High pregnancy rates can be achieved after freezing and thawing human blastocysts

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Objective: To examine the results of a 3-year trial using blastocyst cryopreservation to limit multiple pregnancy and optimize overall pregnancy per cycle.

Design: Retrospective clinical evaluation of pregnancy rates after freezing and thawing human blastocysts.

Setting: Tertiary-care academic center.

Patient(s): Seven hundred fifty-three different patients treated in 783 IVF cycles with blastocysts frozen from July 2000 to June 2003.

Intervention(s): Two thousand, two hundred fifty-nine blastocysts were frozen in cycles in which only blastocysts were cryopreserved (cycles with pronuclear stage oocytes or pre-embryos also cryopreserved were excluded from the analysis). Of these, 628 (27.6%) were thawed in 218 cycles.

Main Outcome Measure(s): Pregnancy rate per cycle with thaw.

Result(s): Four hundred seventy-nine (76.3%) blastocysts survived thawing, and 440 (92.0%) were transferred after exhibiting evidence of survival (most commonly, blastocoele reexpansion). In cycles with a thaw, 211 (96.8%) of 218 underwent intrauterine transfer. An average of 2.09 blastocysts was transferred per replacement. One hundred twenty-five (59.2%) clinical pregnancies were established, which included 23 sets of twins and 5 triplet gestations. Two sets of monozygotic twins were identified after the replacement of a single thawed blastocyst (1.6%). The age of the patient at the time of cryopreservation was an important factor in the establishment of clinical and ongoing pregnancy. The mode of ovarian stimulation, replacement method, and whether blastocysts were frozen on day 5 or day 6 of development did not demonstrate clinical significance.

Conclusion(s): Cryopreserved and thawed blastocysts demonstrated a similar potential for implantation when compared with fresh pre-embryos on day 3. On the basis of these results, the blastocyst stage of development appears to be optimal for clinical freeze–thaw trials. (Fertil Steril 2004;82:1418–27. ©2004 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, freeze, thaw, blastocyst

Twenty years have passed since the very first thawed human pre-embryo led to the birth of a child (1), and it has been 18 years since pregnancy from a thawed human blastocyst was reported (2). During this time, most IVF programs have embraced cryobiology to augment clinical pregnancy from a single ovarian stimulation attempt. As ovulation induction protocols have improved, allowing the recruitment of multiple healthy oocytes, so has the need grown to responsibly manage their numbers. It is common today to harvest in excess of 10, or sometimes even 20, mature oocytes after treatment with agonists and gonadotropins.

Before freezing techniques were routinely used in the laboratory, a woman producing so many gametes either would be forced to limit the number inseminated or risk having to discard healthy pre-embryos because only two to three could be transferred safely and responsibly to the uterus after fertilization. Neither was an attractive option.

It is now apparent that pregnancy rates after thawing are nearly equal to those resulting
from the transfer of fresh pre-embryos, at least in some programs. When the cumulative effect of adding thawed pregnancies (from cycles failing to result in pregnancy after fresh transfer) to fresh pregnancies is examined, delivery outcomes are significantly enhanced (3).

Additionally, patients at risk of ovarian hyperstimulation syndrome are managed more effectively by freezing all conceptuses upfront, thereby reducing, although not eliminating, the likelihood of adverse clinical symptoms (4).

The availability of sequential media has led to an increase in the practice of blastocyst freezing. Several groups have reported freezing blastocysts quite successfully, with some of the earliest investigations using coculture systems to support pre-embryo growth (2, 5–7). Until the mid-1990s, most reports of clinical pregnancy after blastocyst thawing fell in the range of 10%–30% per transfer (8), results that were not significantly improved when compared with the case of thawing earlier stages. This situation has recently changed; pregnancy rates in the range of 40%–60% are now common for thawed blastocysts (9, 10).

Typical pronuclear-stage and pre-embryo slow-freezing protocols in use today are based loosely on the original work of Testart and his colleagues (11, 12), from the mid-1980s. PROH and dimethyl sulfoxide are commonly used as cryoprotectants, with or without sucrose to aid dehydration. On the other hand, most blastocyst slow-freezing protocols have evolved from the published work of Menezo and colleagues (13, 14) and use glycerol and sucrose as cryoprotectants. Although vitrification techniques (rapid-freezing methods) are gaining popularity, few human births have been reported to date (15–18).

**MATERIALS AND METHODS**

Informed consent for cryopreservation and thawing was obtained from all patients. Methods were approved and supervised by an institutional review board at Weill-Cornell Medical School/New York Hospital.

Cycles were selected on the basis that blastocysts alone had been frozen during the IVF attempt. Although other cycles had blastocysts frozen during the same time period, these represented a mixed freezing group (blastocysts frozen in conjunction with earlier pre-embryos or pronuclear-stage oocytes) and thus were excluded from the analysis in an effort to avoid mixed transfers.

Cycles having blastocysts frozen during the study period (20.3% of all patients treated) were represented by a mixed population of patients with various underlying causes of infertility. The group age range was 22 to 44 years, with an average age (±SD) of 34.7 ± 4.8 years. Most of these patients responded appropriately to ovarian stimulation and therefore ultimately had an adequate number of conceptuses available for both fresh transfer and freezing. In this study population, the average number of oocytes collected at hysteroscopy was 13.5; the average number of normally fertilized oocytes exhibiting two pronuclei at 15–18 hours after insemination was 10.7. An average of 2.9 blastocysts was frozen per cycle with freezing.

Ovarian stimulation was achieved after pituitary down-regulation with GnRH agonists and administration of recombinant FSH, with or without hMG, as described elsewhere (19).

The majority of blastocysts frozen were generated after the fresh transfer of day 3 pre-embryos. After choosing the optimal day 3 pre-embryos for intrauterine transfer, remaining viable ones were examined each day for 2 or 3 additional days to evaluate their suitability for freezing. We have termed this the posttransfer observation period.

After extended culture, blastocysts forming on either day 5 (at least one grade 1BB) or day 6 (at least one grade 2BB) were cryopreserved for future use. The grading system used to score blastocysts is shown in Figure 1; individual scores were given to blastocyst expansion, inner cell mass compaction, and trophectoderm morphology.

A blastocyst was defined as having a blastocoele filling greater than half the volume of the conceptus, and early blastocysts possessed cells that suggested the formation of an inner cell mass. Blastocysts were given a numerical score from 1 to 6 on the basis of their degree of expansion and hatching status: 1, an early blastocyst (blastocoele filling greater than half of the volume of the conceptus) but without overall increase in size as compared with earlier stages; 2, a true blastocyst (blastocoele filling greater than half of the volume of the conceptus) with slight expansion in overall size and some thinning of the zona pellucida; 3, a full blastocyst (blastocoele filling greater than half of the volume of the conceptus) with overall size fully enlarged and a very thin zona pellucida; 4, a hatching blastocyst (no artificial manipulation); 5, a fully hatched blastocyst (no artificial manipulation); and 6, a hatching or hatched blastocyst resulting from artificial manipulations that created a substantial hole in the zona pellucida, such as assisted hatching or biopsy.

The development of the inner cell mass was assessed as follows: A, compacted cells; B, larger, loosely grouped cells or formation of a cellular bridge; C, no inner cell mass distinguishable; or D, cells of the inner cell mass appeared degenerative.

The trophectoderm was assessed as follows: A, many healthy cells forming a cohesive epithelium; B, few but healthy cells, large in size, forming a loose epithelium; C, unhealthy, very large, or unevenly distributed cells, or few cells squeezed to the side; or D, cells appeared degenerative.

Glycerol and sucrose were used in the freezing medium to aid in cell dehydration. Specimens were frozen in sterile plastic cryovials (Nunc, Nalge-Nunc International, Rochester, NY) containing 0.3 mL of cryoprotective medium. A Planer Series...
III biological freezer (TS Scientific, Perkasie, PA) was used, allowing specimens to be frozen under computerized control.

Our cryopreservation protocols were amended in several ways from the earlier published work of Menezo et al. (14). Modifications included the following: [1] the base freezing medium was prepared from a standard phase I sequential medium formulation, modified by the addition of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer; [2] extra macromolecules (protein) were added in the form of 5% human serum albumin solution (Irvine Scientific, Santa Ana, CA) and 20% Plasmanate (Bayer Corporation, Elkhart, IN); [3] the glycerol cryoprotectant concentration was elevated to 10%; and [4] additional dilutions were carried out during the thawing process. The base cryopreservation medium is outlined in Figure 2.

Extended culture was accomplished by using a complex, noncommercial sequential medium prepared weekly on site.

Pre-embryos were grown in phase I of this medium until day 3 and in phase II during days 4–6. All pre-embryos were cultured within a Forma water-jacketed incubator (Thermo Electron Corp. [formerly Forma Scientific], Waltham, MA) under conditions of 5.5% CO₂ in humidified air at 37.2°C.

**Blastocyst Freezing**

Blastocysts were exposed to two concentrations of cryoprotective medium at room temperature: 5% glycerol solution for 10 minutes and 10% glycerol–0.2 M sucrose solution for 10 minutes. They were then loaded into cryovials and cooled at a rate of −2.0°C/min until −7.0°C was reached. Cryovials were equilibrated at this temperature for 5 minutes, manual seeding was performed, and an additional 10 minutes was provided for further equilibration. After this period, further cooling was carried out at −0.3°C/min until −38°C was reached. Cryovials were then plunged directly into liquid nitrogen.

**FIGURE 1**

Grading system used to evaluate blastocyst expansion, inner cell mass characteristics, and trophectoderm morphology.

![Grading system](image)

### FIGURE 2

Freezing and thawing media compositions.

<table>
<thead>
<tr>
<th>Component</th>
<th>mM Concentration (7.33 - 7.37 pH; 260-265 mOsm/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (mM)</td>
<td>85.16</td>
</tr>
<tr>
<td>Sigma S5886</td>
<td></td>
</tr>
<tr>
<td>KCl (mM)</td>
<td>5.50</td>
</tr>
<tr>
<td>Sigma P5405</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O (mM)</td>
<td>0.25</td>
</tr>
<tr>
<td>Sigma S9638</td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O (mM)</td>
<td>1.80</td>
</tr>
<tr>
<td>Sigma C7902</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O (mM)</td>
<td>1.00</td>
</tr>
<tr>
<td>Sigma M1880</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃ (mM)</td>
<td>4.00</td>
</tr>
<tr>
<td>Sigma S5761</td>
<td></td>
</tr>
<tr>
<td>Penicillin G (mM)</td>
<td>0.03</td>
</tr>
<tr>
<td>Sigma P3032</td>
<td></td>
</tr>
<tr>
<td>Phenol Red (mM)</td>
<td>0.01</td>
</tr>
<tr>
<td>Sigma P5530</td>
<td></td>
</tr>
<tr>
<td>Sodium Pyruvate (mM)</td>
<td>0.32</td>
</tr>
<tr>
<td>Sigma P5280</td>
<td></td>
</tr>
<tr>
<td>Sodium Lactate (mM)</td>
<td>21.00</td>
</tr>
<tr>
<td>Sigma L4263</td>
<td></td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>0.50</td>
</tr>
<tr>
<td>Sigma G7021</td>
<td></td>
</tr>
<tr>
<td>EDTA (mM)</td>
<td>0.05</td>
</tr>
<tr>
<td>Sigma E6758</td>
<td></td>
</tr>
<tr>
<td>Glutamine (mM)</td>
<td>0.50</td>
</tr>
<tr>
<td>Sigma A8185</td>
<td></td>
</tr>
<tr>
<td>Taurine (mM)</td>
<td>0.10</td>
</tr>
<tr>
<td>Sigma T7146</td>
<td></td>
</tr>
<tr>
<td>HEPES Buffer Solution (mL/L)</td>
<td>23.00 mL/L</td>
</tr>
<tr>
<td>Mediatech 25-060C1</td>
<td></td>
</tr>
<tr>
<td>Non-Essential AA Solution (mL/L)</td>
<td>10.00 mL/L (≈ 0.1 mM each of eight non-essential amino acids)</td>
</tr>
<tr>
<td>Mediatech 25-030-C1</td>
<td></td>
</tr>
<tr>
<td>HSA Solution (mL/L)</td>
<td>50.00 mL/L (≈ 5%)</td>
</tr>
<tr>
<td>Irvine 9988</td>
<td></td>
</tr>
<tr>
<td>Plasmanate Solution (mL/L)</td>
<td>200.00 mL/L (≈ 20%)</td>
</tr>
<tr>
<td>Bayer Corp</td>
<td></td>
</tr>
<tr>
<td>Glycerol (Sigma G9012) added per amounts outlined in text</td>
<td></td>
</tr>
<tr>
<td>Sucrose (Sigma S1888) added per amounts outlined in text</td>
<td></td>
</tr>
</tbody>
</table>

**Blastocyst Thawing**

Cryovials were thawed at room temperature for 60 seconds before being warmed in a 30°C waterbath for 30–90 seconds (until all ice dissipated). Blastocysts were removed from the cryovials and taken through decreasing concentra-

	ions of cryoprotective medium: 10% glycerol + 0.4 M sucrose for 30 seconds, 5% glycerol + 0.4 M sucrose solution for 3 minutes, 0.4 M sucrose solution (no glycerol) for 3 minutes, 0.2 M sucrose solution (no glycerol) for 2 minutes, and 0.1 M sucrose solution (no glycerol) for 1 minute.
Specimens were then washed thoroughly in Phase II sequential medium and incubated until transfer.

**Natural Cycle Replacement (Used Only for Ovulatory Cycles With Normal Luteal Phase P Levels)**

Supplemental P was not administered unless medically indicated after evaluation of the luteal phase P level on the 5th, 7th, and 9th day after the LH surge or unless the patient had experienced a previous pregnancy failure in a non-supplemented attempt. If administered, 200 mg of micronized P4 was given vaginally two or three times per day and was continued until a negative pregnancy test was confirmed 10–12 days after blastocyst replacement or through week 7 if the woman was pregnant, with pregnancy confirmed by ultrasound investigation of a fetal heartbeat. Medrol (16 mg/d; Pharmacia Corporation, Peapack, NJ) and tetracycline (250 mg, four times per day) were administered for 4 days, beginning two days before replacement.

Blastocysts frozen on day 5 of development were thawed 4 days after the LH peak and were transferred the following day; blastocysts frozen on day 6 of development were thawed 5 days after the LH peak and transferred on the same day.

**Programmed Cycle Replacement**

Luteal suppression was accomplished with 0.2 mg of GnRH agonist. Once adequate suppression was verified, this dosage was reduced to 0.1 mg beginning on the predetermined day 1 of the cycle and was maintained until day 15.

Transdermal estrogen patches (Climara, 0.1-mg patch; Berlex Laboratories, Montville, NJ) were administered in the evenings as follows:

- Days 1–4, 0.1 mg every other day
- Days 5–8, 0.2 mg every other day
- Days 9–10, 0.3 mg every other day (variable depending on E2 levels)
- Days 11–14, 0.4 mg every other day
- Days 15 and later, 0.2 mg (two patches every other day, 7 weeks)

Fifty milligrams of P were administered IM beginning on day 15 after estrogen, P, and endometrial thickness parameters were evaluated and judged to be adequate. Progesterone was continued through 12 weeks of gestation if the patient became pregnant and her results were confirmed by ultrasound investigation of a fetal heartbeat. Medrol (16 mg/d) and tetracycline (250 mg, four times per day) were administered beginning on day 17 for 4 days. Blastocysts frozen on day 5 were thawed on day 19 and transferred the following day; blastocysts frozen on day 6 were thawed on day 20 and transferred the same day.

Clinical pregnancy was defined as ultrasound evidence of at least one gestational sac at ≥7 weeks after transfer; the implantation rate was defined as gestational sacs per number of pre-embryos transferred.

**Calculating Total Pregnancy Potential**

The base fresh pregnancy rate was defined as the number of clinical pregnancies established after the transfer of noncryopreserved conceptuses over the number of noncryopreserved (fresh) transfer cycles, that is, (250/500) × 100 = 50%.

The augmented pregnancy rate was defined as the actual number of clinical pregnancies generated by the transfer of noncryopreserved conceptuses, plus the actual number of clinical pregnancies generated by the transfer of thawed conceptuses in cycles failing to achieve pregnancy with fresh transfer, over the number of transfer cycles, that is, (250 + 125/500) × 100 = 75%.

The projected total pregnancy rate was defined as the actual number of clinical pregnancies generated by the transfer of noncryopreserved conceptuses, plus the actual number of clinical pregnancies generated by the transfer of thawed conceptuses in cycles failing to achieve pregnancy with fresh transfer, plus the number of clinical pregnancies expected from the potential transfer of conceptuses still in cryostorage for patients not yet pregnant from fresh or thawed attempts (this last calculation uses the thawed pregnancy rate established to date) over the total number of cycles with a transfer, that is, (250 + 125 + 25/500) × 100 = 80%.

**Statistical Analysis**

Results were analyzed by Fisher’s exact test. P values <.05 were considered significant.
RESULTS

The overall survival rate for thawed blastocysts was very stable at 76.3%. The clinical pregnancy per transfer cycle was 59.2%, the ongoing or delivered rate was 50.2%, and the implantation rate was 38.6%. Twenty-three sets of twins were generated after an average of 2.09 thawed blastocysts was replaced per cycle. Five triplet gestations were established, each after replacing three blastocysts in patients who failed to become pregnant in their fresh-IVF attempts. Although no monozygotic twins were identified after replacing two or three thawed blastocysts, two monozygotic gestations followed the replacement of a single thawed blastocyst, an incidence of 1.6% (2/125).

We analyzed pregnancy outcomes in 211 consecutive patients receiving the transfer of thawed blastocysts. Sixty-nine patients received a transfer of day 5 frozen–thawed blastocysts, and 142 patients underwent transfer with day 6 blastocysts. There were no significant group differences in terms of patient age (33.6 vs. 35.1 years, day 5 and day 6 blastocysts, respectively), average number of blastocysts transferred (2.4 vs. 2.0), or morphology of the blastocysts after thawing. No significant differences were found in the

![Figure 3](http://www.elsevier.es)

**FIGURE 3**

Implantation rates (light green bars; gestational sacs per embryos transferred), clinical pregnancy rates (dark blue bars; pregnancies with ultrasound evidence of implantation, per number of transfers), and ongoing pregnancy rates (dark green bars; pregnancies ongoing >20 weeks or delivered, per number of transfers) in women undergoing transfer of thawed blastocysts, in relation to maternal age. Although selected blastocysts can be replaced, implantation and pregnancy or ongoing pregnancy rates are shown to be reduced with advancing maternal age. Sacs per number transferred, $P<.05$ (<30 years and 30–33 years vs. 37–39 years); clinical pregnancies per transfer, $P<.05$ (<30 years vs. 37–39 years or 42+ years; 30–33 years vs. 37–39 years or 42+ years); ongoing pregnancies per transfer, $P<.05$ (<30 years or 30–33 years vs. 42+ years; 30–33 years and 34–36 years vs. 42+ years).

![Table 2](http://www.elsevier.es)

**TABLE 2**

Impact of number of thawed blastocysts replaced on subsequent pregnancy outcome.

<table>
<thead>
<tr>
<th>Variable</th>
<th>1 Replaced, n (%)</th>
<th>2 Replaced, n (%)</th>
<th>3 Replaced, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical pregnancy/transfer</td>
<td>30/66 (45.5)</td>
<td>46/78 (59.0)</td>
<td>49/67 (73.1)</td>
</tr>
<tr>
<td>Ongoing pregnancy/transfer</td>
<td>25/66 (37.9)</td>
<td>37/78 (47.4)</td>
<td>44/67 (65.7)</td>
</tr>
</tbody>
</table>

Note: 1 vs. 2 replaced, no statistically significant difference in either pregnancy category. $P<.005$, 1 vs. 3 replaced, clinical pregnancy and ongoing pregnancy. $P<.05$, 2 vs. 3 replaced, ongoing pregnancy.

post-thaw survival rates (78.0% vs. 75.1%), clinical pregnancy rates (60.9% vs. 58.5%), or ongoing pregnancy rates (52.2% vs. 49.3%), nor were differences observed in implantation rates (38.0% vs. 39.0%; Table 1). Because no statistical differences were observed in any of these comparisons, day 5 and day 6 data were combined in subsequent analyses.

As shown in Figure 3, successful outcomes with thawed blastocysts were dependent on maternal age, with women under the age of 37 years achieving the highest rates of clinical pregnancy, ongoing pregnancy, and implantation. Although 7 (35.0%) of 20 women aged 42 years or older established clinical pregnancies from thawed blastocysts, their miscarriage rate was extraordinarily high (4/7, 57.1%).

The number of blastocysts replaced played a significant role in subsequent pregnancy success. Although no significant differences were observed in clinical pregnancy or ongoing pregnancy rates between replacing one vs. two thawed blastocysts, statistically higher ongoing pregnancy rates were observed after the replacement of three—although it must be noted that all 5 triplet gestations involved replacement of three blastocysts (Table 2).

Cycles were also examined for the effects of ovarian stimulation (fresh cycles) and uterine preparation for replacement (thawed cycles). No differences were observed when recombinant FSH was used in conjunction with hMG or when recombinant FSH was used alone (Table 3, clinical pregnancy, 58.9% vs. 60.0%, respectively). Nor were clinical pregnancy rates different when blastocysts were replaced in either natural or programmed cycles (61.3% vs. 56.3%, respectively).

Table 4 compares both fresh and frozen-thawed clinical pregnancy rates in cycles with at least one pre-embryo (1–10 cells) or at least one blastocyst frozen. Three striking findings were observed in this comparison: [1] fresh pre-embryos and fresh blastocysts demonstrated higher pregnancy and implantation rates than their frozen-thawed counterparts, [2] thawed blastocysts performed better than thawed pre-embryos despite fewer being replaced, and [3] implantation rates were remarkably similar when thawed blastocysts and fresh pre-embryos were compared.

Cumulative pregnancy results for blastocysts, using the total pregnancy model described earlier in this article, are shown in Table 5. A true increase in overall pregnancy rate per stimulated cycle was observed by virtue of applying the cryopreservation techniques described by the authors (see category labeled True augmented clinical pregnancy/transfer). This augmented pregnancy rate takes into account only those cycles (with either day 3 or day 5 fresh transfer and with blastocysts frozen) that failed to produce a pregnancy after fresh transfer. The numerator is increased by 80 preg-

### Table 3

Effect of fresh-cycle ovarian stimulation and method of replacement on thawed blastocyst implantation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>hMG ± recombinant FSH, n (%)</th>
<th>Recombinant FSH alone, n (%)</th>
<th>Natural cycle, n (%)</th>
<th>Programmed cycle, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sacs/no. transferred</td>
<td>88/226 (38.9)</td>
<td>82/214 (38.3)</td>
<td>94/257 (36.6)</td>
<td>76/183 (41.5)</td>
</tr>
<tr>
<td>Clinical pregnancies/transfer</td>
<td>66/112 (58.9)</td>
<td>59/99 (60.0)</td>
<td>76/124 (61.3)</td>
<td>49/87 (56.3)</td>
</tr>
<tr>
<td>Ongoing pregnancies/transfer</td>
<td>54/112 (48.2)</td>
<td>52/99 (52.5)</td>
<td>65/124 (52.4)</td>
<td>41/87 (47.1)</td>
</tr>
</tbody>
</table>

Note: No statistically significant difference between ovarian stimulation regimes or method of replacement in any category.


### Table 4

Fresh clinical pregnancy and implantation rates in cycles with freezing compared with subsequent thawed clinical pregnancy and implantation rates.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-embryos, fresh</th>
<th>Pre-embryos, thawed</th>
<th>Blastocysts, fresh</th>
<th>Blastocysts, thawed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age at fresh transfer/freeze (y)</td>
<td>34.5 ± 4.8</td>
<td>34.5 ± 4.8</td>
<td>34.7 ± 4.8</td>
<td>34.7 ± 4.8</td>
</tr>
<tr>
<td>Patient age (y)</td>
<td>21–47</td>
<td>21–47</td>
<td>22–44</td>
<td>22–44</td>
</tr>
<tr>
<td>Avg. no. transferred/transfer</td>
<td>3.4 ± 0.7</td>
<td>3.9 ± 1.9</td>
<td>2.0 ± 0.4</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>Clinical pregnancy/transfer, n (%)</td>
<td>1,252/1,801 (69.5)</td>
<td>173/444 (39.0)</td>
<td>178/248 (71.8)</td>
<td>125/211 (59.2)</td>
</tr>
<tr>
<td>Sacs/no. transferred, n (%)</td>
<td>2,331/6,099 (38.2)</td>
<td>262/1,645 (15.9)</td>
<td>280/496 (56.5)</td>
<td>170/440 (38.6)</td>
</tr>
</tbody>
</table>

Note: All comparisons of pregnancy and implantation (sacs) significantly different from one another except the following: clinical pregnancy—pre-embryos, fresh vs. blastocysts, fresh; sacs/no. transferred: pre-embryos, fresh vs. blastocysts, thawed.

nancies to show the additional thawed pregnancies that were realized in cycles failing to establish a fresh pregnancy; the denominator is increased by 7 cycles, which represents patients who had all conceptuses frozen upfront (no fresh transfer but a first transfer on the thawed attempt). Those patients who established both fresh and thawed pregnancies (more than one pregnancy after fresh and thawed transfers) were counted only once and are only included under the category Fresh base clinical pregnancy/transfer.

Also in Table 5, the Projected total clinical pregnancy/transfer group takes into account the anticipated total pregnancy rate per stimulated cycle once all blastocysts are eventually thawed and all patients have had at least one transfer, either fresh or thawed. Although only a theoretical number (682/783, 89.1%), its increased numerator is based on the number of treatment cycles without pregnancy success that still have frozen blastocysts stored and is calculated on the valid pregnancy results of previous thaw cycles. Again here, the denominator increases by a few cycles, representing patients who have not yet returned for a thawed transfer after having all their conceptuses frozen upfront (never had any transfer).

The cumulative pregnancy rate calculation is valuable for determining the efficacy of cryopreservation methods on a per-cycle basis. It assists us in answering the question, “Are patients truly benefiting from undergoing the procedure in terms of increasing the chances of having at least one baby from a single IVF attempt?” It appears so.

**DISCUSSION**

The value of cryopreserving fertilized conceptuses for future thaw and transfer is an important consideration of every IVF program. The techniques offer many benefits related to increasing the number of healthy pre-embryos that are available for current or future transfer.

Blastocysts have the advantage of possessing many cells. The loss of a few during freezing and thawing may not compromise the integrity of the entire specimen. This may be one reason that blastocysts have been frozen and thawed so successfully over the years in domestic animals for both research and commercial purposes. Blastocyst cryopreservation in the human was first reported in 1985 using glycerol in a series of 10 increasing concentrations (2). After that initial report, blastocyst freezing was only occasionally incorporated into clinical protocols because of the difficulties involved with maintaining high rates of blastocyst development in vitro.

Through the 1990s, reports of clinical pregnancy after blastocyst thaw indicated rates well under 30% per transfer. Because of this, the freezing of blastocysts did not appear to present a better cryostorage option when compared with earlier stages. Although several groups reported freezing blastocysts quite successfully, most often relied on coculture systems to support pre-embryo growth. Today, the availability of sequential media has led to a dramatic increase in the practice of blastocyst freezing, and much higher pregnancy rates are being seen after the replacement of thawed specimens.

Few reports have been published detailing the efficiency of blastocyst freezing after culture in sequential media. Langley et al. (20) described a comparison of thawed day 3 pre-embryos vs. blastocysts during a 30-month period. In this study, the survival rate was higher for blastocysts, and the implantation rate was doubled (21.9% vs. 10.1%, 72 blastocyst cycles). In 2002, Behr et al. (21) reported a 36% clinical pregnancy rate and 16% implantation rate for thawed blastocysts from 64 cycles. Given these few peer-reviewed reports generated after extended culture in sequential media, there may not be adequate evidence to support the concept that blastocysts are optimal for human freezing trials.

Nonetheless, the Cornell program has benefited greatly from the adoption of blastocyst-freezing protocols. Although acceptable clinical pregnancy rates of approximately 40% were realized after freezing and thawing cleavage-stage pre-embryos in >700 cycles, higher rates were established using blastocysts (59%) without any concomitant drop in the number or proportion of patients having conceptuses frozen. Nearly one in four women aged younger than 40 years have had blastocysts frozen after undergoing day 3 transfers, and 60% of women undergoing day 5 transfers have had at least one blastocyst cryopreserved on either day 5 or day 6. In our program, nearly 2,000 blastocysts have been frozen in 3 years of trials. Less than one fifth of these have yet been thawed because of the high fresh-pregnancy rate seen in these cycles.

It is generally assumed that blastocysts that develop in a timely manner in vitro are of better quality than those that develop more slowly. However, this study and an earlier retrospective analysis of blastocyst thaw outcomes from our program demonstrate otherwise. In 154 consecutive patients returning for thawed blastocysts, 60 patients received a transfer of day 5 frozen-thawed blastocysts, and 94 patients underwent transfer with day 6 blastocysts. No significant differences were observed between groups for patient age,
blastocyst survival rates, average number of blastocysts replaced, morphology of thawed blastocysts, clinical pregnancy rates, ongoing pregnancy rates, or implantation rates. These findings are identical to those presented in an earlier study from this center (22).

Although it is intuitive to assume that pre-embryos reaching the blastocyst stage faster (day 5) might be healthier than their day 6 counterparts, these data and the data of others suggest that the rate of development may not be crucial to subsequent postthaw success (21). Surprisingly, this is in direct conflict to reports of fresh transfer using day 5 and day 6 blastocysts, in which pregnancy has been observed to be significantly lower with slower-growing day 6 conceptuses (23). Also, in contrast to our work, Marek et al. (24) carried out a study comparing outcomes from 127 thawed blastocyst cycles in which blastocysts were frozen on day 5 or day 6. Survival rates after thaw were good for both groups, but the clinical pregnancy rate per thaw (50% vs. 29%, respectively), ongoing pregnancy rate per thaw (43% vs. 23%), and implantation rate (34% vs. 15%) were all significantly higher for day 5 blastocysts. As well, Shoukir and colleagues (25) demonstrated greater pregnancy and implantation with pre-embryos that were fastest to reach the blastocyst stage in culture. Why these results are so different from our own is not clear.

We, like others, observed that blastocysts with a high probability of survival after thaw acted as perfect osmometers, shrinking, re-expanding, and swelling in accordance with their osmotic environment (26). One uneasy task immediately after thawing was to determine that a blastocyst had indeed survived, because they often presented a contracted state for up to several hours after reincubation in culture medium. It has been our experience that blastocysts that shrink appropriately in response to cryoprotective agents and exhibit contracted, healthy-appearing cells after thaw do quite well in their ability to survive the rigors of freezing and thawing. All pregnancies were established with blastocysts that had at least begun to re-expand. Two cycles involving the freezing of noncavitated morulae failed to generate pregnancies.

Of the many tribulations associated with running a cryopreservation program, one of the most frustrating is that embryologists cannot reap the fruits of their labor (pregnancy after thawing) until months or years have passed. It is common for patients to wait for some time before returning for a thaw attempt after a negative fresh cycle or to delay 2 or more years after the birth of a child. This situation gives rise to special problems in tracking results during a given freezing period and makes it difficult to identify the efficacy of a new protocol. There are two common ways to analyze freezing-thawing results: [1] calculate pregnancy rate per cycle with transfer of thawed conceptuses and [2] calculate a total or augmented pregnancy rate per cycle with freezing, on the basis of fresh-pregnancy plus thawed-pregnancy rates.

This method has been discussed in detail in numerous publications (3, 27–30). In the last analysis, a total or augmented pregnancy rate refers to the actual cumulative pregnancy rate achieved by patients upon combining pregnancies established from both fresh and thawed transfers. Here, we calculated the augmented pregnancy rate and then extended the analysis to estimate a projected cumulative rate. The validity of reporting a projected estimation is open to criticism because of its reliance on past performance and its assumptions that future results will be similar.

It is encouraging to note that cryopreservation techniques have no apparent negative impact on perinatal outcome and do not appear to adversely effect the growth or health of children during infancy or early childhood (31). Furthermore, available data do not indicate an elevation in congenital malformations for children born after freeze-thaw procedures (32–34). Although it remains unclear whether the freezing of blastocysts poses any additional risks over earlier stages, there is no direct evidence to raise concern at this time.

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References


