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Alternative Embryo Transfer on Day 3 or Day 5 for Reducing the Risk of Multiple Gestations[1]

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Purpose: *This study was carried out to reduce the possibility of high-order multiple gestations and the failure of embryo transfer by determining their replacement date based on the number and quality of 2-day embryos.*

Methods: *All zygotes were cocultured with cumulus cells in 10 μl of YS medium containing 10% human follicular fluid (hFF) for 48 or 96 hr. In period I, all embryos were transferred on day 3 (1032 cycles). In period II, the embryos were transferred on either day 3 or day 5 by determining their replacement date based on the number and quality of 2-day embryos: there were 2701 patients in whom embryos were replaced on day 3 (in the case that the number of zygotes was less than eight and the number of good-quality embryos was less than three) and 1952 patients less than 40 years old in whom embryos were replaced on day 5 (in the case that the number of zygotes was eight or more and/or the number of good-quality embryos was three or more). On the other hand, patients who were 40 years old or more were alloted to day 3 transfer cycles, regardless of the number and quality of the 2-day embryos, due to the possibility of their not producing blastocyst-stage embryos in vitro.*

Results: *The number of embryos transferred in period II was 2.9 ± 0.6, while that in period I was 3.7 ± 0.5. The multiple pregnancy rate was significantly decreased in period II (30.7%) compared to that (49.6%) in period I, while the pregnancy and implantation rates in period II (36.1 and 16.4%, respectively) were not lower than those (34.9 and 16.1%, respectively) in period I. The rate of triplet or more gestations was significantly minimized in period II (2.3%) compared to that in period I (26.5%).*

Conclusions: *We propose that determination of the date on which embryos should be transferred based on the number and quality of embryos on day 2 may help to maintain an acceptable pregnancy rate, while minimizing embryo transfer failure and high-order multiple gestations.*

KEY WORDS: coculture; 2-day embryo quality; blastocyst transfer; multiple gestations.

INTRODUCTION

The transfer of early cleavage-stage embryos on day 2 or day 3 is a common practice in assisted reproductive technology programs. This procedure results in implantation rates of 20% or less, so that many clinics transfer two embryos or more to obtain acceptable pregnancy rates (1). The pregnancy rate increases with the number of embryos transferred, but so does the incidence of multiple gestations. Recently, higher pregnancy and implantation rates have been reported with the transfer of blastocyst-stage embryos than with the transfer of cleavage-stage embryos (2,3). These high implantation rates could allow transferring fewer embryos, thus avoiding the risk of multiple pregnancies.

However, the main problem of blastocyst-stage embryo transfer in human IVF-ET programs is the possibility of embryo transfer failure: that is, there were some patients who failed to produce blastocyst-stage embryos and had no embryos to replace. Therefore, it is questionable whether the transfer of

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blastocyst-stage embryos is beneficial in all infertile patients. If the transfer of blastocyst-stage embryos is performed in patients with a poor prognosis, it might significantly reduce the chance of pregnancy. Jones *et al.* (4) reported that the number of blastocyst-stage embryos developing from the zygote stage was positively influenced by the number of oocytes or zygotes and the number of embryos developing to the eight-cell stage or more. Also, good results from 5-day transfer cycles were achieved when the patients had eight or more embryos on day 2 after fertilization (5). Milki *et al.* (6) suggested that the transfer of blastocyst-stage embryos should be performed only in patients in whom the number of eight-cell stage embryos on day 3 was three or more.

Beginning in January 1997, to reduce the possibility of high-order multiple gestations and the failure of embryo transfer, embryo culture extended to day 5 was performed for patients at high risk for multiple gestations. Transfers of blastocyst-stage embryos were selectively performed only in patients who had more than seven zygotes or two or more good-quality 2-day embryos. An early decision on the date of embryo transfer, on day 2, may help to offer patients plenty of time to travel for the replacement of their embryos.

This study was carried out to compare alternative embryo transfer in period II with routine cleavage-stage embryo transfer in period I in terms of the implantation, pregnancy, and multiple gestation rates, resulting from the determination of their replacement date based on the number and quality of 2-day embryos.

MATERIALS AND METHODS

This study was carried out in 5732 cycles that produced more than one cleavage-stage embryo from May 1996 to December 1998. The data were analyzed in two periods. The 3-day embryo transfers (1032 cycles) were routinely performed from May 1996 to December 1996 (period I). In period II (from January 1997 to December 1998), the embryo transfers (4700 cycles) were performed on either day 3 or day 5 (occasionally on day 6) by determination of the embryo transfer date in accordance with the number and quality of 2-day embryos. During both periods, the methods used for ovarian stimulation, embryo culture, and embryo cryopreservation were identical. The study protocol was approved by the Institutional Review Board.

Stimulation Protocol

Ovarian stimulation was performed using FSH (Metrodin; Serono, Geneva, Switzerland) or hMG (Humegon; Organon Pharmaceuticals, Saint Denis, France) with gonadotropin releasing hormone agonist (Superfact, Allemagne, Germany). When two follicles reached 18 mm in diameter, a dose of 10,000 IU of hCG (Profasi; Serono) was administered. Oocytes were retrieved transvaginally 36–38 hr after hCG injection.

Embryo Culture

All zygotes were cocultured with cumulus cells in 10 μl of YS medium (Table I) supplemented with 10% hFF for 48 or 96 hr. The hFF used in the present study was prepared using the method reported by Chi *et al.* (7). Cumulus cell pools were also prepared from cumulus–oocyte complexes which were washed three times immediately after oocyte collection. The portions of the cell mass which were unclear outside the corona radiata of cumulus–oocyte complexes were removed using two pieces of a 30-gauge needle. Then only corona radiator masses from the cleaned cumulus–oocyte complexes were excised using two pieces of a 30-gauge needle. Oocytes with one or more layers were cultured in 1 ml of YS medium containing 10% hFF for insemination. On the other hand, the corona radiator masses were treated with 100 to 150 μl of YS medium containing 10% hFF and 0.003%

Table I. Components of YS Medium Compared to G1, G2, and P1 Media

Chemical	G1	G2	P1	YS[a]
EDTA	0.10	—	—	0.05
$CaCl_2$	1.80	1.80	2.04	1.80
NaCl	85.16	85.16	101.60	110.00
KCl	5.50	5.50	4.70	5.00
NaH_2PO_4	0.50	0.50	—	—
$MgSO_4$	1.00	1.00	0.20	0.80
Na-citrate (mg/L)	—	—	0.15	—
Taurine	0.10	—	0.05	0.20
Na-pyruvate	0.32	0.10	0.33	0.40
Glucose	0.50	3.15	—	—
$KHCO_3$	—	—	—	5.00
$NaHCO_3$	25.00	25.00	25.00	20.00
L-Glutamine	1.00	1.00	—	1.20
Na-lactate (DL)	21.00	11.74	21.40	3.00
MEM nonessential AA	10 ml/L	10 ml/L	—	10 ml/L
RPMI 1640 AA	—	—	—	10 ml/L
MEM essential AA	—	10 ml/L	—	—
MEM vitamins	—	—	—	5 ml/L
Antibiotics	P/S	P/S	Gt	P/S

[a] All components of YS medium were contained in 1 kg of distilled water.

hyaluronidase for lysis of gap junctions, resulting in a change of the corona radiator masses to a pool containing many single cells. The cumulus cell layers used for coculture were made in 10-μl droplets by seeding cumulus cells (1×10^4) obtained from the pool using a pipette. After 4 hr of incubation, YS medium containing 0.003% hyaluronidase was exchanged with a preequilibrated culture medium, and then the drop dishes containing cumulus layers were incubated overnight. All zygotes were cocultured with cumulus cells in 10 μl of YS medium containing 10% hFF for 48 or 96 hr. All droplets for coculture were exchanged for a preequilibrated culture medium every morning until day 6. At the time of each medium change, only 5 μl of medium was removed from the coculture droplet, and then an equal volume of a preequilibrated fresh medium was added to it.

Determination of the Embryo Transfer Date

In period II, embryos were transferred on either day 3 or day 5. The date of embryo transfer was determined by the number of zygotes and the quality of 2-day embryos. On day 2 after insemination, the embryos were classified into four grades: embryos which reached the four-cell or more stage and had no cytoplasmic fragments (grade 1); embryos which reached the four-cell or more stage but had less than 20% cytoplasmic fragments or embryos which did not develop to the four-cell stage but had no cytoplasmic fragments (grade 2); embryos which developed to the four-cell or more stage but had 20–50% cytoplasmic fragments or embryos which did not reach the four-cell stage but had less than 50% cytoplasmic fragments (grade 3); and embryos which did not reach the four-cell stage and had more than 50% cytoplasmic fragments (grade 4). Grade 1 and grade 2 embryos were regarded as good-quality embryos, while grade 3 and grade 4 embryos were considered as poor quality. Day 5 transfer was performed in patients (age, <40 years) who had more than seven zygotes or two or more good-quality embryos regardless of the number of zygotes in period II. The remaining patients were allotted to day 3 transfer cycles. All patients who were more than 40 years old also had 3-day cleavage-stage embryos replaced, regardless of the number and quality of the 2-day embryos, due to the possibility of their not producing blastocyst-stage embryos in vitro. The number of embryos transferred was determined as below by depending upon the age of the patient, the patient history, and the present embryo quality. In general, a maximum of four embryos at the cleavage stage was replaced for day 3 transfer, while two embryos at the blastocyst stage were used for day 5 transfer. In the blastocyst-stage embryos selected for day 5 transfer, the inner cell mass and trophectoderm cells were clear.

After embryo transfer, regardless of the transfer date, surplus embryos were cultured until day 6, and embryos developing to the expanded blastocyst stage were cryopreserved by Menezo's method III (8).

Statistical Analysis

Statistical analysis was performed using the χ^2 test. A P value <0.05 was considered statistically significant.

RESULTS

Five thousand seven hundred thirty-two cycles were included in this study. In the first period, all 1032 patients underwent day 3 transfer. In the second period, 4652 of 4700 patients underwent embryo transfer on day 3 or day 5.

The mean age and the mean number of oocytes or embryos obtained in period I were similar to those in period II (Table II). The mean number of embryos transferred per cycle in the second period was significantly lower than that in the first period (2.9 ± 0.6 and 3.7 ± 0.5, respectively; $P < 0.001$). Although the pregnancy and implantation rates were similar in both periods (Table II), the multiple pregnancy rate was significantly lower in the second period than in

Table II. Embryo Transfer Outcome in Period I Compared to Period II

	Period I	Period II	*P*
No. of cycles	1032	4700	
Mean age ± SD	34.2 ± 4.4	34.7 ± 3.9	NS[a]
Mean No. of oocytes ± SD	10.5 ± 3.1	11.5 ± 7.9	NS
Mean No. of fertilized oocytes ± SD	6.8 ± 4.5	7.1 ± 4.9	NS
ET cycles	1032	4653	
Mean No. of embryos transferred ± SD	3.7 ± 0.5	2.9 ± 0.6	<0.001
Mean No. of blastocysts frozen ± SD	0.6 ± 0.5	1.3 ± 0.9	
Cycles with embryo freezing (%)	6.4	27.6	<0.001
Implantation rate (%)	16.1	16.4	NS
Pregnancy rate (%)	34.9	36.1	NS
Multiple gestations (%)	49.6	30.7	<0.05
Triplet gestations (%)	13.3	2.3	<0.001
Quadruplet gestations (%)	3.2	0	<0.001

[a] Not significant.

the first period ($P < 0.05$). Furthermore, 13.3% of the pregnancies were triplets, and 3.2% were quadruplets, in the first period. In contrast, only 2.3% of the pregnancies were triplets, and there were no quadruplet pregnancies, in the second period. The mean number of embryos frozen per retrieval in the second period was 1.3 ± 0.9, much higher than the 0.6 ± 0.5 in the first period. Consequently, significantly more patients (27.6%) had embryos frozen in the second period than in the first period (6.4%) ($P < 0.001$) .

The outcome of day 3 and day 5 transfers in the second period is shown in Table III. Day 3 transfer cycles were performed in 57% of the patients (2701 cycles), while day 5 transfer cycles were carried out in 43% of the patients (1999 cycles). However, blastocyst-stage embryos were transferred in only 1952 of 1999 cycles after 5 days of culture. The mean age, mean number of oocytes, and mean number of embryos in the day 3 transfer cycles (34.8 ± 4.0, 8.6 ± 3.1, and 5.0 ± 2.1, respectively) were similar, despite the inclusion of patients who were more than 40 years old, to those (34.6 ± 3.9, 15.7 ± 7.0 and 10.0 ± 4.5, respectively) in the day 5 transfer cycles. The rate of embryos developing to the blastocyst stage in the day 5 transfer cycles was 52.8%. The embryos in 33 cycles (1.7%) failed to develop to the blastocyst stage even by day 6, resulting in the cancellation of embryo transfer. In 14 cycles, the day 5 embryo transfer was canceled despite successful embryo development to the blastocyst stage, and embryos reaching the blastocyst stage were frozen due to the risk of ovarian hyperstimulation syndrome (OHSS). The mean number of embryos transferred per cycle in day 5 transfer cycles was significantly lower than that in day 3 transfer cycles (2.3 ± 0.5 vs 3.4 ± 0.9), although the mean numbers of oocytes or embryos per cycle in day 5 transfer cycles were higher than those in day 3 transfer cycles. On the other hand, many more embryos were frozen at the blastocyst stage in day 5 transfer cycles than in day 3 transfer cycles (3.1 ± 1.0 and 0.4 ± 0.6, respectively), although we might consider the proportion to the mean numbers of oocytes or embryos per cycle. The pregnancy and implantation rates in day 5 transfer cycles (47.1 and 23.2%, respectively) were significantly higher than in day 3 transfer cycles (28.6 and 13.1%, respectively), while the multiple pregnancy rate in day 5 transfer cycles was similar to that in day 3 transfer cycles (30.4 and 31.0%, respectively). On the other hand, the triplet pregnancy rate was reduced in day 5 transfer cycles compared to day 3 transfer cycles (0.8 and 3.9%, respectively). Consequently, the rate of pregnancies with triplets or more in period II was significantly decreased compared to that in period I (Table II vs Table III).

Table III. Outcome of Day 5 Versus Day 3 Embryo Transfer in Period II

	Embryo transfer	
	Day 3	Day 5
No. of cycles	2701	1999
Mean age ± SD	34.8 ± 4.0	34.6 ± 3.9
Mean No. of oocytes ± SD	8.6 ± 3.1	15.7 ± 7.0
Mean No. of fertilized oocytes ± SD	5.0 ± 2.1	10.0 ± 4.5
% blastocysts per 2PNs	—	52.8
ET cycles	2701	1952[a]
Mean No. of embryos transferred ± SD	3.4 ± 0.9	2.3 ± 0.5
Mean No. of blastocysts frozen ± SD	0.4 ± 0.6	3.1 ± 1.0
Cycles with embryo freezing (%)	1.3	63.1
Implantation rates (%)	13.1	23.2
Pregnancy rates (% per ET)	28.6	47.1
Multiple gestations (%)	31.0	30.4
Triplet gestations (%)	3.9	0.8

[a] Embryo transfer for 14 patients was canceled despite successful embryo development to the blastocyst stage, and all their embryos developing to the blastocyst stage were frozen for the prevention of OHSS.

DISCUSSION

This study demonstrates that the choice of embryo transfer date is important for maintaining a constant pregnancy rate, while decreasing multiple gestations and embryo transfer failures. Multiple gestations, especially triplets or more, are one of the most serious issues in assisted reproductive technology. According to the American Society of Reproductive Medicine–Society for Assisted Reproductive Technology report (1995), 7% of deliveries are triplet-or-more gestations (9). There is no doubt that blastocyst transfer is a way of eliminating high-order multiple pregnancies, due to the decreased number of embryos transferred. However, the current culture systems may still be suboptimal for supporting later preimplantation development, although new culture systems and media are currently being developed. In previous studies using coculture (10), a single culture medium (11), or sequential media (12), there were no embryos that reached the blastocyst stage in 8.5%, 28%, and 8.9% of patients. Furthermore, Scholtes *et al.* (13) reported that, of 929 patients in their first cycles, 545 (59%) had at least one blastocyst-stage embryo available for transfer, and thus 41% of the patients failed to transfer

a blastocyst-stage embryo. Despite the recent encouraging results from the transfer of blastocyst-stage embryos (2,3,11), embryo transfer failure is the main reason why the transfer of blastocyst-stage embryos cannot become an established protocol. Quinn *et al.* (14) suggested that the embryos should be replaced in the reproductive tract as soon as possible if patients have a small number of embryos. To reduce not only the possibility of high-order multiple gestations but also the failure of embryo transfer, we encouraged patients with more than seven zygotes or two or more good embryos to undergo day 5 transfer and the remainder to undergo day 3 transfer. Only 1.7% of patients in day 5 transfer cycles failed to transfer a blastocyst-stage embryo, and the rates of multiple gestations and triplet gestations were significantly reduced when blastocyst-stage transfer was performed for patients with large numbers of zygotes and good-quality embryos. Furthermore, there were no ongoing quadruplet pregnancies in the second period.

Patton *et al.* (15) reported that blastocyst-stage embryo transfer cycles had higher implantation and pregnancy rates compared with day 3 embryo transfer cycles in similar patient populations. In the present study, it is unclear whether the implantation and pregnancy rates in the blastocyst-stage embryo transfer cycles are higher than those in day 3 embryo transfer cycles in similar patient populations. In the case of a large number of oocytes, zygotes, or good-quality embryos, it might be a natural result that the implantation and pregnancy rates were higher in day 5 than in day 3 embryo transfer cycles. However, we found no difference in the pregnancy and implantation rates between the two periods. There were no statistically significant differences in the mean patient age or the mean number of oocytes or zygotes between the two periods. It is important that similar pregnancy rates were observed despite the transfer of significantly fewer embryos. Therefore, in the traditional day 2 or day 3 embryo transfer cycles, it is possible that embryos with a high implantation potential are replaced to the uterus, increasing the risk of high-order multiples without improving the pregnancy rate.

It is interesting to note that significantly more cycles had embryos frozen in the second period than in the first period. In our previous report (16), the implantation and ongoing pregnancy rates for frozen–thawed blastocysts (21.2 and 38.7%, respectively) were similar to those for fresh blastocysts (23.1 and 36.6%). Consequently, the cumulative pregnancy rate per oocyte collection must be increased by frozen–thawed blastocyst transfer.

Using the culture conditions described in the present study, 52.8% of all zygotes developed to the blastocyst stage, when cultured until day 6. YS medium was designed to support the development of human zygotes to the blastocyst stage in a coculture system with cumulus cells. YS medium contains vitamins, amino acids, and taurine and omits glucose and phosphate (see Table I). It also contains much more potassium and less lactate compared to G1/G2 or P1 media. Coats *et al.* (17) showed that the culture of human embryos in a glucose-free medium versus a medium containing 5.5 m*M* glucose did not improve pregnancy rates. Conaghan *et al.* (18), however, reported that there was an increase in the development and blastocyst cell numbers in human embryos cultured in a glucose-free compared with a glucose-containing media. We also found that 2.5 to 5.5 m*M* glucose was detrimental to the development of human embryos (unpublished data). Ludwig *et al.* (19) found that a low level of glucose (0.5 m*M*), despite causing little overt improvement in development up to the blastocyst stage, significantly increased the proportion of the fetus formed from the transfer embryos. Under our culture conditions, with 10% hFF added to the glucose-free YS medium as a protein source, the embryos were exposed to a lower level of glucose compared to that in other standard culture media. The mean glucose concentration in hFF is in the range of 3.3–5.5 m*M* (20).

We found a positive correlation between the quality of embryos on day 2 or day 3 and their potential to develop to the blastocyst stage in vitro (unpublished data). Similar results have been reported by Sapiro *et al.* (21), who observed that embryos reaching the four-cell stage by day 2 are twice or more as likely to develop to the blastocyst stage than slower-growing embryos. Twin gestations are normally considered acceptable, while triplets are associated with an increase in perinatal morbidity and mortality. Therefore, we carried out blastocyst-stage embryo transfer in patients with more than two good-quality embryos on day 2. The minimum of eight zygotes was based on the results showing 30–50% blastocyst formation (2,13).

In conclusion, determination of the day on which embryos should be transferred based on the number of zygotes and the quality of embryos on day 2 may help to maintain an acceptable pregnancy rate, while minimizing embryo transfer failure and high-order multiple gestations. In the present study, a 36.1% pregnancy rate, a 2.3% triplet rate, and only a 0.7% cancellation rate were obtained. On the other hand, to omit triplet-or-more gestations as well as to

maintain an acceptable pregnancy rate while minimizing embryo transfer failure, it is suggested that a maximum of two viable blastocyst-stage embryos be replaced to the uterus, although we did not investigate the variation of the multiple gestation rate according to the number of blastocyst-stage embryos transferred on day 5.

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