A modified embryo cryopreservation method increases post-thaw survival with a concomitant increase in implantation

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Objective: To achieve maximum post-thaw survival of frozen embryos.

Design: Historical controlled study.

Setting: Hospital-based fertility center.

Patient(s): One hundred forty-five patients whose embryos were frozen and thawed according to the standard method, and 56 patients whose embryos were frozen and thawed according to a modified method.

Intervention(s): Modifications were made to the various steps of cryopreservation: freezing and thawing solutions, loading of embryos into the straws, and warming rates.

Main Outcome Measure(s): Post-thaw survival, implantation, and pregnancy rates.

Result(s): With the modified method, 138 (93%) of the 149 embryos thawed for 56 patients survived freezing, and 79.8% had all their blastomeres intact, which is almost double the result obtained (41.8%) for patients whose embryos were thawed with the standard method. The implantation and pregnancy rates were also significantly higher with the modified method compared with the standard method.

Conclusion(s): Greater post-thaw embryo survival was achieved, with a concomitant increase in implantation and pregnancy rates, by modifying the various steps in the standard cryopreservation methodology. This has important implications in IVF practice. (Fertil Steril 2005;84:1498–504. ©2005 by American Society for Reproductive Medicine.)

Key Words: Embryo cryopreservation, post-thaw survival, blastomeres, IVF, implantation, pregnancy, propanediol

Embryo cryopreservation has been a routine component of clinical IVF programs for more than 2 decades (1) but has a relatively poor outcome in terms of post-thaw survival and pregnancy rates in a majority of IVF programs.

Efficient embryo cryopreservation has several advantages. It helps to reduce costs and increases cumulative pregnancy rates. It can also help in cases of canceled ET in fresh cycles caused by ovarian hyperstimulation syndrome and difficulties in ET.

One of the major concerns in IVF is high-order multiple pregnancies, which result from the transfer of multiple embryos in a given cycle. In the last 5 years, new fertility drugs for better clinical management, improved culture and laboratory systems, and better identification of viable embryos have enhanced the success rates of IVF. During the same period, some clinics have started the practice of transferring two embryos to reduce multiple pregnancy rates without compromising overall pregnancy rates (2–5).

Furthermore, in recent years some European countries, particularly the Scandinavian countries, have taken a lead in performing elective single-embryo transfers and have achieved acceptable pregnancy rates (6–9). This trend is spreading to other countries. This can result in embryos being available for freezing for almost every IVF/intracytoplasmic sperm injection (ICSI) patient aged <40 years and has created the necessity to have an excellent freezing program in routine clinical IVF practice.

Review of the current literature reveals unsatisfactory post-thaw embryo survival, implantation, and pregnancy rates. Post-thaw survival rates vary from 50% to 80% for different embryo stages. Implantation and pregnancy rates have varied from 3% to 15% and 15% to 25% respectively, which is approximately half of the rates achieved for fresh ETs (10–18).
To improve the outcome of frozen ET cycles, we attempted to modify the various steps of the cryopreservation protocol. In a pilot study on arrested and fragmented embryos (grades 3 and 4), we achieved post-thaw survival rates of 80% and 65%, respectively, having all blastomeres intact after making modifications to the various steps of the standard method. Encouraged by the post-thaw survival outcomes of these embryos, we used this modified method in our routine clinical frozen ET program. This report describes the improvements of the modified method, in comparison with the standard method, and the results therefrom.

**MATERIALS AND METHODS**

**Study Design and Patients**

We designed a nonrandomized, historical controlled study. Embryos from 145 patients were frozen and thawed according to the standard method (12). The treatment group consisted of 56 patients whose embryos were frozen and thawed according to a modified method. The outcomes of the freeze-thaw cycles in the treatment group were compared with those of the 145 patients whose embryos were frozen and thawed previously according to the standard method. The mean (±SD) age of the patients in the standard and modified groups was 35.4 ± 3.8 years and 35.8 ± 3.4 years, respectively.

In our routine embryo freezing program, patients sign consent forms to freeze their embryos. Before using the modified method in our clinical IVF programs, we performed a pilot study on arrested and fragmented embryos and obtained better post-thaw survival of embryos compared with the standard method. Hence, approval from the hospital ethics committee was not obtained when we used the modified freezing method.

In the standard method, embryos from 145 IVF/ICSI patients were frozen and thawed from January 1997 to December 2001. In the modified method, embryos from 56 IVF/ICSI patients were frozen and thawed from January 2002 through December 2004.

**Fertilization and Embryo Culture**

Fertilization and embryo culture was performed with culture medium from Medicult (Jyllinge, Denmark) and Cook (Brisbane, Australia), according to our previously published protocol (19). Briefly, fertilized oocytes from IVF/ICSI patients were group-cultured in four-well dishes up to day 3 from the day of oocyte retrieval. The embryos were graded from 1 to 4 according to the criteria of Cummins et al. (20). The clinical outcomes from fresh embryos during the two distinct time periods are given in Table 1.

**Selection of Embryos for Freezing**

It is a standard practice in our laboratory to choose the best embryos for transfer on day 3 and freeze the supernumerary grade 1 and grade 2 embryos with more than five cells on day 3.

**Freezing Solutions**

The freezing solutions used in the standard method were obtained from Medicult and contain 1,2-propanediol and 0.1 mol/L sucrose as cryoprotectants in phosphate-buffered saline (PBS). The freezing solutions used in the modified method were obtained from SureLife Fertility Technologies (SureLife; Singapore). These were similar to the freezing solutions from Medicult, except that the freezing solutions in the modified method were made in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-based cleavage medium instead of PBS, added with 20 mg/mL human serum albumin.

**Thawing Solutions**

The thawing solutions used in the standard and modified methods were again from Medicult and SureLife, respectively. The thawing package from Medicult consists of solutions 1, 2, and 3 with 1.0 mol/L and 0.5 mol/L 1, 2-propanediol and 0.2 mol/L sucrose in PBS. The thawing

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard method group</th>
<th>Modified method group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cycles</td>
<td>742</td>
<td>222</td>
</tr>
<tr>
<td>Total no. of oocytes</td>
<td>7,791</td>
<td>2,264</td>
</tr>
<tr>
<td>Normal fertilization</td>
<td>5,851 (75.1%)</td>
<td>1,693 (74.7%)</td>
</tr>
<tr>
<td>Mean no. of embryos</td>
<td>2.36</td>
<td>2.38</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>360 (48.5%)</td>
<td>105 (47.3%)</td>
</tr>
<tr>
<td>Miscarriage</td>
<td>93 (25.8%)</td>
<td>28 (26.6%)</td>
</tr>
<tr>
<td>Implantation</td>
<td>1,751 (26.0%)</td>
<td>139 (26.3%)</td>
</tr>
</tbody>
</table>

*Note: Data in parentheses are percentages. There was no statistically significant difference between the two groups between any of the parameters.*

package from SureLife contains 0.5 mol/L and 0.2 mol/L of sucrose in HEPES-based cleavage medium without any propanediol.

**Protocol for Embryo Freezing**

In both the methods, the freezing solutions 1, 2, and 3 were dispensed in 0.6–0.7-mL volumes into the corresponding wells of a four-well dish (Nunc, Roskilde, Denmark). The dish was allowed to equilibrate to room temperature (25°C). In both methods, after ensuring that the freezing solutions had attained 25°C, embryos were transferred into the well containing freezing solution 1. After 5 minutes, embryos were transferred into well two, containing freezing solution 2, for 10 minutes. The embryos were then transferred into solution 3 and left for 15 minutes.

**Embryo Loading**

Embryos were frozen in 0.25-mL straws (CryoBioSystem, L’Áigle, Cedex, France). In the standard method, freezing solution 3 (Medicult) and embryos were loaded in the following sequence. Solution 3, containing 1.5 mol/L propanediol and 0.1 mol/L sucrose, was drawn into the straw with a 1-mL tuberculin syringe as an approximately 2-cm column, followed by a similar column of air and then an approximately 2-cm column of solution 3 with the embryos, followed by another column of air and then a small column of solution 3, then the straw was sealed with a plug (Fig. 1). Loading of the straws was completed within 15 minutes from the time embryos were transferred into freezing solution 3.

In the modified method, the straws were filled with freezing solution 3 (SureLife) in the following sequence. With a tuberculin syringe fitted to the straw, a small column (approximately 2 cm) of solution 3 was drawn into the straw, followed by a similarly sized column of air, then an approximately 3-cm column of freezing solution 3, followed by an approximately 2-cm column of air; the remaining portion of the straw was filled with freezing solution 3, with the plunger of the syringe pulled to its maximum (Fig. 1). This ensured that the freezing solution in the straw remained tight.

With a pulled “L”-shaped Pasteur pipette, the embryos were picked up and deposited approximately 2 mm away from the lower meniscus in the column of freezing solution between the two columns of air. Care was taken in removing the pipette from the straw. The straws were sealed with colored plugs. The number of embryos in each straw varied from one to three, depending on their grade and number of blastomeres, as follows; eight-cell grade 1 embryos were loaded one per straw; five- to seven-cell grade 1 embryos were loaded two per straw; five- to eight-cell grade 2 embryos were loaded three per straw.

**Freezing Program**

The freezing program was similar for both the methods. After sealing, the straws were fixed to the straw holder of the Planer Kryo 10-1.7 MRV freezing machine (Planer, Sunbury, United Kingdom) and cooled as follows.

Cooling was started at 25°C at the rate of −2°C/min down to −7°C, at which point seeding was performed by touching the straws for a few seconds at the upper meniscus of the middle column of freezing solution with long forceps fitted with gauze dipped in liquid nitrogen (LN₂). Seeding was confirmed by the appearance of a small white speck of ice crystal in the straw. The straws were held at −7°C for 10

![FIGURE 1](http://example.com/figure1.png)

Comparative diagram of straws containing embryos, loaded differently. In the standard freezing method, embryos are drawn into the straw along with an approximately 2-cm column of cryoprotectant (CPA) solution. In the modified freezing method, a pulled “L”-shaped Pasteur pipette is used to deposit embryos approximately 2 mm away from the lower meniscus in the approximately 3-cm column of CPA.

<table>
<thead>
<tr>
<th>Standard freezing method</th>
<th>Modified freezing method</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPA</td>
<td>air</td>
</tr>
<tr>
<td>embryos</td>
<td></td>
</tr>
</tbody>
</table>

| CPA | air | o o o | air | CPA |
| embryos |

minutes after seeding, and cooling was continued to $-30^\circ C$ at the rate of $-0.3^\circ C/min$ and then cooled rapidly to $-150^\circ C$ at the rate of $-50^\circ C/min$. Embryos were held at $-150^\circ C$ for 10 min before being plunged into LN$_2$.

**Embryo Thawing**

In the standard method, thaw solutions 1, 2, and 3 (Medicult) were dispensed in 0.6–0.7-mL volumes into the corresponding wells of a four-well dish (Nunc) and allowed to equilibrate at room temperature (25°C). After ensuring that the thaw solutions had reached room temperature (25°C), frozen straws were taken out from the LN$_2$ Dewar and held in air for 30 seconds and then immersed in a water bath at 30°C for 45 seconds.

Then the straws were wiped with a paper tissue, and the end opposite to the plugged end was severed and connected to a 1-mL syringe. The plug at the other end was removed gently and carefully, without shaking the straw. The embryos were released into the center of the four-well dish; it was ensured that all the embryos were found. The embryos were then transferred immediately into thawing solution 1 in well 1. After 5 minutes, embryos were transferred into well 2 containing thawing solution 2 for 5 minutes and then into thawing solution 3 for 10 minutes. The embryos were then transferred into equilibrated cleavage medium (Cook).

In the modified method, thaw solutions (SureLife) were dispensed into four-well dishes (Nunc) and allowed to equilibrate as above. The straws were removed from the LN$_2$ Dewar and held in air for approximately 2 minutes, then the propanediol was removed in three steps, with an interval of 5 minutes between the first two thaw solutions and 10 minutes in the last solution.

The embryos in both the methods were subjected to zona thinning with acid Tyrode’s solution to assist hatching and kept in the incubator for at least 1 hour before transfer into the uterus.

**Embryo Transfer**

Embryos were transferred in downregulated hormone replacement cycles. Embryos were transferred into the uterus with a soft transfer catheter (K-Soft-7000; Cook). Serum β-hCG levels were measured in patients’ serum 10 days after ET and then serially to detect any rise in the titer. Implantation was confirmed by the appearance of a gestational sac in the uterus on transvaginal ultrasonography.

Data were expressed as mean ± SEM. The post-thaw survival, implantation, and pregnancy rates were compared between the standard and modified method with the $\chi^2$ test, and $P<.05$ was considered statistically significant.

## RESULTS

**Survival of Cryopreserved Embryos with the Standard Method**

Our results from cryopreservation of embryos with the standard method are shown in Table 2. Seventy-four percent of the embryos had $\geq 50$ of the original blastomeres intact after thawing, and only 41.8% remained fully intact.

### TABLE 2

**Comparison of standard and modified methods for post-thaw survival, implantation, and pregnancy rates.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard method</th>
<th>Modified method</th>
<th>$P$</th>
</tr>
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<tbody>
<tr>
<td>Total cycles</td>
<td>145</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Total embryos thawed</td>
<td>380</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>Post-thaw survival (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>45 (11.8)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1–49</td>
<td>54 (14.2)</td>
<td>11 (7.38)</td>
<td></td>
</tr>
<tr>
<td>50–99</td>
<td>122 (32.1)</td>
<td>19 (13.0)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>159 (41.8)</td>
<td>119 (79.8)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Total no. of embryos transferred</td>
<td>335</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>Mean no. transferred</td>
<td>2.31</td>
<td>2.35</td>
<td>NS</td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>31 (21.3)</td>
<td>23 (41.0)</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Miscarriage</td>
<td>3 (9.68)</td>
<td>2 (8.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation</td>
<td>28 (8.36)</td>
<td>24 (18.1)</td>
<td>&lt;.007</td>
</tr>
<tr>
<td>Singleton</td>
<td>28</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Twins</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Note: Data in parentheses are percentages. NS = nonsignificant.

Survival of Cryopreserved Embryos with the Modified Method
The results obtained after freezing embryos with the modified cryopreservation method are shown in Table 2. The proportion of embryos with >50% of the original blastomeres intact were significantly (P<.0001) higher with the modified method (93%) compared with the standard method (74%). The proportion of embryos undergoing complete lysis was nil in the modified method, compared with 11.8% (P<.0001) in the standard method. Total blastomere survival was significantly higher (P<.0001) with the modified method (79.8% vs. 41.8%).

Although this age-matched comparison with historical controls is not an ideal comparison because the embryos were frozen during different time periods, the differences in post-thaw survival were such that a randomized controlled trial to compare the methods would be unethical. The embryos in both the standard and modified groups were from patients with similar mean ages (35.4 ± 3.8 and 35.8 ± 3.4 years, respectively). The embryo survival in the standard and the modified groups was not affected by the number of embryos in each straw.

Implantation of Embryos Cryopreserved with the Standard Method
One hundred forty-five thaw cycles resulted in 145 transfer procedures, in which a total of 335 embryos were transferred (Table 2). Thirty-one (21.3%) of these transfers resulted in clinical pregnancy. Twenty-eight of these showed fetal heart beats (8.36% implantation rate).

Implantation of Embryos Cryopreserved with the Modified Method
In the modified method, 56 thaw cycles resulted in 56 transfer procedures, in which a total of 132 embryos were transferred (Table 2). Twenty-three of these (41.0%) resulted in clinical pregnancies, which was significantly higher (P<.005) than with the standard method. Twenty-one of these progressed further, with 3 patients showing twin gestational sacs (18.1% implantation rate), which was significantly (P<.007) more than with the standard method.

DISCUSSION
There are several reports on frozen ET (10–18). Most of these studies report an overall post-thaw survival rate of 50%–80%, with 43%–55% having all their blastomeres intact. Thus, on average, only 50% of the embryos that are frozen have all their blastomeres intact. Only 25% of the embryos have 50%–99% of their blastomeres intact. It has been shown that implantation and pregnancy rates of embryos with 100% survival of blastomeres are almost double those of embryos with 50%–99% survival of blastomeres, and embryos with <50% survival of blastomeres do not implant at all (14). Thus, to increase the implantation rate, it is important to improve the cryopreservation method to achieve 100% survival of blastomeres in the maximum number of embryos.

In an effort to improve these results, several aspects of the cryopreservation method were modified in this laboratory. In the modified method, we achieved a post-thaw survival rate of 93%, and 80% of the total embryos survived with all their blastomeres intact, with a concomitant increase in implantation rates. This is double the result reported for the standard method of ours and also reported in the literature. This could be attributed to changes made to the various steps of the cryopreservation protocol.

First, HEPES-based cleavage medium containing cryoprotectants was used in the modified method, as opposed to PBS as basal medium in the standard method. Some of the components in the HEPES cleavage medium, particularly certain salts, amino acids, and human serum albumin, might have some cryoprotective properties. Furthermore, exposure of embryos to cryoprotectants in PBS might compromise their viability, owing to lack of amino acids and other nutrients that are present in cleavage medium. However, further studies are required to substantiate our hypothesis.

Second, we made changes to the method of loading embryos into the straws. In the standard method, during the filling of the straws with freezing solutions, embryos are drawn into the middle column along with the freezing solution. Sometimes the embryos might not stay together, and some might move toward the upper meniscus of the second column, where seeding is performed. It is possible that during seeding on the meniscus, some embryos might be exposed to a sudden change in temperature, resulting in formation of ice crystals in embryos that are only partially hydrated. This might result in lysis of the blastomeres on warming.

In the modified method described here, embryos were prevented from moving to the upper meniscus by filling the freezing straws with the freezing solution first and then loading the embryos with an “L”-shaped pulled Pasteur pipette. This ensures that embryos remain together close to the lower meniscus of the freezing column and do not move within the column, even if the straw is turned upside-down. However, caution must be exercised not to introduce air bubbles and to avoid breakage of the “L”-shaped pipette.

Third, we made changes to the warming rates. The optimal warming rate of embryos depends on the cooling procedure. Some freezing programs terminate the cooling of embryos at −30°C, whereas others continue to −150°C and then plunge into LN₂. In both instances, straws have been warmed by exposure to room temperature for 30 seconds and then immersion in a water bath at 30°C for 30 seconds (14–19). However, each method of cooling achieves a different degree of dehydration, with different requirements of warming rate to ensure minimal damage to blastomeres.
If a small amount of intracellular water is present in the embryos when the cooling is stopped at $-30^\circ$C and the straw is plunged into LN$_2$, small ice crystals can form. Under these circumstances, rapid warming rates are essential (room temperature for 30 seconds, followed by a water bath at $30^\circ$C for 30 seconds). A slow warming rate would allow the small ice crystals time to grow into larger, harmful crystals (20, 21).

If embryos are cooled slowly to $-150^\circ$C, they will be nearly completely dehydrated when they are plunged into LN$_2$. These embryos might be damaged by rapid warming because of extremely rapid changes in osmolarity in the dehydrated embryo. Slow warming, which allows more time for cells to rehydrate, is best for severely dehydrated embryos. Thus, in the present study embryos were cooled to $-150^\circ$C before being plunged into LN$_2$ and warmed by leaving the straws at room temperature for 2 minutes. This gave the embryos more time to rehydrate and minimized damage to the blastomeres (20, 21).

Fourth, in routine practice, cryoprotectant is removed from the embryos in three to four steps of progressively lower concentrations of cryoprotectant. This reduces swelling and subsequent lyses of blastomeres. An alternative and more rapid method for removing cryoprotectant from embryos is to use a high concentration of a nonpenetrating molecule, such as sucrose. The high extracellular concentration of sucrose counterbalances the high concentration of propanediol in the embryo because it reduces the differences in the osmolarity between the inside and the outside of the cell (22).

Embryos containing propanediol shrink when placed in a concentrated sucrose solution (0.5 mol/L), indicating that both propanediol and water are leaving the cell; because of this it was difficult to assess the survival of embryos immediately. The use of 0.5 mol/L and 0.2 mol/L sucrose in two steps each of 5 minutes in the modified thawing solutions achieved this aim here and is simpler than the step-wise reduction of cryoprotectant concentration in the standard method.

The second criterion that is used to assess the efficiency of an embryo-freezing program is the ability of embryos to grow further in vitro and particularly in vivo. Ideally it is better to freeze embryos on day 2 and compare the development of these embryos after thawing to the rate of development of fresh embryos on day 3. However, the practice in this laboratory is to choose the best embryos on day 3 for transfer and freeze the supernumerary embryos.

Indirect evidence of further development of frozen–thawed embryos comes from the development of these embryos in vivo. Of the 56 patients who underwent frozen ET, 23 achieved clinical pregnancy, with an implantation rate of 18.1% and a clinical pregnancy rate of 41%. This further confirms the efficacy of the modified cryopreservation method. The implantation and pregnancy rates reported here are greater than those obtained with our standard method and those reported elsewhere (11, 14–18).

Thus, modifications to various steps in the cryopreservation protocol improve the post-thaw survival, with concomitant increase in implantation rates, and this could be due to higher numbers of embryos with 100% blastomere survival. Consequently, we observed a 13% twinning rate among patients in the modified method group as compared with none in the standard method group. The three patients who had two eight-cell embryos each transferred had all their blastomeres intact.

It is difficult to exactly pinpoint whether the improvement in post-thaw survival was due to the improved freezing or thawing protocol. However, the improvement could be attributed mainly to changes made to both the freezing and thawing protocols, particularly the loading of embryos into the straws and the warming rates at room temperature.

It has been reported that the highest implantation and pregnancy rates come from embryos with all their blastomeres intact (14). Thus, it is important to achieve 100% survival of blastomeres in the maximum number of embryos, which in turn results in higher implantation and pregnancy rates. Obviously, results reported here confirm this.

In conclusion, this is the first report of high post-thaw embryo survival after modification of the standard method. The proper study design would have been a prospectively randomized study, with half of the cycles or half of the embryos per cycle frozen with the standard and half with the modified protocol. However, the differences in post-thaw survival were such that a prospective randomized study was unethical.

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