Cryopreservation of blastocysts is the most feasible strategy in good responder patients

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Objective: To assess on which day to cryopreserve and transfer thawed embryos in good-responder patients by comparing the cycle outcomes of day 3 transfers vs blastocysts formed through extended culture before or after cryopreservation.

Design: Retrospective clinical study.

Setting: Private IVF center.

Patient(s): Frozen-thawed cycles (n = 2,531) who had ETs at day 3, 5, and 6 and post-thawed extended culture of day 3 until day 5 or 6.

Intervention(s): None.

Main Outcome Measure(s): Primary outcomes were implantation and delivery rates. Secondary outcomes were clinical pregnancy and miscarriage rates.

Result(s): In thawing cycles, embryos developing to blastocysts on day 5 through extended culture before or after cryopreservation yielded higher rates of implantation (51.1% and 51.3%, respectively), clinical pregnancy (69.9% and 62.2%, respectively), and delivery per thawing cycle (56.7% and 51%, respectively) accompanied by lower miscarriage rates (15.2%, 16.4%, respectively) compared with day 3 transfers (28.3%, 55.3%, 42.5%, 20.1%, respectively). Late-developing embryos formed before or after cryopreservation resulted in compromised implantation (44.7% and 44.2%, respectively), clinical pregnancy (59.9% and 45.9%, respectively), delivery per thawing cycle (42.8% and 32.4%, respectively) and higher miscarriage rates (25.7% and 23.5%, respectively) than day 5 embryos.

Conclusion(s): The feasible strategy in good responder patients appears to be the cryopreservation of blastocysts in the fresh cycle. Retardation in development results in a compromised outcome because of reduced inherent capacity of embryos. (Fertil Steril® 2011;96:1121–5. ©2011 by American Society for Reproductive Medicine.)

Key Words: Blastocysts, frozen embryo transfer, late-developing embryo

Cryopreservation of embryos is essential in many situations during assisted reproduction treatments but particularly in good-responder patients where supernumerary embryos are obtained. High cumulative pregnancy and low multiple gestation rates can be achieved through the establishment of a successful cryopreservation program (1) minimizing risks associated with the infertility treatment (2).

Cryopreservation of embryos requires considerable space in the laboratory and a demand in the work load of embryologists. Furthermore, expectations of the couple are usually high during a thawing cycle as they cover the cost of cryopreservation as well as being prepared for success emotionally because of a more receptive endometrium compared with an induced cycle (3). Therefore, feasible strategies are needed to depict embryos to cryopreserve when they are developmentally most competent and least vulnerable. Although several studies that assessed frozen embryo transfer (FET) cycles showed improved success rates with transfer of blastocysts compared with cleavage-stage (4–9), the outcome of normal- and late-developing blastocyst transfers formed before or after cryopreservation was conflicting (9, 10). Because of a lack of conclusive data on the eventual superiority of different freezing and transfer strategies, cryopreservation of both cleavage-stage embryos and blastocysts is a common practice.

The aim of the present study was to assess the outcomes of thawed day 3, day 5, or day 6 transfers and post-thawed extended culture of day 3 embryos until day 6. These results are expected to contribute to increasing the overall efficiency of cryopreservation programs.

MATERIALS AND METHODS

Patient Population and Preparation

Frozen ET cycles performed from 2004 to 2009 were retrospectively evaluated. During these years, the cryopreservation and transfer strategies have gradually shifted from cleavage to blastocyst stage (the proportion of blastocyst transfers in total thawing cycles increased from 5% in 2004 to 47% in 2009). The patient preparation protocols and laboratory procedures remained similar over this period. The indications for blastocyst culture were [1] two or more previous failed IVF attempts after transfer of cleavage-stage embryos and/or [2] four or more good-quality (as described below) embryos at day 3.

The criterion for cryopreservation was embryo morphology. Day 3 embryos were cryopreserved when they displayed 6–10 blastomeres and <20% difference between cell volumes with a maximum of 20% fragmentation. Blastocysts with a score of ≥2 according to Gardner et al. (11) at days 5 and 6 were chosen for cryopreservation. Blastocysts displaying inner cell mass and/or trophoectoderm with very few cells (scored as C according to Gardner scoring) have not been cryopreserved.

All frozen embryos were from nondonor cycles obtained from good-responder patients who had six or more follicles on ultrasound larger than 13 mm on the day of hCG administration. Embryos obtained from poor

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responders had been frozen and thaw-transferred on day 2 (12) and have been excluded from analysis. Cycles with preimplantation genetic diagnosis were also not assessed. As the procedures associated with the present study involved routine clinical and laboratory treatments, institutional review board approval was not required.

Thawed embryos were transferred in hormone replacement cycles. Preparation of the endometrium was initiated in the first or the second day of menstruation. After 12 days of E2 administration as a transdermal therapeutic system (Estraderm TTS 100; Novartis) or oral form (Estrafen 2 mg; Novo Nordisk), ultrasonography was performed to determine endometrial status (regular three layers and ≥ 7 mm). The level of E2 and P in blood was examined. If appropriate, daily IM injections of P in oil (Progynox, 50 mg; Farmako) or 8% P vaginal gel (Crinone, Serono, Turkey) was administered until the pregnancy test. If the level of E2 and/or endometrium quality was not appropriate, administration of E2 was extended until proper clinical and hormonal parameters were established.

The synchronization of the embryo and the endometrium was established by administration of P when the state of the endometrium, the blood levels of E2 and P was appropriate as described above. The P administration day was accepted as day zero = oocyte retrieval day in a fresh cycle. Hence, for example, transfer of thawed day 3 embryos corresponded to the fourth day of P administration.

Cyropreservation-Transfer Strategies
The study population was classified into groups, and patient demographics, embryo parameters, and cycle outcomes were analyzed: group 1 [3-3], thawed day 3 embryos transferred to endometrium synchronized to day 3; group 2 [5-5], thawed day 5 blastocysts transferred to endometrium synchronized to day 5; group 3 [6-5]: day 6 blastocysts transferred to endometrium synchronized to day 5; and thawed day 3 embryos exposed to prolonged culture either to day 5 (group 4 [3-5]) or 6 (group 5 [3-6]) until they displayed a score of ≥2 (11) before they were transferred to endometrium synchronized to day 5 or 6, respectively.

Embryo Cyropreservation and Thawing
All embryos were cryopreserved in 1.8-mL cryovials (Nunc, CryoTube) in a programmable controlled-rate freezing machine (Planar Series III Kyro 10; T.S. Scientific). A maximum of three cleavage-stage embryos and two blastocysts were cryopreserved in each vial according to patient and embryo characteristics. Procedures regarding culture, freezing, and thawing of embryos were performed in one type of media (SAGE IVF) according to manufacturer’s instructions. Cleavage-stage embryos were frozen under room temperature. The embryos were washed three to four times in 0.7 mL Cryokit Diluent solution and were immersed in 0.7 mL 1.5 mol/L propanediol for 10 minutes. Embryos were then washed in 0.7 mL solution of 1.5 mol/L, propanediol and 0.1 mol/L sucrose and were immediately placed in a vial containing the same solution. Blastocyst freezeg was performed under 37°C. Blastocysts were first immersed in 0.7 mL Cryokit Diluent solution for 5 minutes and then in 0.7 mL 5% glycerol for 10 minutes. Blastocysts were then washed in 0.7 mL 9% glycerol containing 0.2 mol/L sucrose solutions and were immediately placed in a vial containing the same solution. Cyropreservation continued under a controlled program and seeding was performed manually.

Thawing of cleavage-stage embryos and blastocysts was performed by placing the vials into a 30°C water bath for 1 to 2 minutes until the solution was completely thawed. The vial was then kept in room temperature for 5 minutes. The contents were removed and taken through 10 minutes of consecutive washes of 0.5 and 0.2 mol/L sucrose. Then they were washed in the Cryokit Diluent solution for 5 minutes and were placed in Quinn’s Advantage Blastocyst medium until transfer.

The frozen-thawed embryos were considered to have survived if ≥50% of the initial number of blastomeres were intact. Blastocyst survival was evaluated as the proportion of dead cells to survived cells and the ability of the blastocoele to reexpand before transfer. None of the embryos have been subjected to assisted hatching after thawing.

All transfers except extended culture groups (groups 4 and 5) have been performed on the same day as thawing. Embryos/blastocysts were thawed in the morning (8:00-9:00 AM) and transferred in the afternoon (14:30 to 16:00 pm). Thawing sustained until the number of survived embryos/blastocysts reached to the number of embryos/blastocysts planned for transfer.

All transfers were performed under ultrasound guidance to patients with full bladder. One to three embryos were transferred depending on patient’s age, clinical history, and number and quality of available embryos.

Main Outcome Measures and Statistical Analysis
Primary outcomes were implantation and delivery rates. Secondary outcomes were clinical pregnancy and miscarriage rates. A clinical pregnancy was defined as the observation on ultrasound scanning of a gestational sac with fetal heart beat between 4 and 5 weeks after the positive pregnancy test. The clinical pregnancy rate per transfer and per thawing cycles was the ratio of cycles with clinical pregnancy to the total number of ET or thawing cycles, respectively. Implantation rate was defined as the number of gestational sacs divided by the number of embryos transferred. The miscarriage rate was determined by dividing the number of cycles with a spontaneous abortion before 12 weeks’ gestation by the total number of ET cycles resulting in a clinical pregnancy. Delivery rate per ET and per thawing cycles was determined by dividing the number of cycles resulting in live births by the total number of ET or thawing cycles, respectively.

For comparison of numerical values, data were analyzed by Fisher’s exact test or χ² test with Yates’ correction, where appropriate. Differences between continuous variables were assessed with analysis of variance followed by a Tukey-Kramer test. A P value <.05 was considered significant.

RESULTS
A total of 2,531 FET cycles were evaluated. The number of cycles in each group was as follows: group 1 = 1,761; group 2 = 282; and group 3 = 292. Embryos from 196 cycles were thawed at day 3 and subjected to prolonged culture (groups 4 and 5).

The clinical results for the five groups are summarized in Supplemental Table 1 (available online). A similar proportion of cycles in these groups were those from the “freeze-all” cycles, that is, cyropreservation of all embryos without transfer (13). In post-thawed extended culture groups, a total of 196 day 3 FET cycles resulted in 139 day 5 (group 4) and 25 day 6 (group 5) transfers (81% and 93.9%, respectively). In 6.1% of cycles (n = 12), transfer was cancelled because none of the day 3 embryos reached the criteria for transfer after day 6.

The clinical pregnancy rate per thawing cycle of group 2 was significantly higher than other groups (Fig. 1). Group 4 displayed a higher clinical pregnancy rate per thawing cycle than groups 1 and 5. The implantation rate of group 1 was significantly lower than other groups (Fig. 2). Groups 2 had significantly higher implantation rates than group 3. The miscarriage rate (Fig. 3) of group 3 was significantly higher than group 2 and 4. As shown in Figure 4, delivery rate per thawing cycle was similar between groups 2 and 4, which was significantly higher than other groups.

DISCUSSION
The conclusions (with regard to clinical pregnancy and delivery rates) drawn from the findings of this study were based on outcomes per thawing (not on per transfer) cycles as this ratio depicted total embryo loss until the transfer procedure and included post-thaw injuries. Although sample sizes in some groups were still low to reach to statistical significance, tendencies in several parameters were perceptible to get to conclusions.

The results indicate that cyropreservation of fresh embryos when they reach the blastocyst stage (day 5 or 6) is a feasible strategy. The following findings supported this approach: (1) The highest delivery (Fig. 4) and implantation (Fig. 2) rates accompanied by the lowest miscarriage (Fig. 3) rates were obtained after transfer of embryos
The clinical pregnancy rate per ET (black columns) and per thawing (white columns) cycle among groups (group 1: 3-3; group 2: 5-5; group 3: 6-5; group 4: 3-5; group 5: 3-6). *Significantly different from groups 2 (P < .0001), 3 (P = .04), and 4 (P < .0001).
**Significantly different from groups 2 (P < .04) and 4 (P = .004).
††Significantly different from others (P < .0001 vs group 1, P = .04 vs group 3, P = .05 vs group 4, P = .04 vs group 5).
†††Significantly different from groups 1 (P < .05) and 5 (P = .04).

The miscarriage rate per clinical pregnancy among groups (group 1: 3-3; group 2: 5-5; group 3: 6-5; group 4: 3-5; group 5: 3-6). *P = .06 vs group 3 and P = .07 vs group 2. **Significantly different from groups 2 (P = .01) and 4 (P = .03).

The delivery rate per ET (black columns) and per thawing cycle (white columns) among groups (group 1: 3-3; group 2: 5-5; group 3: 6-5; group 4: 3-5; group 5: 3-6). *Groups 1 and 3 were significantly different from groups 2 (P < .0001 and P = .03, respectively) and 4 (P < .0001 and P = .005, respectively). †Groups 2 (vs 1, P < .0001; vs 3, P = .01; vs 5, P = .008) and 4 (vs 1, P < .0001; vs 3, P = .04; vs 5, P = .03) were significantly different from others.

The implantation rates were similar (Fig. 2) between blastocysts developed to blastocysts at day 5 before (group 2) or after (group 4) cryopreservation. This finding was in accordance with those observed in fresh cycles (14). A significantly higher clinical pregnancy (Fig. 1) and better delivery rate (Fig. 4) have been found in group 2 compared with group 4. From this perspective, cryopreservation of blastocysts in a fresh cycle appears to be more logical, because besides better outcome measures, the latter requires more laboratory space and results in higher workload and cost than the former. [2] The implantation rates were similar (Fig. 2) between blastocysts formed through extended culture either before or after cryopreservation, both in normal- (group 2 vs group 4) and late-developing embryos (group 3 vs group 5). This finding indicated that procedures and manipulations associated with freezing did not diminish embryo viability, which constituted further support to the feasibility of cryopreservation of competent embryos in a fresh cycle. [3] When blastocysts were frozen during the fresh cycle, cancellation of transfer was caused merely by poor embryo survival after thawing (groups 2 and 3), whereas embryo development was an additional detrimental factor in prolonged culture (groups 4 and 5). The impact of damage to the embryos was observed in the difference of the outcome measures between per transfer and per thawing cycles (Figs. 1 and 4). Therefore, cryopreservation of cleavage-stage embryos for extended culture purposes could be considered as an inefficient way of...
laboratory space and staff utilization. [4] After slow freezing, a diminished implantation capacity has been shown in thaw-survived cleavage-stage embryos even with some damage to blastomeres (i.e., ≤50%) compared with those with intact cells (15, 16). Use of vitrification technique could improve the outcome compared with slow freezing by increasing the number of embryos with intact blastomeres after thawing (17). However, despite higher survival rates of cleavage-stage embryos and blastocysts using vitrification technique, pregnancy rates have been found to remain similar to that with slow freezing (18). Furthermore, Lieberman and Tucker (19) did not find any difference between vitrification and slow-freezing of blastocysts with respect to survival, implantation, and pregnancy rates. Taken together, the impact of vitrification technique on the outcome measures and cryopreservation strategies as has been presented in the current study remains to be determined. [5] Utilization of late-developing embryos in FET cycles before (group 3) and after (group 5) thawing resulted in compromised implantation, clinical pregnancy, and delivery rates than day 5 embryos (groups 2 and 4, respectively). Transfer of thawed cleavage-stage embryos (group 1) resulted in similar clinical pregnancy (Fig. 1) and delivery rates to late-developing blastocysts (groups 3 and 5). Taken together, instead of cleavage-stage embryos, these clinical parameters favor blastocyst cryopreservation in a fresh cycle (normal- or late-developing), because the former will require less laboratory space and decrease workload and cost.

Data on the impact of the rate of embryo development on the outcome of FET cycles are conflicting. Some studies suggested that the rate of embryo development was not crucial to the success of a thawing cycle by demonstrating similar outcome measures between transfers of blastocysts that were frozen on day 5 to 7 (10, 20–26). According to these authors, slow-growing embryos do not implant within the window of endometrial receptivity in a fresh cycle, despite their similar inherent viability to day 5 blastocysts. On the contrary, other studies proposed that embryo development rate may, in part, predict implantation and subsequent frozen blastocyst transfer outcomes (9, 27–29) and showed significantly higher implantation rates and improved pregnancy outcomes after transfer of cryopreserved day 5 than day 6 blastocysts. Furthermore, Lieberman and Tucker (19) have found a slightly, but not significantly, higher implantation and clinical pregnancy rates with the use of day 5 blastocysts vs day 6 and suggested cryopreservation of slow-growing embryos for subsequent usage. The findings of the present study are in accordance with the latter observations that proposed diminished cleavage rates could be a manifestation of the inherited reduced viability of late developing embryos.

The impact of reduced viability in late-developing embryos has also been demonstrated in the increased incidence of miscarriages; in the present study, a higher miscarriage rate was found after day 6 transfers (groups 3 and 5) compared with other groups. Day 3 (group 1) embryos demonstrated a higher miscarriage rate than day 5 transfers (groups 2 and 4). This was an expected finding as day 3 transfers were a heterogeneous population of embryos composed of normal- (day 5) and slow-growing (day 6) embryos. Considering the similar survival rate among groups, the increased incidence of early pregnancy losses at day 6 may be a reflection of compromised viability of embryos on their developmental rate. Hence, it can be speculated that late-developing embryos possess inherent defects that delay retardation in subsequent cleavages that is not detectable by morphology alone, and such errors may account for the high incidence of early pregnancy losses observed in cryopreserved day 6 ETs.

In conclusion, the feasible strategy in good responder patients appears to be the cryopreservation of blastocysts in the fresh cycle. If the couple has slow-growing embryos they should be informed about realization of a compromised outcome compared with another couple with normal-developing blastocysts.

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## SUPPLEMENTAL TABLE 1

### Group demographics.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of thawing cycles</td>
<td>1,761</td>
<td>282</td>
<td>292</td>
<td>196</td>
<td></td>
</tr>
<tr>
<td>% Freeze-all cycles</td>
<td>13.5</td>
<td>14.5</td>
<td>15.1</td>
<td>17.3</td>
<td></td>
</tr>
<tr>
<td>% Secondary infertility</td>
<td>23.2</td>
<td>34.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.6</td>
<td>22.9</td>
<td></td>
</tr>
<tr>
<td>No. of transfer cycles</td>
<td>1,719</td>
<td>276</td>
<td>277</td>
<td>159</td>
<td>25</td>
</tr>
<tr>
<td>% Thawing cycles reaching transfer</td>
<td>97.6</td>
<td>97.9</td>
<td>94.9</td>
<td>81.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>% Survival rate (no. of thawed embryos)</td>
<td>85.9 (5,945)</td>
<td>85.1 (684)</td>
<td>85.3 (616)</td>
<td>83.7 (468)</td>
<td></td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>32.8 ± 5.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.2 ± 4.6</td>
<td>31.6 ± 4.9</td>
<td>32.4 ± 4.3</td>
<td>32 ± 4.4</td>
</tr>
<tr>
<td>Mean no. of transfer embryos ± SD (n)</td>
<td>3.0 ± 0.7&lt;sup&gt;a&lt;/sup&gt; (5,210)</td>
<td>2.2 ± 0.6 (595)</td>
<td>1.9 ± 0.6&lt;sup&gt;d&lt;/sup&gt; (537)</td>
<td>2.2 ± 0.7 (351)</td>
<td>2.0 ± 0.7 (51)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from others (P<.05).

<sup>b</sup> Proportion of cycles reaching transfer on day 5 (group 4) and day 5 plus 6 (group 5).

<sup>c</sup> Significantly different from groups 2 and 3 (P<.05).

<sup>d</sup> Significantly different from groups 2 and 4 (P<.05).