Selection of Embryos for Day-3 Transfer at the Pronuclear-Stage and Pronuclear-Stage Cryopreservation Results in High Delivery Rates in Fresh and Frozen Cycles¹

Diane G. Hammitt,^{2,4} Christopher A. Sattler,³ Misty L. Manes,² and Anita P. Singh²

Submitted December 19, 2003; accepted May 4, 2004

Purpose: Evaluate IVF-ET outcome data for a unique culture and cryopreservation strategy.

Methods: Retrospective study of 92 patients. Embryos for day-3 transfer were selected at pronuclear-stage; all extra zygotes were cryopreserved at pronuclear-stage.

Results: Delivery rates for Anonymous Oocyte Donation (Group I), patients <35 years (Group II), and 35–38 years (Group III) were 52.9%, 61.5%, and 51.7% for fresh and 38.5%, 33.3%, and 40.0% for frozen transfer. Deliveries per retrieval were 82.3%, 71.8%, and 58.6%. Only 0.88, 0.80, and 0.61 more zygotes were cultured than what were used for fresh transfer. Singleton, twin, and triplet rates were 64.6%, 31.2%, and 4.2% for fresh and 69.2%, 30.8%, and 0% for frozen.

Conclusions: Selection of day-3 transfer embryos at the pronuclear-stage and cryopreservation of extra zygotes results in high delivery rates in fresh and frozen cycles. This approach optimizes deliveries per retrieval and provides many patients with more than one pregnancy per retrieval.

KEY WORDS: Embryo cryopreservation; embryo grading; IVF-ET; pregnancy rate; zygote grading.

INTRODUCTION

To optimize IVF-ET delivery rates per oocyte retrieval requires the use of culture and cryopreservation methods that maximize the innate implantation potential of each two-pronuclear fertilized (2PN) oocyte in the retrieval cohort. Embryos need to be transferred in the fresh cycle before there is any loss in their innate implantation potential from prolonged in vitro culture. Fertilized oocytes in excess of the number required for fresh transfer need to be cryopreserved using techniques that maximize post-thaw

survival and implantation rates in subsequent frozen embryo transfer (FET) cycles.

Embryos with certain pronuclear-stage (PN-S) features, such as the presence of a halo and alignment of nucleoli within the PN, have better day-3 to day-6 cleavage rates and morphology and also implant at higher rates than embryos from zygotes lacking these features (1–7). Zygotes cryopreserved at the PN-S have high rates of cryosurvival and result in high pregnancy rates after post-thaw culture to the 4- to 6-cell stage (8–10). Based on these observations, we began using a unique IVF-ET culture and cryopreservation strategy that may result in higher delivery rates per oocyte retrieval compared to other culture and cryopreservation strategies that are more commonly used. Our approach involves selection of embryos for day-3 fresh transfer early on the day after retrieval at the PN-S and PN-S cryopreservation of zygotes in excess of the maximum number selected by the patient and physician for fresh transfer. The purpose of this study was to examine reproductive outcomes for

¹ Presented in part at the 2003 Pacific Coast Fertility Society, Rancho Mirage, California, 2003.

² Division of Reproductive Endocrinology and Infertility, Mayo Clinic, Scottsdale, Arizona.

³ Present address: Department of Laboratory Genetics, Mayo Clinic Rochester, Rochester, Minnesota.

⁴ To whom correspondence should be addressed at Center for Reproductive Medicine, Mayo Clinic Scottsdale, Thunderbird Women's Health Center, 13737 North 92nd Street, Scottsdale, Arizona 85260; e-mail: dhammitt@mayo.edu.

fresh and frozen embryo transfers and deliveries per retrieval using this unique culture and cryopreservation strategy.

MATERIALS AND METHODS

This study was conducted on patients undergoing an oocyte retrieval for IVF-ET between January 1, 2001 and December 31, 2001. All patients younger than 39 years of age were enrolled in this study except those with all embryos frozen due to risk of ovarian hyperstimulation syndrome ($N = 15$), no 2PN fertilization ($N = 3$), blastocyst transfer ($N = 1$), and GnRH analogue, (Lupron; TAP Pharmaceuticals, Deerfield, Illinois) flare stimulation ($N = 6$). This study was approved by our Institutional Review Board.

Patients were divided into three groups for analysis: anonymous oocyte donation (AOD) recipients (Group I), patients using their own oocytes who were less than 35 years of age at stimulation (Group II), and those 35–38 years of age at stimulation (Group III). Prior to the initiation of the IVF cycle, written consent was obtained for the number of embryos to transfer and for cryopreservation of PN-S zygotes in excess of the maximum number chosen for fresh transfer. Patients in Groups I and II consented to have 2 to 3 embryos transferred in the fresh cycle. Patients in Group III consented to transfer 3 to 4 embryos.

Our ovarian stimulation and PN-S zygote cryopreservation protocols have been previously reported (8–10). Patients undergoing standard ovarian stimulation were downregulated with GnRHa initiated on day 21 of the previous menstrual cycle or overlapped with oral contraceptives. Once pituitary downregulation was confirmed, the GnRH analogue dosage was reduced and ovarian stimulation was initiated using recombinant FSH (Gonal-F; Serono Laboratories, Randolph, Massachusetts or Follistim; Organon, Inc., West Orange, New Jersey) at a dose of 150–450 IU per day. Follicular development was monitored using serial transvaginal ultrasonography and serum E_2 measurements from the sixth day of gonadotropin therapy onward. 10,000 IU hCG (Profasi; Serono Laboratories) was administered when at least two follicles had reached a diameter of 18 mm. Transvaginal oocyte retrieval was performed 36 h after administration of hCG.

Oocytes were inseminated by standard insemination in organ culture dishes or by intracytoplasmic sperm injection (ICSI), as previously described (8–

10). Oocytes were cultured in Human Tubal Fluid (Irvine Scientific, Santa Ana, California) with 10% Synthetic Serum Substitute (Irvine Scientific) from retrieval until insemination. Oocytes for standard insemination were cultured for an additional 3 h with sperm, then transferred to microdrops for overnight culture. After injection, oocytes for ICSI were cultured overnight in G1.2 (Vitrolife Fertility Systems; Goteborg, Sweden).

Oocytes were observed for fertilization and assigned PN-S grades 17.5 to 18 h after standard insemination or 18.5 to 19 h after ICSI. Top-quality zygotes for fresh transfer were selected on the basis of PN-S morphological features (1,2). Top-quality zygotes were those with equal PN size, PN juxtaposed in the center of the zygote, nucleoli aligned at the PN interface, and a cytoplasmic “halo” or clearing around the zygote periphery. All remaining 2PN zygotes were immediately cryopreserved at the PN-S (8–10). Zygotes selected for fresh transfer were cultured together in one microdrop.

On day 2 of the fresh cycle, 24 h after PN-S selection, embryos were checked for multinucleation (11–13). On day 3, embryos were assessed for blastomere number and assigned embryo quality grades based on blastomere uniformity and percent fragmentation (8–10). If there were 2 or more good quality embryos on day-2 and day-3, the lower number on the IVF consent was transferred. Good quality embryos were those that displayed no multinucleation, had 2 to 4 uniform cells and <20% fragmentation on day-2 and 6 to 8 uniform cells and <20% fragmentation on day-3. If there were less than 2 good quality embryos on day-2 or day-3, or for repeat failed cycles, the higher number on the IVF consent was transferred.

Frozen embryo transfer cycles were included in this data set if all of the patient's frozen embryos being transferred were derived from a single oocyte retrieval. Patients consented in writing to the number of embryos for thaw and transfer. Patients usually agreed to have an equal number, or one to two more embryos, thawed than planned for transfer. For the FET cycle, Group I and II usually consented to transfer 2 or 3 embryos and Group III consented to transfer 3 or 4.

Uterine embryo transfer was performed on day 3 of culture using a Wallace (Irvine Scientific) or Casmed (TS Scientific, Perkasi, Pennsylvania) embryo transfer catheter. For luteal phase support, patients received daily IM progesterone (50 mg) initiated in the evening the day following oocyte retrieval. Clinical pregnancy was confirmed by one or more fetal sacs at

Table I. Infertility Diagnoses for Study Population^a

Diagnosis	Group I (N = 17)	Group II (N = 43)	Group III (N = 32)
Male-factor (%)	4 (23.5) ^b	22 (51.2)	21 (65.6) ^c
Endometriosis (%)	2 (11.8)	9 (20.9) ^b	2 (6.2) ^c
Polycystic ovarian disease (%)	0	1 (2.3)	0
Diminished ovarian reserve (%)	17 (100) ^b	10 (23.2) ^c	4 (12.5) ^c
Tubal factor (%)	3 (17.6)	10 (23.2)	10 (31.2)
Uterine factor (%)	0	0	0
Unexplained (%)	0	6 (13.9)	4 (12.5)

^a All male and female diagnoses were entered for each cycle; therefore, total diagnoses do not match the total number of cycles.

^{b,c} Values with different superscripts are significantly different ($P < 0.05$).

transvaginal ultrasound 3 weeks after a positive pregnancy test.

Continuous variables were analyzed with a 1-way ANOVA model. A P value of $<.05$ was considered statistically significant. Pair-wise contrasts also were made with the 1-way ANOVA model with the Tukey–Kramer adjustment for multiple comparisons. Categorical variables were analyzed using the exact Pearson χ^2 -test. Subgroup analysis also used the exact Pearson χ^2 -test with the Bonferroni adjustment for multiple comparisons.

RESULTS

Infertility diagnoses for the three treatment groups are shown in Table I. Significantly more patients with diminished ovarian reserve occurred in Group I compared with Groups II and III. The average age of the female partner (Table II) at the time of ovarian stimulation was 29.2 years (donor age, Group I), 31.4 years (Group II), and 37.2 years (Group III). The serum E_2 measurement and number of follicles greater than 15 mm on the day of hCG administration were significantly higher for Group I than for Groups II and III. The dose of FSH administered was significantly lower

for Group I compared with Groups II and III and for Group II compared with Group III.

The mean number of oocytes retrieved, mature oocytes, 2PN zygotes, retrievals with cryostored embryos, and embryos cryopreserved were significantly higher for Group I compared with Groups II and III (Table III). The percentage of ICSI cycles was significantly higher for Group III than for Group I. The mean number of 2PN cultured to day-3 and embryos transferred in the fresh cycle were significantly higher for Group III compared with Groups I and II. The implantation rate was significantly higher for Group I compared with Group III.

Fresh cycle clinical pregnancy and delivery rates (Table III) were 70.6%, 52.9% (Group I), 69.2%, 61.5% (Group II), and 58.6%, 51.7% (Group III). One triplet pregnancy occurred in Group II and one in Group III for a total triplet rate of 3.6% for fresh transfer cycles (56 fresh clinical pregnancies). The remaining clinical pregnancies were either singleton (60.7%) or twin (35.7%). The highest percentage of deliveries for fresh transfer cycles were singleton (64.6%), followed by twin (31.2%) and triplet (4.2%) deliveries. There were no monozygotic twin pregnancies.

Table II. Stimulation and Retrieval Data for Fresh Cycles

Variable	Group I	Group II	Group III
Stimulations, no.	17	43	32
Retrievals, no. (%)	17 (100)	39 (91)	29 (91)
Age at stimulation, (y) ^d	29.2 ± 3.1 ^a	31.4 ± 2.2 ^{b,c}	37.2 ± 1.2 ^b
Day of hCG ^d			
E_2	2435.5 ± 783.1 ^a	1760.4 ± 822.0 ^b	1674.8 ± 970.5 ^b
Follicles >15mm	11.7 ± 5.4 ^a	7.6 ± 3.2 ^b	7.0 ± 3.6 ^b
Total IU's FSH	1666.8 ± 405.9 ^a	2401.6 ± 1067.7 ^{b,c}	3131.4 ± 1036.7 ^b

^{a–c} Values with different superscripts are significantly different ($P < 0.05$).

^d Values are means ± SD.

Table III. Embryology and Fresh Cycle Outcome Data

Variable	Group I	Group II	Group III
Oocytes retrieved, no. ^c	19.2 ± 5.7 ^a	13.4 ± 6.1 ^b	10.8 ± 5.9 ^b
Mature oocytes, no. ^c	16.8 ± 5.2 ^a	10.9 ± 5.4 ^b	8.8 ± 5.4 ^b
ICSI cycles, no. (%)	8/17 (47.1) ^a	27/39 (69.2)	25/29 (86.2) ^b
2PN ^c			
Total no.	13.1 ± 5.4 ^a	8.3 ± 4.5 ^b	7.1 ± 4.4 ^b
Cultured for fresh transfer, no.	3.1 ± 0.4 ^a	3.2 ± 0.7 ^a	3.6 ± 0.8 ^b
Embryos transferred, no. ^c	2.2 ± 0.5 ^a	2.4 ± 0.6 ^a	3.0 ± 0.9 ^b
Retrievals with frozen embryos, no. (%)	17/17 (100.0) ^a	28/39 (71.8) ^b	17/29 (58.6) ^b
Frozen embryos, no. ^c	10.0 ± 5.3 ^a	5.1 ± 4.6 ^b	3.7 ± 4.2 ^b
Clinical pregnancies per retrieval, no. (%)	12/17 (70.6)	27/39 (69.2)	17/29 (58.6)
Implantation rate, no. (%) ^d	19/37 (51.4) ^a	37/93 (39.8)	24/88 (27.3) ^b
Fetal heart beats/pregnancies	19/12 (1.6)	34/27 (1.3)	22/17 (1.3)
Clinical pregnancies			
With 1 sac, no. (%)	5/12 (41.7)	18/27 (66.7)	11/17 (64.7)
With 2 sacs, no. (%)	7/12 (58.3)	8/27 (29.6)	5/17 (29.4)
With 3 sacs (%)	0 (0)	1/27 (3.7)	1/17 (5.9)
Deliveries per retrieval (%)	9/17 (52.9) ^e	24/39 (61.5)	15/29 (51.7)
Total deliveries			
Singleton, no. (%)	5/9 (55.5)	17/24 (70.8)	9/15 (60.0)
Twin, no. (%)	4/9 (44.4)	6/24 (25.0)	5/15 (33.3)
Triplet, no. (%)	0 (0)	1/24 (4.2)	1/15 (6.7)

^{a,b} Values with different superscripts are significantly different ($P < 0.05$).

^c Values are means ± SD.

^d No. sacs/no. embryos transferred.

^e One patient terminated ongoing pregnancy for psychosocial reasons.

The mean number of 2PN zygotes held in culture for the fresh cycle was 3.06 for Group I, 3.18 for Group II, and 3.64 for Group III. The mean number of fresh embryos transferred for these three groups was 2.18, 2.38, and 3.03, respectively (Table III). On average only 0.88 (Group I), 0.80 (Group II), and 0.61 (Group III) more embryos were cultured than transferred.

Embryologic and outcome data for FET cycles are summarized in Table IV. To date, 30 FET cycles have been performed in this study group. All FET deliveries came from couples that did not deliver from the fresh transfer. Only first FET deliveries were included in this data set. Therefore, no patient had two deliv-

eries per retrieval in this study. The mean number of embryos thawed was 3.7 for Groups I and II and 3.8 for Group III. Clinical and delivery rates per thaw were 46.1%, 38.5% for Group I, 41.7%, 33.3% for Group II and 40.0%, 40.0% for Group III. The number of fetal heartbeats per clinical pregnancy was similar for Groups I, II, and III. Frozen embryo transfer cycles resulted in no triplet pregnancies. The percentage of singleton and twin clinical pregnancies for FET cycles was 69.2% and 30.8%, respectively.

Delivery rates per oocyte retrieval after the transfer of fresh embryos and some of the cryostored embryos were 82.3% for Group I, 71.8% for Group II,

Table IV. Frozen Embryo Transfer Embryology and Cycle Outcome Data

Variable	Group I	Group II	Group III
Thaw cycles, no.	13	12	5
2PN thawed, no. ^a	3.7 ± 1.2	3.7 ± 1.0	3.8 ± 1.3
Cleavage to 2-cell, (%)	86.5%	78.4%	84.2%
Embryos transferred, no. ^a	3.0 ± 0.7	2.3 ± 0.8	2.8 ± 0.8
Clinical pregnancies per thaw, no. (%)	6/13 (46.1)	5/12 (41.7)	2/5 (40.0)
Implantation rate, no. (%)	8/39 (20.5)	6/28 (21.4)	3/14 (21.4)
Fetal heart beats/pregnancies, no. (mean)	8/6 (1.3)	6/5 (1.2)	3/2 (1.5)
Clinical pregnancies			
With 1 sac, no. (%)	4/6 (66.7)	4/5 (80.0)	1/2 (50.0)
With 2 sacs, no. (%)	2/6 (33.3)	1/5 (20.0)	1/2 (50.0)
With 3 sacs, no. (%)	0	0	0
Deliveries per thaw, no. (%)	5/13 (38.5)	4/12 (33.3)	2/5 (40.0)

^a Values are means ± SD.

Table V. Cryoaugmented Ongoing Pregnancy or Delivery Rate per Oocyte Retrieval for Study Population

Patient group	Retrievals, no.	Fresh delivery rate, No. (%)	FET delivery rate, No. (%)	Cryoaugmented ongoing pregnancy or delivery rate per retrieval, no. (%) ^a
I	17	9/17 (52.9%) ^b	5/13 (38.5%)	14/17 (82.3%)
II	39	24/39 (61.5%)	4/12 (33.3%)	28/39 (71.8%)
III	29	15/29 (51.7%)	2/5 (40.0%)	17/29 (58.6%)

^aAll FET deliveries came from couples that did not deliver from the fresh transfer. Only first FET deliveries were included in this data set. Therefore, no patient had two deliveries per retrieval in this study.

^bOne patient terminated ongoing pregnancy for psychosocial reasons.

and 58.6% for Group III (Table V). One patient in Group I terminated an ongoing pregnancy for psychosocial reasons. There were no selective reductions in the fresh or FET pregnancies conceived in this study. Of the patients who have and have not yet delivered from a fresh or FET cycle, 74% and 44% still have two or more embryos in cryostorage.

DISCUSSION

Various embryo culture and cryopreservation strategies are used by IVF-ET programs throughout the world. Transfer and cryopreservation at the zygote, cleavage, morula, and blastocyst stages have all been used with success. However, there is still considerable debate regarding which culture and cryopreservation strategy is optimal.

We have several years of experience with cryopreserving patient's zygotes in excess of the number selected for fresh transfer at the PN-S (8–10). The introduction in 1998 of blastocyst culture caused us to reevaluate our embryo culture and cryopreservation strategy. The initial studies on blastocyst transfer showed that the two possible advantages of blastocyst culture were an improved rate of pregnancy in the fresh cycle and a decrease in high-order multiple pregnancies (14–16). We sought to determine whether additional strategies could enhance our long-standing approach of cryopreserving extra embryos at the PN-S. We were interested in determining whether this enhanced approach might be an effective alternative to the more commonly used strategy of leaving all embryos in culture for selection of the best embryos at the cleavage or blastocyst stages.

We made two changes to our previous embryo culture and cryopreservation strategy. First, we adopted a new PN-S grading method (1) to improve our ability to select the best embryos for fresh transfer. Previously, we had selected PN-S zygotes with uniformly-sized PN, no vacuoles, even cytoplasmic granularity, and a small perivitelline space for continued culture

and transfer in the fresh cycle (10). Second, instead of specifying the exact number of embryos for transfer before stimulation and then having the laboratory leave this exact number in culture (8–10), a range of embryos for transfer, plus or minus one, was decided on before stimulation, with the final number determined on the day of the transfer. We hypothesized that culturing one more zygote than the minimum number planned for transfer might boost fresh cycle delivery rates because PN-S morphology is a good, but not absolute, predictor of day-3 embryo morphology and implantation potential.

Fresh-cycle clinical pregnancy rates in our study were 70.6% for Group I, 69.2% for Group II, and 58.6% for Group III. These were higher than those reported in most blastocyst studies even though only 0.88, 0.80, and 0.61 more embryos were cultured for Groups I, II, and III, respectively, than the number transferred. Pregnancy and implantation rates with blastocyst transfer have ranged from 21 to 77% and 9–62%, respectively (2,5,6,14–28). With blastocyst transfer, all embryos are retained in culture to ensure at least two high-quality blastocysts are available for transfer. Similarly, the most common culture strategy for cleavage-stage embryo transfer is to leave all 2PN zygotes in culture for selection of top-quality embryos on the day of transfer.

The results of our study confirm previous studies (8–10) demonstrating that it is not necessary to leave all zygotes in culture for cleavage-stage embryo transfer to obtain high fresh cycle delivery rates. The higher delivery rates observed in the present study, using the new approach of culturing one more zygote than the minimum number selected for fresh transfer, were most likely due to the increased flexibility this new approach allows at the time of transfer. Our new strategy allows us to do some preselection on the day of transfer or to transfer one more embryo if embryo quality grades are poor on day-2 and day-3.

We believe this culture and cryopreservation strategy offers some advantages over retaining all embryos

in culture. These include high FET delivery rates, high delivery rates per oocyte retrieval, a chance for a second delivery from one retrieval in a greater percentage of patients, reduction of high-order multiples without an increase in monozygotic twins, and cryopreservation at the PN-S. The advantage of PN-S cryopreservation over later stages is that cryopreservation takes place at a stage when the female and male genomes are still physically separated. Our approach circumvents ethical concerns about cryostorage and possible eventual destruction of embryos at later stages of development. PN-S zygotes generally are not considered to be embryos since the female and male genomes are still separated at this stage of development.

Leaving all the fertilized zygotes in culture for selection of the best embryos for fresh transfer may decrease pregnancy rates per oocyte retrieval because extra embryos must be cryopreserved at the cleavage or blastocyst stages. With current cryopreservation techniques, human embryos survive and implant at higher rates when cryopreserved at the PN-S (8–10) compared with the cleavage or blastocyst stages (6,19,29,30). The cohort of fertilized oocytes from a retrieval cycle has better nuclear synchrony at the PN-S than do the embryos as they progress to later stages of development. At the cleavage and blastocyst stages, loss of this nuclear synchrony makes it difficult to cryopreserve each embryo at its optimal stage.

In this study, 73% of patients had embryos cryopreserved, 82% of thawed zygotes cleaved to the 2-cell stage or greater, and the delivery rate per thaw was 43%. Of the patients who have and have not delivered, 74% and 44%, respectively, have at least 2 embryos remaining in cryostorage. Transfer of these additional cryostored zygotes could potentially increase delivery rates per retrieval further.

The percentage of patients with frozen embryos and number of embryos frozen are generally lower when all 2PN zygotes are held in culture and extra embryos are frozen at the time of transfer (6,17,22,29) than when extra zygotes are cryopreserved at the PN-S (8–10). With current culture techniques, only 30–50% of zygotes typically develop into normal blastocysts which results in fewer patients having cryopreserved embryos and fewer numbers of embryos frozen per cycle (2,5–7,15,17,18,20–22,25,27,29).

With current culture methods, there may be some loss of embryonic viability with extended culture (2,6,13,28). Culture of embryos to day-5 or day-6 allows the top-quality embryos in a cohort to develop to good quality blastocysts. However, medium-to-poor

quality embryos appear to require the *in vivo* environment for optimal development beyond day 3 (25,26,28). We have noted that medium-to-poor quality day-3 embryos rarely develop to blastocysts *in vitro* but often result in pregnancy after day-3 transfer with assisted hatching. Scott *et al.* (2) showed that zygotes with certain PN-S features usually failed to form blastocysts and any that did had too few cells and failed to implant. However, embryos from zygotes with these same features that were transferred on day 3 resulted in a 55% implantation rate (6).

For Groups I, II, and III, delivery rates per retrieval were 82.3%, 71.8%, and 58.6%, respectively. Only minimal data of this type are available for the more common IVF-ET culture strategy in use today, which is to leave all 2PN oocytes in culture until the day of transfer with cryopreservation of extra embryos at the cleavage or blastocyst stage. Rienzi *et al.* (6) reported lower cryoaugmented rates per retrieval with blastocyst transfer than with day-3 embryo transfer, because fewer embryos were cryopreserved and implanted with blastocyst cryopreservation.

PN-S morphology appears to be predictive of cleavage, fragmentation, chromosomal abnormalities, and blastocyst development (2,5,7). If PN-S morphology can be used to predict blastocyst development, then technical and ethical concerns with blastocyst culture can be avoided. These include greater cost and workload with extended culture, ethical concerns with freezing advanced stage embryos, possible skew in sex ratio due to male embryos developing more quickly with selection of fastest growing embryos on day 5 and risk of having no blastocysts for transfer (5,13,28).

During the past decade, increasing emphasis has been placed on limiting IVF-ET multiple pregnancies (15–27,29,31–34). Culture of embryos to the blastocyst stage has been advocated as a method for reducing multiple births while maintaining high fresh cycle pregnancy rates (15,16). To date, blastocyst transfers have reduced high-order multiple pregnancies, that is triplet or greater, but not overall multiple rates (16,21,31). Monozygotic twinning also appears to be higher with blastocyst transfer (24). In our study, 66.1% of deliveries were singletons and 30.5% were twins. There were only 2 triplet pregnancies in 59 deliveries (3.4%) and no monozygotic pregnancies.

The primary PN-S morphological selection criteria used in this study were the presence of polarized nucleoli within the PN and cytoplasmic clearing around the zygote periphery (1,2). Top-quality zygotes also were required to be free of vacuoles, to possess similarly sized PN and to have a regular shape with

no excessive granularity. Embryos were selected for transfer on the basis of lack of multinucleation, number of blastomeres, fragmentation, and uniformity in cell size. Our day 2 and day 3 embryo grading methods were similar to those used by Van Royen *et al.* (13) who defined a type of day 3 embryo with a 49% implantation rate. These embryos had no multinucleation, 4 or 5 cells on day 2, 7 or more cells on day 3, and fewer than 20% fragments.

Several oocyte, zygote, and cleavage-stage grading methods that were not used in this study have been shown to correlate with an embryo's implantation potential (1–3,5–7,11,12,28,30–37). Tesarik *et al.* (1) showed that the relationship between the number of embryos transferred and the multiple rate depended on PN-S morphology; transfer of 2 embryos from pattern zero zygotes usually resulted in a twin pregnancy. Pattern zero zygotes had a difference of less than four in the number of nucleoli between PN, the distribution were the same in both PN (either random or polarized), and each PN had at least 3 nucleoli.

We are currently investigating whether the number of embryos transferred can be further reduced by using several additional grading methods that were not used in this study. New methods under evaluation include number, pattern, and distribution of nucleoli within the PN (1,2,5,7), size and shape of PN-S halos (7), early cleavage to the 2-cell stage (32,36,37), day-3 fragmentation pattern (35), and variation in the thickness of the zona pellucida.

We performed PN-S, day-2, and day-3 embryo evaluations on static embryos under direct microscopic observation. Zygotes were not rotated during evaluation and the numbers of nucleoli in each PN were not counted. Rotating zygotes and embryos during grading and recording the images for a more detailed review after embryos are returned to the incubator may allow us to select top-quality zygotes and cleavage embryos with greater accuracy (7).

In conclusion, the results of this study demonstrate that selection of embryos for day-3 fresh transfer at the PN-S and cryopreservation of PN-S zygotes in excess of the maximum number selected for fresh transfer results in high delivery rates in fresh and frozen cycles. This approach also optimizes the chance of a delivery per retrieval, results in a high percentage of retrievals with cryostored embryos, and provides many patients with more than one pregnancy per retrieval. This culture and cryopreservation strategy may offer the best method for preserving the innate implantation potential of each fertilized oocyte since embryos are transferred on day 3 before loss of

viability or reduction in implantation potential from prolonged culture and all extra 2PN oocytes are cryopreserved at a cell-stage which results in high post-thaw survival and implantation rates.

REFERENCES

1. Tesarik J, Junca AM, Hazout A, Aubriot FX, Nathan C, Cohen-Bacrie P, Dumont-Hassan M: Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of pronuclear morphology. *Hum Reprod* 2000;15:1396–1399
2. Scott L, Alvero R, Leondires M, Miller B: The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Hum Reprod* 2000;15:2394–2403
3. Wittemer C, Bettahar-Lebugle K, Ohl J, Rongieres C, Nisand I, Gerlinger P: Zygote evaluation: An efficient tool for embryo selection. *Hum Reprod* 2000;15:2591–2597
4. Salumets A, Hyden-Granskog C, Suikkari AM, Tiitinen A, Tuuri T: The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. *Hum Reprod* 2001;16:2177–2181
5. Balaban B, Urman B, Isiklar A, Cengiz A, Aksoy S, Mercan R, Mumcu A, Nuhoglu A: The effect of pronuclear morphology on embryo quality parameters and blastocyst transfer outcome. *Hum Reprod* 2001;16:2357–2361
6. Rienzi L, Ubaldi F, Iacobelli M, Ferrero S, Minasi MG, Martinez F, Tesarik J, Greco E: Day 3 embryo transfer with combined evaluation at the pronuclear and cleavage stages compares favorably with day 5 blastocyst transfer. *Hum Reprod* 2002;17:1852–1855
7. Kahraman S, Kumtepe Y, Sertyel S, Donmez E, Benkhalifa M, Findikli N, Vanderawalmen P: Pronuclear morphology scoring and chromosomal status of embryos in severe male infertility. *Hum Reprod* 2002;17:3193–3200
8. Damario MA, Hammitt DG, Galanits TM, Stevens SA, Session DR, Dumesic DA: Anonymous oocyte donation performed exclusively with embryos cryopreserved at the pronuclear stage. *Fertil Steril* 1999;71:830–835
9. Damario MA, Hammitt DG, Galanits TM, Session DR, Dumesic DA: Pronuclear stage cryopreservation after intracytoplasmic sperm injection and conventional IVF: Implications for timing of the freeze. *Fertil Steril* 1999;72:1049–1054
10. Damario MA, Hammitt DG, Session DR, Dumesic DA: Embryo cryopreservation at the pronuclear stage and efficient embryo use optimizes the chance for a liveborn infant from a single oocyte retrieval. *Fertil Steril* 2000;73:767–773
11. Pelinck MJ, De Vos MD, Dekens M, Van der Elst J, De Sutter P, Dhont M: Embryos cultured in vitro with multinucleated blastomeres have poor implantation potential in human in-vitro fertilization and intracytoplasmic sperm injection. *Hum Reprod* 1998;13:960–963
12. Jackson KV, Ginsburg ES, Hornstein MD, Rein MS, Clarke RN: Multinucleation in normally fertilized embryos is associated with an accelerated ovulation induction response and lower implantation and pregnancy rates in in vitro fertilization-embryo transfer cycles. *Fertil Steril* 1998;70:60–66
13. Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W, Gerris

- J: Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* 1999;14:2345–2349
14. Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB: Culture and transfer of human blastocysts increases implantation rate and reduces the need for multiple embryo transfers. *Fertil Steril* 1998;69:84–88
 15. Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J: A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum Reprod* 1998;13:3434–3440
 16. Milki AA, Hinckley MD, Fisch JD, Dasig D, Behr B: Comparison of blastocyst transfer with day 3 embryo transfer in similar patient populations. *Fertil Steril* 2000;73:126–129
 17. Alves da Motta EL, Alegretti JR, Baracat EC, Olive D, Serafini PC: High implantation and pregnancy rates with transfer of human blastocysts developed in preimplantation stage one and blastocyst media. *Fertil Steril* 1998;70:659–663
 18. Jones GM, Trounson AO, Lolatgis N, Wood C: Factors affecting the success of human blastocyst development and pregnancy following in vitro fertilization and embryo transfer. *Fertil Steril* 1998;70:1022–1029
 19. Rijnders PM, Jansen CAM: The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. *Hum Reprod* 1998;13:2869–2873
 20. Shapiro BS, Harris DC, Richter KS: Predictive value of 72-hour blastomere cell number on blastocyst development and success of subsequent transfer based on the degree of blastocyst development. *Fertil Steril* 2000;73:582–586
 21. Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan R: Blastocyst quality affects the success of blastocyst-stage embryo transfer. *Fertil Steril* 2000;74:282–287
 22. Shapiro BS, Richter KS, Harris DC, Daneshmand ST: A comparison of day 5 and day 6 blastocyst transfers. *Fertil Steril* 2001;75:1126–1130
 23. Richter KS, Harris DC, Daneshmand ST, Shapiro BS: Quantitative grading of a human blastocyst: Optimal inner cell mass size and shape. *Fertil Steril* 2001;76:1157–1167
 24. de Costa ALE, Abdelmassih S, de Oliveira FG, Abdelmassih V, Abdelmassih R, Nagy ZP, Balmaceda JP: Monozygotic twins and transfer at the blastocyst stage after ICSI. *Hum Reprod* 2001;16:333–336
 25. Racowsky C, Jackson KV, Cekleniak NA, Fox JH, Hornstein MD, Ginsburg ES: The number of eight-cell embryos is a key determinant for selecting day 3 or day 5 transfer. *Fertil Steril* 2000;73:558–564
 26. Coskun S, Hollanders J, Al-Hassan S, Al-Sufyan H, Al-Mayman H, Jaroudi K: Day 5 versus day 3 embryo transfer: A controlled randomized trial. *Hum Reprod* 2000;15:1947–1952
 27. Huisman GJ, Fauser BC, Eijkemans MJ, Pieters MH: Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture. *Fertil Steril* 2000;73:117–122
 28. Kovacic B, Vlasisavljevic V, Reljic M, Lovrec VG: Clinical outcome of day 2 versus day 5 transfer in cycles with one or two developed embryos. *Fertil Steril* 2002;77:529–536
 29. Shoukir Y, Chardonens D, Campana A, Bischof P, Sakkas D: The rate of development and time of transfer play different roles in influencing the viability of human blastocysts. *Hum Reprod* 1998;13:676–681
 30. Vilska S, Tiitinen A, Hyden-Granskog C, Hovatta O: Elective transfer of one embryo results in an acceptable pregnancy rate and eliminates the risk of multiple birth. *Hum Reprod* 1999;14:2392–2395
 31. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerse M, Valkenburg M: Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: A prospective randomized clinical trial. *Hum Reprod* 1999;14:2581–2587
 32. Bos-Mikich A, Mattos ALG, Ferrari AN: Early cleavage of human embryos: An effective method for predicting successful IVF/ICSI outcome. *Hum Reprod* 2001;16:2658–2661
 33. De Neubourg D, Mangelschots K, Royen EV, Vercruyssen M, Ryckaert G, Valkenburg M, Barudy-Vasquez J, Gerris J: Impact of patients' choice for single embryo transfer of a top quality embryo versus double embryo transfer in the first IVF/ICSI cycle. *Hum Reprod* 2002;17:2621–2625
 34. Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Vercruyssen M, Barudy-Vasquez J, Valkenburg M, Ryckaert G: Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme. *Hum Reprod* 2002;17:2626–2631
 35. Alikani M, Cohen J, Tomkin G, Garrisi J, Mack C, Scott RT: Human embryo fragmentation in vitro and its implications for pregnancy and implantation. *Fertil Steril* 1999;71:836–842
 36. Shoukir Y, Campana A, Farley T, Sakkas D: Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: A novel indicator of embryo quality and viability. *Hum Reprod* 1997;12:1531–1536
 37. Fenwick J, Platteau P, Murdoch AP, Herbert M: Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Hum Reprod* 2002;17:407–412