Comparison of Pregnancy Outcome of Pronuclear- and Multicellular-Stage Frozen–Thawed Embryo Transfers

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Purpose: *Our purpose was to determine if supernumerary embryos generated by in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) should be frozen (using 1,2-propanediol) at the pronuclear or multicellular stage.*
Methods: *The study was a retrospective analysis conducted at the Dubai Gynaecology & Fertility Centre of the Department of Health & Medical Services, Dubai, U.A.E. One hundred forty-one women undergoing frozen–thawed embryo replacement cycles with IVF generated embryos and 84 women undergoing the same with ICSI generated embryos.*
Results: *Supernumerary, IVF-generated embryos frozen at the multicellular stage had a significantly higher rate of survival on thawing (73.9%) than embryos frozen at the pronuclear stage (64.4%). The morphological grades of the embryos in the two groups were similar, but a significantly higher pregnancy rate was obtained with embryos frozen at the multicellular stage (22.8%) than with pronuclear-stage embryos (14.8%). Similarly, with ICSI-generated embryos, significantly higher survival was seen with multicellular-stage frozen embryos (74.8%) than pronuclear-stage embryos (64.4%). The morphological grades of the embryos and pregnancy outcomes of the two groups were similar.*
Conclusions: *Supernumerary embryos generated by IVF and ICSI should be frozen at the multicellular stage so as to allow selection of the best embryos for transfer and embryo freezing of only robust embryos.*

KEY WORDS: frozen–thawed embryo transfer; 1,2-propanediol; pronuclear embryos; multicellular embryos.

INTRODUCTION

Since the first successful pregnancy following the transfer of a frozen–thawed human embryo in 1983, a large number of babies have been born from this procedure. The initial results were obtained using a slow-freezing protocol with dimethyl sulfoxide (DMSO) (1), but more recently good results have been reported using glycerol for blastocyst-stage embryos (2) and 1,2-propanediol for pronuclear and multicellular embryos (3,4). Most in vitro fertilization (IVF) programs have adopted their own freezing protocols with varying degrees of success (4–10). However, the best success rates have been obtained with a protocol using 1,2-propanediol for freezing good-quality pronuclear and multicellular embryos (4,5,11,12).

The success of cycles in which frozen–thawed embryos are replaced depends on the age of the woman, the quality of the endometrium, and the quality and morphological appearance of the embryos at the time of transfer (4,13). Generally, embryos that have regular blastomeres and an absence of fragmentation at the time of the initial freezing survive the freezing–thawing process better than embryos showing fragmentation (14,15).

With the advent of intracytoplasmic sperm injection (ICSI), supernumerary embryos from this procedure have become available for freezing and subsequent transfer. In this study we compare the pregnancy outcome using frozen–thawed embryos generated by IVF and ICSI and frozen at the pronuclear and multicellular stages of development.

MATERIALS AND METHODS

Between October 1991 and July 1997, patients undergoing IVF and ICSI received ovarian stimulation using a long down-regulation protocol using Decapeptyl, 3.75 mg (Beufor; Ipsen, France), and urinary gonadotropins. Follicular growth was monitored by serial ultrasound vaginal examination and the human chorionic gonadotropin (hCG) trigger (10,000 IU) was administered when at least three follicles were at a

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diameter of 18 mm or greater. Ultrasound-guided vaginal oocyte retrieval was performed under local anesthesia and intravenous sedation.

The retrieved oocytes were cultured in Medicult IVF medium (Denmark). Oocytes undergoing IVF had routine insemination. Oocytes that were subjected to microinjection were initially treated with hyaluronidase to remove the cumulus and corona cells and then microinjected within 2 hr of collection. All oocytes were assessed for evidence of fertilization 18 hr later. Oocytes that contained two pronuclei were transferred to fresh culture medium. Some of the embryos were frozen at this stage if a large number (more than eight) of pronuclear embryos had been generated. Any good-quality supernumerary embryos available after embryo transfer (i.e., 48 hr after oocyte retrieval) were frozen at the multicellular stage (two- to six-cell stage).

Freezing Protocol

A slow-freezing protocol (16) with 1.5 *M* 1,2-propanediol was used throughout the study period. The freezing medium consisted of modified Dulbecco's phosphate-buffered saline (PBS) with 5% human serum albumin (HSA) and 1,2-propanediol. Embryos were passed separately through the following solutions for 5 min each:

(a) Dulbecco's PBS with 5% HSA,
(b) 1.5 *M* 1,2-propanediol, and
(c) 1.5 *M* propanediol and 0.1 *M* sucrose.

Embryos were than transferred to glass ampoules containing 300 μl of a 1.5 *M* 1,2-propanediol and 0.1 *M* sucrose solution and frozen in a Planer series II Kryo 10 programmable freezer. Embryos were cooled from room temperature to −7°C at −2°C/min, at which temperature manual seeding was done. The embryos were then cooled at a rate of −0.3°C/min to −30°C and then −50°C/min to −140°C, at which temperature the ampoule was immersed in liquid nitrogen.

Embryo thawing was done manually by removing an ampoule from the liquid nitrogen and transferring it immediately to a 30°C water bath for 40 sec. The ampoule was emptied and the embryos were transferred to a solution of 1.5 *M* 1,2-propanediol and 0.2 *M* sucrose for 5 min. The embryos were then transferred to 0.5 *M* 1,2-propanediol and, next, to 0.2 *M* sucrose, each for 5 min. Thereafter, the embryos were rinsed in culture medium and transferred to an equilibrated culture dish.

The pronuclear embryos were cultured for 24 hr before replacement and were judged to have survived if they showed no lysis on thawing and went on to cleave. Only cleaved embryos were transferred. Multicellular embryos were cultured for 3–4 hr before replacement and were judged to have survived if at least 50% of the initial blastomeres were intact. Multicellular embryos were graded according to their morphological appearance as follows.

Grade 4: Embryos with equal- or unequal-sized blastomeres and no anucleate fragments.
Grade 3: Embryos with up to 20% of their volume filled with anucleate fragments.
Grade 2: Embryos with between 20 and 50% of their volume filled with anucleate fragments.
Grade 1: Embryos with greater than 50% of their volume filled with anucleate fragments.

Frozen–thawed embryos were transferred during natural or hormone replacement cycles. [Pituitary down-regulation using 3.75 mg of Decapeptyl was followed by endometrial stimulation using estradiol valerate, 4 mg per day, till the endometrial thickness had reached 10 mm. At this point progesterone (Cyclogest pessaries, 400 mg twice daily) was commenced.] Embryo transfer was done using a Wallace catheter and a serum β-hCG assay was performed 10 days afterward. The observation of a gestational sac by means of ultrasonographic screening at 6 weeks of gestation indicated a clinical pregnancy.

A χ^2 statistical analysis was used to analyze the data for significance.

RESULTS

The results following the transfer of frozen–thawed embryos generated between October 1991 and July 1997 during previous IVF cycles are presented in Table I. Five hundred four pronuclear embryos were thawed for transfer in 82 cycles and 223 multicellular embryos were thawed for transfer in 59 cycles. The survival rates for the pronuclear and multicellular embryos were 64.4 and 73.9%, respectively. This difference was statistically significant ($P < 0.02$). Of the 268 pronuclear embryos that cleaved and were available for transfer, 110 (41.1%) were grades 4 and 3 and 158 (58.9%) were grades 2 and 1. Similarly, 56 of the 156 surviving multicellular embryos (35.8%) were grades 3 and 4 and 64.2% were grades 1 and 2. These differences were not significant.

Twelve pregnancies (positive β-hCG on day 10 after embryo transfer) resulted from frozen embryo transfer

Table I. Outcome After Transfer of Frozen-Thawed Pronuclear (PN) and Multicellular Embryos Obtained from IVF

	PN	Multicellular
No. cycles resulting in freezing of supernumerary embryos	127	108
No. embryos frozen	921	450
No. frozen–thawed embryo replacements	82	59
No. embryos thawed	504	223
No. embryos surviving	325 (64.4%)*	165 (73.9%)
No. embryo transfer cycles	81	56
No. embryos transferred	268	156
No. embryos transferred (mean ± SD)	3.2 ± 1.76	2.7 ± 1.48
Quality of embryos transferred		
Grades III & IV	110 (41.1%)	56 (35.8%)
Grades I & II	158 (58.9%)	100 (64.2%)
No. positive hCG measurements		
Per thawing cycle	12 (14.6%)	13 (22.0%)
Per embryo transfer	12 (14.8%)**	13 (22.8%)
No. preclinical abortions	0	2
No. miscarriages	1	3
No. clinical pregnacies		
Per thaw cycle	12 (14.6%)	11 (18.6%)
Per ET	12 (14.8%)	11 (19.2%)
Singletons	8	6
Twins	3	2

* $P < 0.02$.
** $P < 0.01$.

Table II. Outcome After Transfer of Frozen–Thawed Pronuclear (PN) and Multicellular Embryos Obtained from ICSI

	PN	Multicellular
No. cycles resulting in freezing of supernumerary embryos	55	103
No. embryos frozen	380	457
No. frozen–thawed embryo transfer cycles	33	51
No. embryos thawed	197	219
No. embryos surviving	127 (64.4%)*	164 (74.8%)
No. embryo transfer cycles	31	50
No. embryos transferred	104	158
No. embryos transferred (mean ± SD)	3.2 ± 1.43	3.09 ± 1.18
Quality of embryos transferred		
Grades III & IV	47 (46.5%)	77 (48.7%)
Grades I & II	54 (53.5%)	81 (51.2%)
No. positive hCG measurements		
Per thawing cycle	5 (15.1%)	9 (17.6%)
Per embryo transfer	5 (16.1%)	9 (18.0%)
No. preclinical abortions	1	1
No. miscarriages	1	1
No. clinical pregnacies		
Per thaw cycle	4 (12.1%)	8 (15.6%)
Per ET	4 (12.9%)	8 (16.0%)
Singletons	2	7
Twins	1	0

* $P < 0.01$

using pronuclear embryos (14.8% per embryo transfer), whereas there were 13 pregnancies following the use of multicellular embryos (22.8% per embryo transfer). The difference was highly significant ($P < 0.01$).

Eleven clinical pregnancies were obtained following the transfer of embryos frozen at the multicellular stage (19.2% per embryo transfer). This was higher than the clinical pregnancy rate of 14.8% following the transfer of embryos frozen at the pronuclear stage, however, the difference was not significant.

Between August 1994 and July 1997, 837 supernumerary embryos were frozen during 158 ICSI cycles. Of these, 197 pronuclear and 219 cleaved embryos were thawed in 33 and 51 frozen embryo replacement cycles, respectively (Table II). Sixty-four percent of the embryos frozen at the pronuclear stage survived, compared to 74.8% of the embryos frozen at the multicellular stage ($P < 0.01$). The quality of embryos available for transfer in the two groups was similar. A positive pregnancy test was obtained in five women (16.1% per embryo transfer) from embryos frozen at the pronuclear stage, compared to a pregnancy rate of 18% per embryo transfer (nine women) using embryos frozen at a multicellular stage. The clinical pregnancy rates in the two groups were 12.9 and 16.0% per embryo transfer, respectively. These differences were not significant.

When the embryos generated from conventional IVF and subsequently frozen at the pronuclear or multicellular stage were compared with the corresponding embryos generated by ICSI, no difference was found in the survival rate, embryo quality, or pregnancy rate per transfer.

DISCUSSION

The ability to cryopreserve supernumerary embryos and transfer them following an unsuccessful outcome with fresh embryos enhances the overall pregnancy rate per cycle of oocyte retrieval. In the past we have cryopreserved supernumerary embryos at both the pronuclear and the multicellular stages. The objective of this study was to determine which stage of embryo development is compatible with better embryo survival and pregnancy following freezing and thawing. The data clearly show significantly better survival of frozen multicellular embryos obtained from both the IVF and the ICSI procedures. It is possible that since only good-quality multicellular embryos were frozen, this consti-

tutes a selection bias. It is difficult to grade the quality of pronuclear embryos and thus there is a tendency to freeze all supernumerary pronuclear embryos. It is also possible that the higher number of cells in a multicellular embryo may lessen the likelihood of freezing embryos with changes that are detrimental to subsequent survival. Even if one or two of the cells are damaged by the freezing process, other blastomeres are still capable of providing a viable embryo. In embryos generated by the IVF procedure a significantly higher pregnancy rate per embryo transfer (22.8%) was obtained following the transfer of embryos frozen at the multicellular stage compared to those frozen at the pronuclear stage (14.8%). This was despite the fact that fewer embryos (2.7 ± 1.48) were transferred per cycle compared to embryos frozen at the pronuclear stage (3.2 ± 1.76). However, two preclinical abortions occurred in the pregnancies obtained from frozen multicellular embryos, thereby reducing the clinical pregnancy rate per embryo transfer to 19.5%. Thus, the difference in clinical pregnancies between the two groups was not statistically significant. Our results are not in agreement with earlier reports by Demoulin *et al.* (17) and Fugger (18) which indicated better pregnancy rates following the transfer of IVF-generated embryos frozen at the pronuclear stage. However, Demoulin *et al.* admit that the majority (69%) of the multicellular embryos that they transferred were from clomiphene citrate and human menopausal gonadotropin (hMG) stimulation cycles, whereas most (65%) of the pronuclear-stage frozen embryos were derived from gonadotropin releasing hormone agonist and hMG protocols. Their data showed that the latter drug regime resulted in embryos with a significantly higher pregnancy rate per frozen embryo transfer cycle, and this could have led to a biased observation in favor of pronucleate embryos. In contrast, Van den Abbeel *et al.* (19) have reported a higher pregnancy rate with embryos frozen at the multicellular stage.

Analyzing the data from the ICSI-generated supernumerary embryos, once again, significantly better survival (74.8%) was seen in embryos frozen at the multicellular stage compared to embryos frozen at the pronuclear stage (64.4%). Similar numbers of embryos were transferred in the two groups and the overall pregnancy and clinical pregnancy rates in the two groups were not significantly different, though they tended to be higher following the transfer of embryos frozen at the multicellular stage.

Doubts about the ability of ICSI-generated embryos to withstand the freeze–thawing process have been put to rest. Both Al-Hasani *et al.* (20) and Hoover *et al.* (21) have shown that ICSI-generated embryos frozen at the pronuclear stage have survival and pregnancy rates similar to those of conventionally generated embryos. A more recent study (22), using embryos frozen at the multicellular stage, has shown comparable survival rates for ICSI- and conventionally generated embryos, though in this study, the clinical pregnancy rate per transfer was significantly higher for IVF-generated embryos. Our own results indicate that cryopreservation is not detrimental to ICSI-generated embryos and that the survival and pregnancy rates of both pronuclear and multicellular frozen embryos are similar to those of corresponding embryos derived from conventional IVF.

Embryo freezing using 1,2-propanediol is presently the most popular method, with DMSO being used on rare occasions. Our unit has been using 1,2-propanediol exclusively. However, the 1995 study by Van den Abbeel *et. al.* (23) suggests that this may need to be reevaluated. They reported a significantly higher survival and implantation rate per transfer using DMSO compared to 1,2-propanediol. They suggest that 1,2-propanediol is less suitable for freezing multicellular embryos because of the changed permeability characteristics and surface-to-volume ratios of these embryos as opposed to pronuclear embryos. The rapidity with which 1,2-propanediol permeates the cell may cause rehydration during the prefreeze equilibration and this may enhance the chance of intracellular freezing.

Andre *et al.* (24) have shown a higher incidence of preclinical abortions in embryos generated by the ICSI technique compared to those generated by IVF. This has not been our experience. It seems sensible to allow embryo development to continue for 48 hr and avoid freezing embryos at the pronuclear stage. This would give the embryologist a greater choice to select the best embryos for transfer. Additionally, embryo quality could be better judged at the multicellular stage, and only robust embryos likely to withstand the freeze–thaw procedure frozen. This would reduce the total number of freezing cycles, which are both time-consuming and laborious.

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