Comparison of Cryopreservation Outcome Following Intracytoplasmic Sperm Injection and Conventional In Vitro Fertilization

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Purpose: Our purpose was to compare the success rate of transferring frozen-thawed embryos generated from either intracytoplasmic sperm injection (ICSI) or conventional in vitro fertilization (IVF).

Methods: A retrospective review of all frozen-thawed embryo transfer (ET) cycles between January 1995 and April 1997 was performed. There were 83 and 204 transfer cycles of frozen-thawed multicellular embryos generated from conventional IVF (group A) and ICSI (group B), respectively. The survival rate of frozen-thawed embryos and the outcome following ET in both groups were assessed.

Results: The groups did not differ in age (31.7 ± 4.6 and 30.6 ± 6.0 ; mean $\pm$ SD) or number of embryos transferred (3.5 ± 1.1 and 3.8 ± 1.3 for groups A and B, respectively). An acceptable pregnancy rate per ET was achieved in both groups, but the rate was significantly higher ($P = 0.04$) for group A than group B, 32.5 and 20%, respectively. Group A included frozen embryos of a higher quality than those of group B, but the proportion of embryos surviving after thawing was significantly higher for group B than group A (92.5 and 85.6%, respectively; $P = 0.0004$). The abortion rate did not differ between the two groups: 22 and 26.8% for groups A and B, respectively.

Conclusions: Although an overall high pregnancy rate was achieved following frozen-thawed ET, it was lower for cycles in which embryos had been generated from ICSI. This difference may be attributed to a lower prefreezing embryo quality in the ICSI group. Embryos originating from ICSI were not vulnerable to cryopreservation and, when implanted, resulted in a comparable abortion rate to thawed embryos of conventional IVF.

KEY WORDS: embryo cryopreservation; embryo freezing; embryo thawing; in vitro fertilization; intracytoplasmic sperm injection; pregnancy.

INTRODUCTION

The use of gonadotropin-releasing hormone agonist (GnRH-a) in in vitro fertilization (IVF) eliminated the concern of premature ovulation and enabled us to extend the length of ovarian stimulation. As a result, a higher number of follicles could be recruited, yielding an increased number of oocytes obtained during a single pickup. Concomitantly, improvements in embryo culture conditions resulted in an increased number of embryos available for transfer. In addition, to decrease the multiple pregnancy rate that is associated with a poor perinatal outcome, many IVF programs limited the transfer to only two or three embryos (1). These developments led to an increased number of extra embryos that were not used for immediate transfer. The ability to cryopreserve these supernumerary embryos and obtain viable pregnancies after thawing and embryo transfer (ET) served as an excellent solution to the problem of surplus embryos and increased the overall pregnancy rate from one oocyte retrieval attempt (2,3). Cryopreservation of embryos has additional advantages as it may be used in cases in which ovarian hyperstimulation syndrome is anticipated to occur and fresh ET is not recommended (4). In addition, although pregnancy rate for frozen-thawed embryos is somewhat lower than for fresh ET, the expenses for such ET cycles is far below the total cost incurred by a repeat fresh cycle. Taken together, these considerations emphasize the role of cryopreservation as an indispensable tool in any assisted fertilization program.

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During the past few years, intracytoplasmic sperm injection (ICSI) emerged as an effective solution for male-factor infertility resulting in high fertilization rates and an excess of embryos available for cryopreservation. For fresh ET the pregnancy rate for ICSI cycles seems to be comparable to that of conventional IVF attempts (5,6). This rate is somewhat lower for frozen-thawed ET cycles of both ICSI and conventional IVF and is reported to range between 12.9 and 21% (7–13).

Because ICSI may breach the zona pellucida integrity and interfere with the protective mechanisms acting during cryopreservation, it is important to gain information about the safety and efficiency of cryopreservation in these cycles. However, only a small number of studies (7–9) compared the efficacy and safety of cryopreservation of ICSI and conventional IVF embryos in the same clinical set-up.

The present study was undertaken to compare the survival rate and clinical results of frozen-thawed ET following ICSI and conventional IVF during the same period and under similar clinical protocols.

MATERIALS AND METHODS

Patients

Records of all patients who underwent frozen-thawed ET between January 1995 and April 1997 were retrospectively analyzed and divided into two groups. Group A included 62 patients who underwent conventional IVF and subsequently had 83 cycles of frozen-thawed ET. Group B consisted of 133 patients who were treated by ICSI and had 204 frozen-thawed ET. The increased proportion of ICSI cycles represents a shift in the main indication for IVF occurring in the past few years in our IVF unit, in which male infertility comprises over two-thirds of the total number of assisted reproductive treatment cycles performed.

For both conventional IVF and ICSI treatment cycles, patients underwent a similar treatment of ovulation induction that entailed the use of a midluteal desensitizing protocol with GnRH-a D-Trp-6-LHRH (Decapeptyl, Ferring, Malmo, Sweden) followed by human menopausal gonadotropin (hMG) stimulation as reported previously (14). Transvaginal oocyte retrieval was performed 34 to 36 hr after human chorionic gonadotropin (hCG) administration using ultrasound guidance. For ICSI cycles, the procedure was performed as described previously (7), and ET was carried out 48 to 72 hr after ovum pickup. Supernumer-

ary embryos not used for fresh transfer were cryopreserved.

Embryo Freezing and Thawing Protocols

Embryos of both group A and group B were scored before freezing according to the blastomere symmetry, the presence of anucleate fragments, and the extent to which they filled the embryo's content. Four types of embryos were considered: type A embryos had regular blastomeres with no fragmentation; type B embryos had irregularity and asymmetry of blastomeres but no fragmentations; type C embryos had either regular or irregular blastomeres, with $\leq 20\%$ of their surface filled with anucleated fragments; and type D embryos had $\geq 20\%$ and up to 50% of their surface filled with anucleated fragments. Embryos with $> 50\%$ of their surface filled with anucleated fragments were not eligible for freezing or for transfer and were discarded.

Embryos were cryopreserved 48 or 72 hr after ovum pickup (two- to eight-cell stage) according to a protocol described previously (15). Cryopreservation was performed using a slow freezing protocol in a programmed biological freezer (Planer Kryo 10; Planer Products Ltd., Middx, England, PA). Propanediol (1,2 propanediol; Sigma Chemical Co, St. Louis, MO) was used as the cryoprotective supplement of the freezing medium. Embryos were placed in 1.5 M propanediol in Dulbecco's phosphate-buffered saline (dPBS; Sigma Chemical Co., St. Louis, MO, USA) enriched with 20% maternal serum and allowed to equilibrate at room temperature for 15 min. Subsequently, specimens were transferred into cryotubes (Nunc, Roskilde, Denmark) containing 0.25 ml of freezing medium consisting of 1.5 M propanediol and 0.1 M sucrose in dPBS enriched with maternal serum. Cryotubes were then placed in the freezing apparatus and frozen in a predetermined program as follows: the temperature was lowered in the freezer at a rate of $-2^{\circ}\text{C}/\text{min}$ until a temperature of -7°C was reached. Manual seeding was performed after the specimen was held in this temperature for 5 min. Seeding was done by lifting the freezing cane out of the unit and grasping the cryovial at the level of the medium meniscus with a forceps that was frozen in liquid nitrogen. The freezing cane was gently lowered back into the freezer after the visualization of an ice crystal within the vial. The specimen was held for another 5 min at -7°C after seeding before allowing the biological freezer to drop further in temperature. Subsequently, the temperature within the freezer was lowered at a rate of $-0.3^{\circ}\text{C}/\text{min}$ until a temperature of -30°C was reached. At this time cryotubes were

dropped into a liquid nitrogen container and subsequently mounted on prelabeled storage canes always under the level of liquid nitrogen. The labeled storage canes were then transferred to a storage tank and kept at -196°C under liquid nitrogen until thawing.

To thaw the embryos, cryotubes were transferred directly into a water bath at a temperature of 35°C . After complete thawing, embryos were taken through a series of decreasing concentrations of propanediol and 0.2 M sucrose for 5 min in each dilution (1.5, 1, 0.5, and 0.2 M sucrose without propanediol in serum-enriched dPBS) before being washed three times in dPBS and placed in fresh equilibrated, warmed culture medium. After thawing, only embryos surviving the freezing procedure ($\geq 50\%$ of their initial number of blastomeres intact) were transferred within 30 min.

Intrauterine transfer of thawed embryos was scheduled using an artificial endometrial preparation.

Endometrial Preparation and Embryo Transfer

ET was carried out using an artificial endometrial preparation. All patients underwent medical hypophysectomy by using controlled-release GnRH-a (decapeptyl CR; 3.75 mg) given im prior to estrogen (E) stimulation. In regularly menstruating patients, the injection was given on day 21 of the cycle, and in oligomenorrheic patients, from day 1 of the cycle. Fourteen days following the injection, patients were evaluated for a hypogonadotrophic state (E_2 levels < 100 pmol/ml; conversion factor to SI units, 3.761) and E stimulation was then initiated. The standard E replacement was estradiol valerate, 4 mg/day, in two divided doses (Estrofem; Novo Industry A/S, Copenhagen, Denmark) for 7 days. At that time the patient's E_2 level was measured and the endometrial thickness evaluated. The dose of Estrofem was increased to 6 or 8 mg/day whenever the E_2 level was < 1000 pmol/mL or the endometrial thickness < 8 mm. This dose was continued for another 5–10 days as needed and when the endometrial thickness reached at least 8 mm, micronized progesterone (P) tablets (Utrogestan; Besins, Iscovesco, France) were concomitantly given. Utrogestan was introduced vaginally at a dose of 300 mg three times a day, for a total dose of 900 mg/day. Uterine transfer of thawed embryos took place 48 to 72 hr after Utrogestan initiation, depending on the stage at which they were frozen. Embryos of two to four cells and six to eight cells were transferred 48 and 72 hr after Utrogestan initiation, respectively. Patients were instructed to proceed with hormonal treatment and perform serum β -hCG 14 days following ET. If

the pregnancy test was positive, patients were followed with serial ultrasounds to determined fetal viability. Clinical pregnancy was defined as the presence of a gestational sac on transvaginal ultrasound. When pregnancy occurred, the hormonal treatment was continued until 12 weeks of gestation.

Statistical Analysis

Embryo survival rate and laboratory and clinical outcome of the ET cycles were compared between the groups. Data are presented as mean $\pm$ standard deviation (SD). The results were analyzed using the χ^2 test or Fisher's exact test when the expected frequency was less than 5. Student's *t* test was used when needed, and a *P* value of less than 0.05 was considered as statistically significant. The power of these tests was calculated using a two-tailed significance level of 5%.

Statistical analyses were performed using a standard computer program of Microsoft Excel 5 for Windows.

RESULTS

There were 287 cycles of frozen-thawed ETs during the period that was evaluated. Eighty-three transfers (62 patients) were of frozen-thawed embryos that were generated from conventional IVF (group A). The other 204 transfers (133 patients) were of embryos originating from an ICSI procedure (group B). The two groups did not differ in age [31.7 ± 4.6 and 30.6 ± 6 years (mean $\pm$ SD) for groups A and B, respectively]. Twenty-seven pregnancies were achieved in group A and 41 in group B, for a pregnancy rate per ET of 32.5 and 20% in groups A and B, respectively. Although this pregnancy rate differs significantly between the groups ($P = 0.04$), the implantation rate was similar for both group A and group B (10.6 and 7.4%, respectively) (Table I). However, the power of the test was 40% considering the number of embryos used for transfer in both groups. Similarly, although the two groups did not differ in pregnancy rate per patient (Table I), the power of analysis was only 42%. Moreover, it was calculated that the number of patients in each group had to be increased threefold to reach a power of 80%.

The mean number of embryos that was transferred in group A did not differ from that transferred in group B (3.5 ± 1.1 and 3.8 ± 1.3 for groups A and B, respectively).

The survival rate as well as the quality of the frozen embryos in both groups is shown in Table II. Of 835

Table I. Pregnancy and Implantation Rates After Transfer of Frozen-Thawed Embryos Obtained from Conventional In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI)

	Conventional IVF (group A)	ICSI (group B)	P
No. of patients	62	133	
Age	31.7 ± 4.6 ^a	30.6 ± 6.0	NS ^b
No. of frozen-thawed ET cycles	83	204	
No. of clinical pregnancies	27	41	
Singleton	21	27	
Twin	4	10	
Triplet	0	3	
Ectopic	2	1	
Pregnancy/patient (%)	43.5	30.8	NS
Pregnancy/ET (%)	32.5	20	0.04
Implantation rate (%)	10.6	7.4	NS

^a Values are means ± SD.^b Not significant.

embryos that were frozen in group B, 773 (92.5%) survived the procedure and were suitable for transfer. This survival rate was significantly higher ($P = 0.0004$) than in group A, where only 293 (85.6%) of the initially frozen embryos were eventually transferred. When the two groups are compared as to the quality of embryos initially frozen, a significantly higher percentage of embryos graded as having quality A were frozen in group A, while a significantly higher number of embryos graded as D were frozen in group B.

Of 27 clinical pregnancies in group A, 6 (22%) ended in abortions and 2 were ectopic pregnancies necessitating laparoscopic surgery. In group B a similar rate was observed, with 11 abortions (26.8%) and 1 ectopic pregnancy, which was also treated laparoscopically.

When each group was assessed separately (Tables III and IV), comparing cycles in which pregnancy was

achieved to those in which conception did not occur, it seems that conception cycles were associated with an initial cohort of better-quality embryos subjected for freezing. For both groups, the percentage of grade D embryos was significantly higher in cycles in which pregnancy was not achieved compared to those resulting in conception (21.5 versus 6.3% and 24 versus 16% for groups A and B, respectively).

In both groups, the patient's age and the mean number of embryos transferred for successive cycles did not differ from those in cycles in which pregnancy was not achieved (Tables III and IV).

DISCUSSION

The present study demonstrates that multicellular embryos generated from ICSI are not adversely affected by cryopreservation and that an acceptable pregnancy rate of 20% can be achieved after the transfer of such thawed embryos. This pregnancy rate is in agreement with that of 16–21% reported by others for ICSI multicellular embryos (11–13) and is comparable to that reported for the transfer of frozen-thawed embryos originating from conventional IVF (16). However, to assess adequately the potential vulnerability of ICSI-derived embryos to freezing and whether comparable results could be obtained from the transfer of these embryos, one should compare the outcome of ICSI-derived thawed embryos to those of conventional IVF performed during the same period and under identical clinical conditions. Only a small number of studies directly address this question. Al-Hasani *et al.* (8) reported a pregnancy rate of 17% (204 transfers) and 20% (89 transfers) for transfers of frozen-thawed pronuclear-stage embryos resulting from ICSI and conventional IVF, respectively. Van Steirteghem *et al.* (7)

Table II. Embryo Quality Before Freezing and Survival Rate After Thawing of Embryos Obtained from Conventional In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI)

	ET/cycle ^a	No. of embryos by grade of quality before freezing ^b				No. of embryos frozen	No. of embryos transferred
		A	B	C	D		
Conventional IVF (group A)	3.5 ± 1.1	36 (10.5)	126 (37)	123 (36)	57 (16.5)	342 (100)	293 (85.6)
ICSI (group B)	3.8 ± 1.3	58 (7)	355 (42.5)	225 (27)	187 (22.5)	835 (100)	773 (92.5)
P	NS ^c	0.05	NS	0.003	0.03		0.0004

^a Values are means ± SD.^b Values in parentheses are percentages.^c Not significant.

Table III. Embryo Quality Before Freezing and Survival Rate After Thawing of Embryos Obtained from Conventional In Vitro Fertilization (IVF) in Successive and Failed Frozen–Thawed Embryo Transfer Cycles

	No. of cycles	Age ^a	ET/cycle ^a	No. of embryos by grade of quality before freezing ^b				No. of embryos frozen	No. of embryos transferred
				A	B	C	D		
Cycles resulting in conception	27	32.2 ± 4.7	3.6 ± 1.0	16 (14.4)	45 (40.5)	43 (38.7)	7 (6.3)	111 (100)	98 (88)
Failed cycles	56	31.4 ± 4.5	3.5 ± 1.2	20 (8.4)	81 (35)	80 (35)	50 (21.5)	231 (100)	195 (84.5)
<i>P</i>		NS ^c	NS	NS	NS	NS	<0.001		NS

^a Values are means ± SD.^b Values in parentheses are percentages.^c Not significant.

reported a comparable clinical pregnancy rate of 12.9 and 14.6% for transfers of multicellular frozen–thawed embryos derived from either ICSI (101 cycles) or conventional IVF (253 cycles), respectively. This rate is lower than that reported in our study and may stem from differences in the freezing protocol and in endometrial preparation for ET. The former study used dimethyl sulfoxide as cryoprotectant, while we used propanediol. In addition, ET was performed in natural or clomiphene citrate-stimulated cycles, whereas we used artificially prepared cycles. For natural or artificially manipulated cycles, the pregnancy rate for frozen–thawed ET is comparable. However, the use of clomiphene citrate may adversely affect the endometrium interfering with implantation. We, therefore, refrain from using clomiphene citrate for endometrial preparation in a frozen–thawed ET cycle. Although previous studies (7,8) have not indicated what the power of their analysis was, our results suggest that larger samples than that being reported have to be assessed to reach a firm and clinically significant conclusion.

The clinical pregnancy rate for transfers of embryos originating from conventional IVF (32.5%) was significantly higher than that for ICSI-derived embryos (20%). However, from the clinical point of view and taking into account the size of the study groups, such a difference may not be of clinical significance. Nevertheless, we have attempted to look into the reasons leading to the difference found between the two groups. To do so, we considered factors that affect the outcome of frozen–thawed ET cycles. These factors include endometrial preparation, patient age, embryo stage, and embryo quality before and after thawing (10). In the present study, factors such as freezing and thawing protocols and endometrial preparation for ET were identical in all cycles. In addition, groups A and B did not differ in age or mean number of embryo transferred per cycle. Thus, the differences in pregnancy rates found between the groups could not be attributed to these factors. On the other hand, a higher proportion of embryos of good quality before freezing was found in conventional IVF cycles compared to ICSI cycles.

Table IV. Embryo Quality Before Freezing and Survival Rate After Thawing of Embryos Obtained from Intracytoplasmic Sperm Injection (ICSI) in Successive and Failed Frozen–Thawed Embryo Transfer Cycles

	No. of cycles	Age ^a	ET/cycle ^a	No. of embryos by grade of quality before freezing ^b				No. of embryos frozen	No. of embryos transferred
				A	B	C	D		
Cycles resulting in conception	41	29.8 5.3	4.0 ± 1.2	15 (8)	86 (46)	57 (30)	30 (16)	188 (100)	181 (96)
Failed cycles	163	30.8 6.0	3.7 ± 1.3	45 (7)	271 (42)	170 (26)	161 (25)	647 (100)	582 (90)
<i>P</i>		NS ^c	NS	NS	NS	NS	0.02		0.01

^a Values are means ± SD.^b Values in parentheses are percentages.^c Not significant.

Similarly, a significantly higher percentage of embryos graded as D was frozen in the ICSI group. Therefore, it seems that this variation in embryo quality is the main factor contributing to the higher success rate found in IVF-derived frozen-thawed ETs. Because for any fresh ET our policy is to use the embryos of best quality, the results suggest that we were left for cryopreservation with embryos of lower quality in the ICSI group compared to those in the IVF group. This difference may result from a less optimal development of ICSI-derived embryos in our laboratory. However, using embryos of the best quality, the pregnancy rate for fresh ET cycles in our clinic is similar for ICSI or conventional IVF patients: 22 and 22.6% per ET, respectively.

The significance of embryo quality for success rate was further emphasized when each group of patients was assessed separately. In each group, when treatment cycles were divided as successful and failed cycles, the subgroups did not differ by patients' age or number of embryos transferred. On the other hand, cycles resulting in pregnancy were associated with the freezing of a significantly lower percentage of grade D embryos. Our results, therefore, confirm previously reported data indicating that prefreezing embryo quality is a crucial factor in determining postthaw survival and implantation rates. Camus *et al.* (17) reported an implantation rate of 21% per ET for excellent-grade embryos with regular blastomeres without any fragmentations. The implantation rate decreased to 14.5% for embryos with up to 20% fragmentations and no pregnancies were achieved after the transfer of embryos with >20% of their content fragmented.

Although postthaw embryos were not graded again to assess the effect of freezing, a high survival rate (>50% of the initial number of blastomeres intact after thawing) of 85.6 and 92.5% was found for groups A and B, respectively. This rate is higher than that of 53% reported by Van Steirteghem *et al.* (7) for multicellular embryo freezing. However, as mentioned previously, the two studies differ in their methodology of freezing and thawing, which may account for such differences. Al-Hasani *et al.* (8) reported a survival rate of 78 and 76.5% for zygotes derived from conventional IVF and ICSI, respectively. A higher survival rate for freezing of pronuclear-stage zygotes, 94.8 and 93.2% for IVF- and ICSI-derived zygotes, was recently reported by Hoover *et al.* (9). These reported rates as well as our results suggest that cryopreservation is not detrimental to ICSI-derived embryos of any stage, yielding a survival rate comparable to that of embryos originating from conventional IVF.

A previous report (7) showed that the preclinical wastage of pregnancies is significantly higher for cryopreserved ICSI embryos compared to conventional IVF: 40.9 and 27%, respectively. Our experience could not substantiate this observation, as our preclinical abortion rates for the same groups were only 10 and 9% (data not shown). The abortion rate of clinical pregnancies was 22% for frozen-thawed conventional IVF embryos and 26.8% for ICSI-derived embryos. These rates were comparable and corroborate previous studies demonstrating that the abortion rates for frozen-thawed ET cycles ranged between 20 and 23% for conventional IVF embryos and 18 and 25% for ICSI-derived embryos (8,9,13).

In conclusion, our study strongly suggests that cryopreservation has no adverse affect on ICSI-derived embryos and that the option of freezing supernumerary embryos can be a safety precaution suggested to patients undergoing ICSI procedures.

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