.* (1992) except that T6 medium was used in place of Ménézo's B2. At the time of transfer the blastocysts were thawed rapidly at room temperature. The cryoprotectant was removed using seven steps, with decreasing concentrations of glycerol and each step lasting 5 min. After thawing, the blastocysts were allowed to recover for 3–4 h in co-culture before transfer. In normally ovulating patients, the transfer was performed during a natural cycle monitored by ultrasound echography and hormone assay. The transfer was timed according to the LH peak. In patients with anovulation, ovarian stimulation was performed using human menopausal gonadotrophin and ovulation was triggered by HCG. Plasma HCG was measured 14 days after LH peak to determine whether pregnancy had occurred.

The statistical evaluations used were analysis of variance followed by Scheffé's *F*-test for comparisons of mean values and Fisher's exact test for comparison of pregnancy rates.

Results

Blastocyst development and viability after freezing

Figure 1 presents the number of early and expanded blastocysts frozen in relation to the number of days in culture prior to freezing. In most cases, blastocysts were frozen at the earliest opportunity once they reached the expanded blastocyst stage. In some cases early blastocysts were frozen for laboratory convenience. In total, of 423 supernumerary embryos, 200 developed to the blastocyst stage (47.3%). A total of 28 embryos were not frozen as they vacuolated after forming a blastocoelic cavity and were not considered suitable for freezing. Of 172 frozen blastocysts, 57.0% had reached the blastocyst stage on day 6 and were frozen on that day compared to 30.2% on day 7. Of the spare embryos initially cultured in T6 + 10% serum until day 2, 85/173 (49.1%) formed blastocysts, while 115/250 (46.0%) of the spare embryos that were co-cultured from the 2 PN stage formed blastocysts. Therefore the medium used to culture embryos initially did not have a significant bearing upon blastocyst development of the spare embryos.

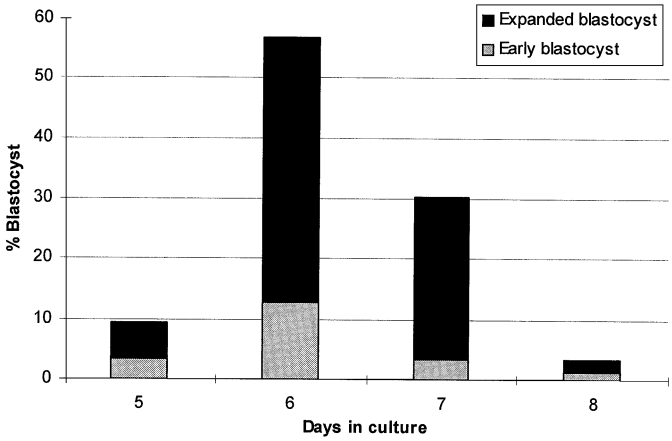

Figure 1. The percentage of expanded and early blastocysts frozen in relation to the number of days in culture.

Table I. Results of the transfer of frozen blastocysts

Number of cycles with frozen blastocysts	64[a]
Total number of blastocysts (mean ± SD)	200 (2.9 ± 2.2)
Total number of frozen blastocysts (mean ± SD)	172 (2.5 ± 2.1)
Number of transfer cycles	44[a]
Number of transferred blastocysts (mean ±SD)	84[b] (1.9 ± 0.9)
Mean number of days after the LH peak (±SD)	5.9 ± 1.0
Mean number of days in the cycle (±SD)	19.2 ± 3.4
Clinical pregnancy rate (%)	8 (18.2)
Implantation rate (%)	10
(fetal hearts/number of blastocysts transferred)	(11.9)

[a]Of the 64 cycles with frozen blastocysts, 20 have yet to have embryos thawed for transfer.
[b]All blastocysts survived thawing and were transferred.
LH = luteinizing hormone.

The results of the transfers of thawed blastocysts are shown in Table I. In 44 cycles, 84 frozen–thawed blastocysts were transferred, resulting in a pregnancy rate and implantation rate of 18.2% and 11.9% respectively. When one, two, three or four blastocysts were transferred, the incidence of clinical pregnancy per transfer was 2/17 (11.8%), 3/16 (18.8), 2/9 (22.2%) and 1/2 (50.0%) respectively. Our current policy is to limit the number of the transferred thawed blastocysts to two.

Table II. The influence of the day of freezing and the day of transfer (after LH peak) on pregnancy rate

Day of transfer from LH peak	Day of freezing		Total (%)
	Day 5 and 6 blastocysts	Day 7 and 8 blastocysts	
4	0/2	0/3	0/5 (0)
5	1/4	1/4	2/8 (25)
6	4/10	0/7	4/17 (23.5)
7	1/1	0/2	1/3 (33.3)
9	1/1	-	1/1 (100)
Total (%)	7/18 (38.9)	1/16 (6.2)[a]	8/34 (23.5)

[a]$P = 0.04$ comparing day 7–8 blastocysts to day 5–6 blastocysts.
LH = luteinizing hormone.

The influence of the day of blastocyst freezing and day of transfer on the pregnancy rate

When the clinical pregnancy rate was assessed in relation to the day of transfer after the LH peak, we found that the pregnancy rate when thawed blastocysts were transferred on day 4, 5, 6, 7 and 9 post LH peak, irrespective of the day on which the cryopreservation was performed, was 0/6, 2/11, 4/22, 1/3 and 1/1 respectively. These results suggest that the transfer of thawed blastocysts early in the cycle, e.g. day 4 post LH peak, gives the least successful results, while implantation can occur from day 5 up to day 9 post LH peak.

Table II presents the influence of the day of freezing and the day of transfer on pregnancy rate. At the time these data were collected, it was not known that the timing of blastocyst development was crucial and transfers were being done on different days according to weekends. Therefore the transfer dates varied. However, Table II includes only those cases where transfer of blastocysts frozen on day 5–6 or day 7–8 was performed but excludes cases where, for example, day 6 and 7 or day 5 and 8 blastocysts were transferred. Although the number of the transferred blastocysts in the different groups is low, it is apparent that the majority of pregnancies take place when day 5–6 frozen blastocysts are transferred.

Development of supernumerary embryos to the blastocyst stage

In the second part of the study, we investigated whether development to the blastocyst stage could be used as an indicator of the overall viability of a patient's embryos in the initial treatment cycle. We therefore compared the pregnancy rates in patients who had and did not have blastocysts developed from their supernumerary embryos (Table III). In cycles in which the embryos were transferred on day 2 or day 3 there were 85 blastocysts out of 173 spare embryos (49.1%) and 115 blastocysts out of 250 spare embryos (46.0%) respectively. There was no significant difference in the age of the couples, number of oocytes, number of 2 PN embryos, embryos transferred and spare embryos between the patients with and without blastocysts. In addition, when examining if there was an influence of the husband's semen parameters we found that there was no significant difference in sperm concentration, motility and morphology between the groups (data not shown). The pregnancy and implantation rates are shown in Table III. The clinical pregnancy rate in the fresh cycle was not affected by the day of transfer. When comparing the clinical pregnancy and implantation rates between groups of patients who did and did not have blastocysts, there was no significant difference.

To evaluate whether the cohort of embryos on the day of the transfer can affect the development of the supernumerary embryos to the blastocyst stage, we calculated the mean cell number and quality for all embryos, transferred embryos and supernumerary embryos on the day of the transfer (Table IV). When comparing the supernumerary embryos of patients who did and did not have blastocysts, we found that on day 2 there was no significant difference in the mean cell number/embryo; however the mean quality/embryo was significantly different. On day 3 the differences between patients with and without blastocysts were significantly different for both cell number and quality. The appearance of a significant difference when embryos were cultured until day 3 is indicative that the longer embryos remain in culture the more pronounced is the effect of the culture on embryo development and blastocyst formation.

Discussion

The cryopreservation of human embryos fertilized *in vitro* has become an established clinical procedure since the initial pregnancy was established in the early 1980s (Trounson and Mohr, 1983). Subsequently, pregnancies have been achieved by the transfer of thawed embryos at various developmental stages (Trounson and Mohr, 1983; Lassale *et al.*, 1985; Testart *et al.*, 1986; Cohen *et al.*, 1988). Cryopreservation allows the storage of supernumerary embryos such that the cumulative pregnancy rate increases for every cycle of successful ovarian recovery and transfer.

Blastocyst development in vitro

A number of improvements in culture techniques have taken place over the last decade that allow acceptable rates of development of human embryos to the blastocyst stage. Although the question of which is the best culture method remains unanswered, it is clear that several culture techniques are able to provide a good percentage of blastocysts after culture. In this study and our prior reports (Sakkas *et al.*, 1994) we have used Vero cells in a co-culture system. In the human, various types of co-culture systems have been used and it seems that the cell type used is not species-specific. Weimer *et al.* (1989a, b) used fetal bovine fibroblast, Ménézo *et al.* (1990) Vero cells, Bongso *et al.* (1991) human ampullary cells, Birkenfeld and Navot (1991) homologous endometrial cells, Plachot *et al.* (1993) granulosa cells and Quinn and Margalit (1996) proposed the use of cumulus cells. When comparing the percentage of blastocysts obtained with culture media alone and co-culture in the same study, Bongso *et al.* (1989) found the proportion was 33% with T6 medium versus 69% with ampullary cells. Ménézo *et al.* (1990) reported 3% with B_2 medium versus 61% with Vero cells and Plachot *et al.* (1991) had 9% with B_2 versus 40% when using granulosa cells. A number of studies have also reported excellent blastocyst development rates using complex medium only without the

Table III. Development of spare embryos and pregnancy rates in patients receiving embryo transfer on days 2 or 3

	Day 2 transfer with no blastocyst	Day 2 transfer with blastocyst	Day 3 transfer with no blastocyst	Day 3 transfer with blastocyst
No. of cycles	10	35	10	35
No. of patients	10	33	10	29
Age of female	35.4 ± 4.0	33.2 ± 3.1	34.2 ± 4.2	32.8 ± 4.3
Age of male	38.0 ± 10.4	38.0 ± 7.8	37.6 ± 7.6	36.6 ± 6.1
No. of oocytes	85	426	138	492
(mean ± SD)	(8.5 ± 2.84)	(12.2 ± 6.3)	(13.8 ± 8.2)	(14.1 ± 7.3)
2 PN	59	288	67	327
(mean ± SD)	(5.9 ± 1.4)	(8.2 ± 3.6)	(6.7 ± 2.5)	(9.3 ± 3.7)
Embryos on day of transfer	51	255	66	317
(mean ± SD)	(5.1 ± 1.52)	(7.3 ± 2.8)	(6.6 ± 2.6)	(9.1 ± 3.7)
No. of embryos transferred	30	103	30	103
(mean + SD)	(3.0 ± 0.0)	(2.9 ± 0.2)	(3.0 ± 0.0)	(2.96 ± 0.2)
No. of spare embryos	21	152	36	214
(mean ± SD)	(2.1 ± 1.5)	(4.3 ± 2.7)	(3.6 ± 2.6)	(6.1 ± 3.7)
No. of blastocysts	0	85	0	115
(mean ± SD)		(2.44 ± 1.6)		(3.3 ± 2.6)
Number of clinical pregnancies	2	8	1	10
(%)	(20.0)	(22.8)	(10.0)	(28.6)
Implantation rate	2/30	10/103	1/30	14/103
(%)	(6.7)	(9.7)	(3.3)	(13.6)

2PN = 2 pronuclei.

Table IV. Mean cell number and quality of all embryos on the day of the transfer, the transferred embryos and the supernumerary embryos. The number of embryos for each group is in parentheses

	Day 2 transfer with no blastocyst	Day 2 transfer with blastocyst	*P* value	Day 3 transfer with no blastocyst	Day 3 transfer with blastocyst	*P* value
Mean cell number (±SD) of:						
all embryos on the day of transfer	2.9 ± 1.0 (51)	3.4 ± 1.2 (239)	0.09	4.7 ± 1.9 (66)	5.4 ± 1.5 (317)	0.005
embryos transferred	3.1 ± 1.1 (30)	3.8 ± 1.2 (103)	0.01	5.5 ± 1.0 (30)	6.0 ± 0.8 (103)	0.09
supernumerary embryos	2.7 ± 0.8 (21)	3.2 ± 1.2 (136)	0.48	4.1 ± 2.3 (36)	5.1 ± 1.6 (214)	0.003
Mean quality (±SD) of:						
all embryos on the day of transfer	2.3 ± 0.8 (51)	2.6 ± 0.6 (239)	0.01	2.1 ± 0.7 (66)	2.4 ± 0.7 (317)	0.02
embryos transferred	2.6 ± 0.6 (30)	2.8 ± 0.5 (103)	0.5	2.5 ± 0.5 (30)	2.8 ± 0.4 (103)	0.03
supernumerary embryos	1.8 ± 0.8 (21)	2.5 ± 0.6 (136)	0.0001	1.7 ± 0.7 (36)	2.1 ± 0.7 (214)	0.0001

use of co-culture. For example, Desai *et al.* (1997) reported that 45.1% of spare embryos developed to the blastocyst stage in alpha minimal essential medium supplemented with a synthetic serum substitute. In addition, more promising results of over 50% blastocyst development and implantation rates of >40% have been reported using medium G1 and G2 supplemented with human serum albumin (D.K. Gardner, personal communication).

Factors influencing blastocyst development

Development to the blastocyst stage is influenced by a number of factors. Although Janny and Ménézo (1996) reported a reduction in both blastocyst formation and expansion rates with increasing age, contributing to the age-related decline in female infertility, we did not find a difference in maternal age between those patients with and without blastocyst development. The same applies to a paternal effect on blastocyst development. While we found no difference in sperm concentration, motility and morphology between both groups, Janny and Ménézo (1994) found that when the usual parameters of sperm quality are good, there is a strict linear relationship between cleavage and blastocyst formation rates. For spermatozoa with both poor motility and poor morphology, the cleavage rate did not correlate with further embryonic development. Most importantly, we noted that the strongest indicator that a patient would achieve blastocyst formation was the difference in the mean cell number and quality of supernumerary embryos on day 3, pointing to a selection process during the culture of embryos. The fact that we found no such difference in the mean cell number of supernumerary embryos at day 2 transfer indicates that the longer the embryos remain in culture, the greater the selection pressure. There was however a significant difference in the mean quality of supernumerary embryos on day 2 in patients with and without blastocyst

formation, supporting the report by Dokras *et al.* (1993) who found that the probability of an embryo developing to the blastocyst stage varied with the grade of that embryo on day 2.

It has also been previously suggested that only the 'hardier' embryos would reach the blastocyst stage *in vitro*, and therefore would have an increased implantation potential (Edwards and Hollands, 1988). In this study, blastocysts were not transferred in fresh cycles; however, we wanted to investigate whether a difference in pregnancy rate between patients with and without blastocyst formation existed. We hypothesized that the ability of a patient to achieve blastocyst formation could be an indicator of an overall higher viability of the cohort of embryos from a single patient. However, there was no significant association between whether a patient became pregnant after transfer of sibling embryos and whether a fully expanded blastocyst developed from her supernumerary embryos left in culture. A similar observation was reported by Quinn and Margalit (1996).

The ability to evaluate the viability of blastocysts accurately is important for increasing the pregnancy rate with blastocyst transfers. Hartshorne *et al.* (1991) reported that no morphological markers of frozen–thawed human blastocysts were identified which were prognostic for their survival or implantation. According to their report, assessing morphological criteria after thawing may not be sufficient to determine the viability of blastocysts. However, Cohen *et al.* (1985) and Fehilly *et al.* (1985) suggested that only blastocysts with a distinct inner cell mass should be selected for cryopreservation and only those where the blastocoelic cavity re-expanded after thawing should be transferred. This morphological selection of blastocysts might contribute to the high implantation rate observed in their studies. Weimer *et al.* (1995), however, proposed that when evaluating blastocysts from sub-optimal embryos, more emphasis should be placed on criteria other than the visualization of the inner cell mass, such as the length of time in culture.

Rates of blastocyst development and pregnancy

Although we observed no association between the ability of patients' supernumerary embryos to become blastocysts and pregnancy rates, we did see an association when examining the rate of development of blastocysts in culture. The rate of blastocyst development is perhaps more important than the percentage of embryos that attain this stage. We found that seven out of 18 pregnancies, following the transfer of frozen–thawed blastocysts, were obtained when blastocysts frozen on day 5 and 6 were transferred, compared to only one out of 16 pregnancies for blastocysts frozen on day 7 and 8. Lelaidier *et al.* (1995) found that when pregnancy rates were compared with the duration of embryo development in co-culture required to reach the blastocyst stage, an inverse correlation was found, indicating that the faster the embryos reached the blastocyst stage, the higher were their chances of implantation. Their transfers were performed on day 19 (on the 5th day of progesterone supplementation) whereby the pregnancy rate per transfer of blastocysts frozen on day 5, 6 and 7 were 25%, 14% and 0% compared to our results of pregnancy rate of 50%, 44% and 7% respectively. The observation that pregnancy rates were lower when embryos had not reached the blastocyst stage by day 6 indicates that these embryos have a reduced viability.

The timing of blastocyst transfer

The question arises as to whether an implantation window exists when transferring human blastocysts. When the pregnancy rate per transfer was assessed in relation to the day of transfer of fresh blastocysts, Olivennes *et al.* (1994) found a pregnancy rate of 41.0% and 36.1% for day 5 and day 6 post-retrieval respectively, while no pregnancies were obtained when blastocysts were transferred on day 7. When we sought a relationship between the pregnancy rate and synchrony between the day of freezing of the transferred blastocysts and number of days post LH peak endometrium, we found no difference, but this may have been influenced by the small number of cases. Our results do, however, show that the implantation window is wide, as pregnancies were established after transfer of frozen–thawed blastocysts from day 5 to day 9 after the LH peak. This finding is further supported by a pregnancy reported when the embryo transfer was carried out 10 days after the onset of the luteal phase (Edwards, 1988). Interestingly, in the limited number of cases when the transfer was carried out early in the cycle (day 4 from the LH peak) no pregnancies were obtained. Data from a number of studies (Hartshorne *et al.*, 1991; Olivennes *et al.*, 1994; Lelaidier *et al.*, 1995) suggest that a synchrony may exist between blastocyst formation and endometrial receptivity. The results presented in this study, however, indicate that the day of transfer is probably less important than the rate of blastocyst formation.

This further supports the theory that culture to the blastocyst stage allows, firstly, selection against poorer embryos, i.e. those that cannot reach the blastocyst stage. Secondly, when a time limit is put on development to the blastocyst stage, a further selection of high quality embryos is made. Hence if an expanded blastocyst is obtained by day 7 it indicates that the embryo may have an irregular pattern of growth but one that is not as poor as embryos that are not able to form blastocysts. Hardy *et al.* (1989) have previously reported that day 6 or 7 blastocysts may display abnormalities, particularly in their cell number.

Conclusion

The transfer of thawed blastocysts is now a well established procedure in assisted reproductive technologies. Every attempt should be made to increase the development rate of the supernumerary embryos to the blastocyst stage, and to improve the results of the transfer of blastocysts in fresh cycles and after freezing. Here, we have shown that the viability of blastocysts is compromised when their development is retarded. It appears that the rate of blastocyst development plays a more crucial role than that of the timing of transfer. Consequently, from our results, a good quality blastocyst is not only the one that has a well expanded blastocoelic cavity and well defined inner cell mass, but in addition, it should have attained this stage by day 5 or 6. Further investigation of the faster and slower developing human blastocysts by assessing either cell numbers or metabolic parameters is warranted.

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