

Transfer of refrozen twice-thawed embryos do not decrease the implantation rate

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Summary

Purpose: To assess the ability of twice frozen/thawed multi-cell embryos to implant in the human uterus.

Method: Fourteen frozen embryo transfer (ET) cycles, in which at least one twice-frozen embryo was thawed for transfer, were matched to frozen ET cycles in which no twice-frozen embryos were thawed. The number of embryos thawed at the pronuclear stage and at the multi-cell stage were matched.

Results: Multi-cell embryos frozen once had a 76.3% survival rate after thaw and those frozen twice had a 74.0% survival rate. For frozen ET cycles that had no twice-frozen embryos, the viable pregnancy and implantation rates were 58.3% and 29.8%, respectively. The corresponding rates for cycles involving at least one twice-frozen embryo were 50.0% and 25.5%, respectively.

Conclusion: The inclusion of twice-frozen embryos in the embryo pool did not reduce the implantation rate.

Key words: Cryopreservation; Embryo deselection; Refreezing.

Introduction

Most in vitro fertilization (IVF) centers employ some type of embryo selection process to transfer the best embryos the first time. Ideally, the best embryos should have the most blastomeres with the least fragmentation. Those embryos not transferred on the retrieval cycle are generally cryopreserved.

The same type of selection process can be used for frozen embryo transfer (ET). The question arises as to the value of refreezing an already once-frozen, once-thawed embryo. Anecdotal case reports have been published of human pregnancies that have been achieved following frozen ET of refrozen twice-thawed embryos [1-3].

The study presented herein attempted to determine if the chance of implantation was as likely with refrozen, twice-thawed embryos versus once-frozen, once-thawed ones. Since transfer of all refrozen twice-thawed embryos is rare, the study tried to answer this question by comparing implantation rates when all once-frozen, once-thawed embryos were transferred versus situations when at least one twice-frozen, twice-thawed embryo was used.

Materials and Methods

Fourteen patients undergoing frozen ET elected to use embryos that had been refrozen following a previous frozen ET. These patients comprised the study group. In the previous frozen ET cycle, the patient had more embryos thawed than she intended to transfer. Selection of embryos for transfer was based on number of blastomeres and degree of fragmentation.

Each patient was matched retrospectively to another patient in the database who had the same frozen embryo pool, i.e.,

same number of embryos thawed that had been frozen at the pronuclear and multi-cell stage. For example, if a patient had five embryos thawed, three frozen at the pronuclear stage and two frozen at the multi-cell stage, she was matched to a couple who also had five embryos thawed, three pronuclear and two multi-cell. The matched patients made up the control group.

Source of Frozen Embryos

Embryos used for transfer were either frozen at the 2PN stage or multi-cell stage. The 2PN embryos may have been from IVF cycles where twice as many embryos as intended to transfer were allowed to reach multi-cell stage and the rest of the embryos were cryopreserved at the 2PN stage. Alternatively, in some individuals considered at risk for developing ovarian hyperstimulation syndrome, or those whose endometrium on the day of hCG injection was considered inappropriate by ultrasound criteria, all embryos were cryopreserved at the 2PN stage.

The source of multi-cell embryos used for frozen ET could be either the remaining multi-cell embryos not chosen for fresh ET, or could be the multi-cell embryos that developed from thawing 2PN embryos that were not selected for the first frozen ET.

Freezing Procedure

The embryos were frozen using a simplified method in which a slow cooling program is started at the seeding temperature of -6°C in an alcohol-bath controlled-rate freezer. 1,2 propanediol was used as the cryoprotectant [4].

The thawing procedure for refrozen embryos was the same as that used for once-frozen embryos. They were thawed using a one-step dilution of the cryoprotectant [4]. The straws were left in air for two minutes, after being removed from the liquid nitrogen, to warm to room temperature. The columns were shaken down, and the straws were inverted in a 37°C water bath for three minutes. The straws were inverted again in room temperature water for one minute before the contents were expelled.

Revised manuscript accepted for publication February 18, 2001

led into a petri dish. The thawed embryos were placed in a dish of phosphate-buffered saline (PBS) with 0.3% BSA for ten minutes and then placed in an organ culture dish containing one milliliter of HTF +10% synthetic serum substitute covered with mineral oil. The embryos were placed in this dish immediately after it was removed from an incubator at 37°C with 5% CO₂. They were subsequently placed in an incubator to remain in culture.

The embryos included in this study were refrozen at the multi-cell stage. After being thawed the second time, they were kept in culture anywhere from two to 24 hours before being transferred, depending on what stage they were refrozen. If they were refrozen at two or three cells they were kept in culture for 24 hours after thawing, before being transferred. Embryos that were refrozen with four cells or more were thawed two to three hours before the transfer.

Decision on Which Embryos to Transfer

Whenever once-frozen never-thawed 2PN embryos were available, they were chosen for transfer. If twice as many of these 2PN embryos as the number intended to transfer were not available, then frozen multi-cell embryos were also thawed to make up the balance. These multi-cell embryos would either be those rejected during fresh ET or those rejected from frozen ET using thawed 2PN embryos. Once-frozen, once-thawed multi-cell embryos were used in preference to twice-frozen, twice-thawed multi-cell embryos. Assisted embryo hatching using acidic Tyrode's solution was performed prior to the transfer of the three-day-old frozen/thawed embryos [5].

Outcome Measures and Statistical Analysis:

The main outcome measures were survival rate following thaw and pregnancy and implantation rates. An embryo thawed at the pronuclear stage was said to survive if it remained intact after the thaw. A multi-cell embryo was said to survive, if at least 50% of the cells were intact after the thaw. A clinical pregnancy was defined as the presence of a gestational sac in the uterus. A viable pregnancy was defined as a pregnancy that was ongoing at the end of the first trimester. Implantation rates were defined as the number of gestational sacs per embryo transferred. Rates were compared by groups using chi-square analysis. A p-value of .05 was used.

Results

The average age of the 14 patients who used twice-frozen embryos was 36.2±7.2 years, as compared to 33.4±4.4 years for their matched controls. Seventy-nine embryos were thawed in the study group, 77 in the control group; 46.7% of the embryos were thawed at the pronuclear stage in the control group and 53.3% were thawed at the multi-cell stage. In the study group, 43.0% were thawed at the pronuclear stage, 22.8% were once-frozen embryos thawed at the multi-cell and 34.2% were twice-frozen embryos thawed at the multi-cell stage.

All of the embryos that were frozen twice were originally frozen at the pronuclear stage. Following the initial thaw and ET, supernumerary embryos were refrozen at the multi-cell stage; two were refrozen at the 2-cell stage; two at the 3-cell stage, 13 at the 4-cell stage, three at the 5-cell stage, and seven at the 6-cell stage.

Of the embryos thawed at the pronuclear stage 95.7%

survived. The survival rates for the embryos were the same in the group with some twice-frozen vs the group with only once-frozen embryos (Table 1). The survival rates for once frozen and twice frozen multi-cell embryos did not differ (76.3% vs 74.0%).

In two cycles, none of the refrozen embryos thawed were used for transfer. These cycles and their match were therefore excluded from the analysis of the transfer outcome data. The average cell stage of the embryos transferred in the study group was 4.7±1.7 cells as compared to 5.6±2.4 cells in the control group ($p = .045$). On average, 3.9±1.2 embryos were transferred in the study group and 3.9±1.9 in the control group ($p = \text{NS}$). The viable pregnancy rate was 58.3% in the control group and 50.0% in the study group ($p = \text{NS}$, Table 2). The implantation rate was 29.8% for the controls and 25.5% for the study group.

There was only one cycle in which all the embryos transferred were frozen twice. This cycle resulted in a viable pregnancy.

Table 1. — Comparison of Survival Data.

	Control Group (n=14 cycles)	Study Group (n=14 cycles)
Embryos Thawed	77	79
Pronuclear	36 (46.7%)	34 (43.0%)
Multi-cell (1) ^a	41 (53.3%)	18 (22.8%)
Multi-cell (2) ^b		27 (34.2%)
Survival Rates		
Pronuclear ^c	94.4% (34/36)	97.0% (33/34)
Multi-cell (1) ^d	80.5% (33/41)	66.7% (12/18)
Multi-cell (2) ^d		74.0% (20/27)

^aEmbryo frozen once at the multi-cell stage; ^bEmbryo refrozen; ^cEmbryo intact after thaw; ^dAt least 50% of cell intact after thaw.

Table 2. — Comparison of Outcome Measures.

	Control Group (n=12 cycles)	Study Group (n=12 cycles)
Positive pregnancy test	7 (58.3%)	8 (66.7%)
Clinical pregnancy	7 (58.3%)	7 (58.3%)
Viable pregnancy rate	7 (58.3%)	6 (50.0%)
Implantation rate	29.8% (14/47)	25.5% (12/47)

Discussion

Multi-cell embryos do not have the same survival rate as 2PN embryos, but twice-frozen, twice-thawed embryos fare as well as once-frozen thawed embryos. Part of the explanation for lower survival may be a selection of the worst quality multi-cell embryos for freezing.

To evaluate whether a twice-frozen-thawed embryo is as hearty as a once-frozen-thawed embryo or whether its chance of implanting was very poor we thought the best method for evaluation would be comparison of implantation rates rather than pregnancy rates of transfers of all once-frozen-thawed vs those including twice-frozen-thawed embryos since it is rare to have a transfer of

exclusively twice-frozen twice-thawed embryos. We reasoned that if the implantation rates were similar there would be no question that freezing and thawing twice does not weaken the embryo. If there was a decreased implantation found with twice-frozen-thawed embryos, the if they were almost non-viable, the implantation rate should be reduced by the percentage of the total number of embryos transferred that were represented by the twice-frozen twice-thawed ones. Implantation rates between the two extremes would suggest some efficacy, but reduced, in twice-frozen twice-thawed embryos.

We matched the study group to a control group with not only the same number of embryos thawed but the same stage (i.e., 2PN vs multi-cell). As it turned out the number of embryos transferred were identical in the study and control groups. Though two cases from each group were dropped because the twice-frozen twice-thawed embryos (n=2) were not used for transfer, they did in fact survive the thaw but the preference was to always use only once-frozen-thawed embryos if they were sufficient to provide the requested number of embryos for transfer.

The fact that there were statistically more blastomeres in the control group than in the study group could either be the result of the fact that these twice-frozen twice-thawed embryos were also twice deselected vs one time deselection for the control group, or possibly twice freezing and thawing may damage some blastomeres. However, the lower number of blastomeres did not seem to negatively influence the implantation rates.

One confounding variable could have been the difference in ages of the groups since that was not a matching variable. However, as it turned out, the control group was younger which should have favored a higher implantation rate for the control group.

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