

Clinical outcome of cryopreserved human pronuclear stage embryos resulting from intracytoplasmic sperm injection*

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Objective: To compare the survival rate and pregnancy rate (PR) of embryos from intracytoplasmic sperm injection (ICSI) or conventional IVF, which were cryopreserved at the pronuclear stage in cycles where fresh transfer was deferred.

Design: Comparative observational study.

Setting: University-associated IVF center.

Patient(s): Ninety-nine patients who deferred ET and had all their embryos cryopreserved at the pronuclear stage after 153 oocyte retrievals. Thirty-nine patients had their oocytes inseminated by ICSI and 60 patients had conventional IVF insemination.

Intervention(s): All embryos were frozen-thawed at the two pronuclear stage and allowed to cleave for 2 days before transfer.

Main Outcome Measure(s): Survival rate (morphologically intact after thaw), cleavage rate (cleaved by time of transfer), and the clinical PR after frozen ET.

Result(s): In the ICSI group, 205 embryos were thawed for use in 57 frozen ETs; in the IVF group, there were 527 embryos thawed for use in 149 frozen ETs. There was no significant difference in any of the outcome measures by insemination method: survival rates (ICSI, 93.2%; IVF, 94.8%); cleavage rates (ICSI, 95.2%; IVF, 94.7%), and clinical PR (ICSI, 14.0%; IVF, 17.4%).

Conclusion(s): Pronuclear embryos resulting from ICSI can be cryopreserved successfully, thawed, and the survival rate and PR are comparable to conventional IVF. (Fertil Steril® 1997;67:621-4. © 1997 by American Society for Reproductive Medicine.)

Key Words: Micromanipulation, ICSI, cryopreservation, IVF, pregnancy

The development of intracytoplasmic sperm injection (ICSI) has allowed many couples to achieve fertilization through IVF where they would have experienced no or little fertilization using standard IVF insemination methods (1). There have been many publications demonstrating high fertilization and pregnancy rates (PRs) with ICSI after fresh ETs at various centers around the world (2). However, there

is little information regarding the effect of cryopreservation of these embryos.

One center reported the survival rate and PR of frozen-thawed multicell human embryos using a multistep cryopreservation protocol. They reported a 53% survival rate and an overall PR of 21.8% per transfer. The clinical PR was 12.9% and the delivery rate was 5.9%. The abortion rate was 40.9% (3). More recently, Al-Hasani et al. (4) have reported that supernumerary embryos frozen at the pronuclear stage have survival rates and PRs similar to embryos from conventional embryos.

The current study compares the survival rates and PRs from pronuclear embryos resulted from ICSI or conventional insemination in cases in which the

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patients have elected to defer ET after retrieval either for medical or personal reasons and have had all their embryos cryopreserved at the pronuclear stage using a one-step cryopreservation method.

MATERIALS AND METHODS

It is the policy of our IVF center to offer the patient the option of cryopreserving all the embryos resulting from either conventional IVF or ICSI at the pronuclear stage and to defer ET if either the patient is at risk of ovarian hyperstimulation ($E_2 > 4,000$ pg/mL on day of hCG [conversion factor to SI unit, 3.671] and/or >30 oocytes retrieved); there is inadequate endometrial development (lining < 10 mm, echo pattern is homogeneous hyperechogenic); or if special circumstances arise, such as exposure to rubella, job commitment, or personal emergency, that require a delay in transfer. Ovarian stimulation protocols for the oocyte retrieval for both ICSI and conventional IVF included luteal phase leuprolide acetate (LA) followed by hMG, a short-flare (follicular phase LA days 2 to 4 followed by LA and a mixture of hMG and pure FSH) or clomiphene citrate (CC) and hMG. When the size of two lead follicles was ≥ 20 mm in diameter, 10,000 IU of hCG was given. Oocyte retrieval was carried out by transvaginal ultrasound-guided puncture of follicles 36 hours after hCG.

Insemination was performed by ICSI if the sperm count and/or motility was substandard for conventional IVF insemination, there were antisperm antibodies, or a history of poor fertilization with conventional IVF. One of the patients having ICSI used frozen sperm from a previous epididymal aspiration, and one had a testicular biopsy performed to obtain sperm.

If needed, the ICSI technique was performed on all mature oocytes (1). For each oocyte, a motile sperm was immobilized with an injection pipet in a drop of polyvinylpyrrolidone (Scandinavian IVF Science AB, Goteberg, Sweden) and then injected into the ooplasm. The injected oocytes were placed in human tubal fluid (HTF; Irvine Scientific, Irvine CA) and 10% synthetic serum substitute (Irvine Scientific) and incubated for ≥ 16 hours before evaluation for signs of fertilization (two pronuclei).

For standard insemination, semen was prepared using the Percoll gradient technique. Oocytes were inseminated with 50,000 sperm per oocyte and incubated for ≥ 16 hours before evaluation for signs of fertilization.

Pronuclear embryos were cryopreserved using a one-step addition of cryoprotectant. The embryos

were equilibrated in 1.5 M 1,2 propanediol and frozen in 0.25 mL straws in an alcohol bath controlled rate freezer. The straws were preloaded with 0.12 mL of 1 M sucrose followed by 1 cm of air and a column of 1,2 propanediol containing the embryos. Seeding was performed at -6°C . The straws were cooled at -0.4°C per minute down to -40°C and then plunged into liquid nitrogen (5).

Thawing the embryos involved a one-step dilution of the cryoprotectant (5). The straws were warmed to room temperature in air for 2 minutes and the columns were shaken down. The straws were then inverted in 37°C water bath for 3 minutes. The straws were inverted again at room temperature for 1 minute before the contents were expelled into a petri dish. The thawed embryos were placed in a dish of phosphate-buffered saline for 10 minutes before being rinsed and placed in an organ culture dish containing 1 mL of warmed gassed HTF + 10% synthetic serum substitute covered with mineral oil. The embryos were allowed to cleave for another 48 hours before transfer.

Clinical management of frozen ET included either natural cycles, hMG cycles, estrogen supplementation in the form of oral E_2 (Estrace, Mead Johnson, Princeton, NJ) or E_2 valerate IM, or down-regulation with LA followed by estrogen supplementation. Natural cycles were monitored with serum levels of E_2 and P and ultrasound until the natural LH surge. Estrogen supplementation started on day 2 of the cycle. In down-regulated cycles, LA was started on day 21 of cycle, and estrogen therapy was initiated 10 days later.

The outcomes of all frozen ET cycles using embryos from retrieval cycles in which all embryos were cryopreserved at the pronuclear stage were compared by insemination method (ICSI versus conventional IVF) using χ^2 analysis. The main outcome measures were survival rate (i.e., percent of embryos that were morphologically intact after thawing), implantation rate (number of embryos implanted per embryos transferred), and clinical PR (sonographic evidence of gestational sac). All statistical tests were done using a P value of 0.05.

RESULTS

There were 153 retrieval cycles in which 99 patients elected to cryopreserve all of the embryos at the pronuclear stage and defer ET, including 43 ICSI cycles (39 patients) and 110 conventional IVF cycles (60 patients). A total of 1742 pronuclear stage embryos were cryopreserved (1,400 conventional IVF and 342 ICSI).

In the ICSI group, the patients ranged in age from 23 to 43 years. Twenty-two patients underwent ICSI for male factor only and 17 had male and female factors. Ovarian stimulations in the 43 oocyte retrieval cycles were LA and hMG in 23 cycles (53.5%), short-flare in 19 cycles (44.2%), and CC and hMG in 1 cycle (2.3%). Eight hundred ninety-five oocytes were retrieved, of which 695 were mature and were inseminated via ICSI. The number of mature oocytes retrieved per patient ranged from 5 to 38, with a mean \pm SD of 16.2 ± 7.7 and a median of 8. Three hundred forty-two oocytes were fertilized (49.2%) and the number of oocytes fertilized per patient ranged from 1 to 18, with a median of 8, a mean of 7.9 ± 4.0 . Sixty-six (9.4%) oocytes were damaged during the ICSI procedure.

In the IVF group, the age of the patients ranged from 23 to 45 years. Five patients underwent IVF for male factor, 89 for female factors, 11 for both male and female factors, and 5 for unexplained infertility. There were 69 (62.7%) LA and hMG stimulations, 25 (22.7%) short-flare stimulations, and 16 (14.5%) CC and hMG stimulations. The number of oocytes retrieved per patient ranged from 2 to 51, with a mean of 19.2 ± 11.3 and a median of 18. Of 2,118 oocytes retrieved, 2,101 were inseminated and 1,400 were fertilized (66.6%). The fertilization rate per patient ranged from 21% to 100%, with a median of 67% and a mean of $66.3\% \pm 19.8\%$.

The reason for freezing all embryos and deferring ET were similar in the two groups. In the ICSI group, 74.4% of patients opted to freeze their embryos because of risk of hyperstimulation, 13.9% for inadequate endometrial development, 4.6% for elevated P levels, and 7.0% for personal choice. In the IVF group, the percentages were 65.4%, 20.0%, 5.4%, and 9.1%, respectively.

To date, 761 embryos have been thawed for use in 206 frozen ETs. Of the 205 thawed in the ICSI group, 191 (93.2%) were morphologically intact after thawing, 81.7% cleaved after 24 hours in media, and 95.2% cleaved after 48 hours in media. Of 556 embryos thawed in the IVF group, 527 (94.8%) were morphologically intact after thawing, 88.2% cleaved after 24 hours, and 94.7% cleaved after 48 hours. There were no statistical differences in the survival or cleavage rates by type of insemination.

In the 57 frozen ETs in the ICSI group, a total of 190 embryos were transferred, 8 of which were not cleaved but transferred at patients' request for moral or religious reasons. Ten pregnancies resulted, two were chemical, eight were clinical (six singletons, one twin, and one triplet). Of the eight clinical pregnancies, two ended in spontaneous abortion and six are

Table 1 Results of Cryopreservation of Pronuclear Stage Embryos

	ICSI	IVF
Retrieval cycles	43	110
No. of embryos cryopreserved at pronuclear stage	342	1,400
No. thawed	205	556
No. morphologically intact*	191 (93.2)†	527 (94.8)
No. of frozen ET	57	149
Implantation rate* (%)	5.8	6.7
Clinical pregnancies*	8 (14.0)	26 (17.4)
Ongoing-delivered*	6 (10.5)	10 (13.4)
Spontaneous abortions	2 (25.0)	6 (23.1)

* Not significantly different between groups.

† Values in parentheses are percentages.

ongoing or delivered. In the IVF group, a total of 522 embryos were transferred, 23 of which had not cleaved at time of transfer. As a result of the 149 frozen ETs in the IVF group, there were 32 pregnancies, 2 were chemical, 4 were ectopic, and 26 were clinical (19 singleton, 5 twins, and 2 triplets). Of the 26 clinical pregnancies, 20 are ongoing or delivered and 6 ended in spontaneous abortion. The implantation rates were 5.8% for ICSI, 6.7% for IVF ($P =$ not significant; χ^2). A summary of the results of cryopreservation are presented in Table 1.

DISCUSSION

Cryopreservation of pronuclear embryos has been shown to be an effective method for the utilization of supernumerary embryos. It also has been shown to be useful in the cases in which ET has to be deferred either for medical or personal reasons. These data demonstrate that the cryopreservation of pronuclear embryos resulting from ICSI using the simplified freeze-thaw protocol yielded comparable survival and PRs to similar embryos cryopreserved after conventional IVF.

These results are in agreement with those reported by other centers using supernumerary pronuclear embryos (4) and supernumerary multicell embryos (3). However, this is the first report of the results of cryopreservation in cases in which ET was deferred and all embryos are cryopreserved for transfer in an unstimulated or mildly stimulated cycle.

These data further demonstrate that this simplified cryopreservation-thawing technique does not have an adverse effect on embryos resulting from ICSI. Therefore, embryos can be cryopreserved safely for future transfer and the patient need not undergo another expensive and invasive oocyte re-

trieval. Although the PRs obtained from all three studies are comparable, the survival rates differ, with our simplified technique having the highest survival rates. Further randomized studies should be undertaken to determine the cryopreservation-thawing technique that is most effective.

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