Equivalent blastocyst rates after freezing murine embryos in Cryo Bio System high security or standard Instruments-Medicine-Veterinarian straws

David L. Walker, B.S., a Diane G. Hammitt, Ph.D., a, b Phillip A. Dumesic, a and Alan R. Thornhill, Ph.D. a

Division of Reproductive Endocrinology and Infertility, Mayo Clinic, Rochester, Minnesota

Objective: To validate the Cryo Bio System (CBS) straw in our current cryopreservation system before using it in clinical practice.

Design: A prospective comparison of blastocyst development rates in 278 murine embryos after refreezing and thawing at the two-cell stage against the standard Instruments-Medicine-Veterinarian (IMV) straw used in our cryopreservation program.

Setting: Private IVF laboratory.

Patient(s): No human subjects or material was used in this study.

Intervention(s): Frozen two-cell murine embryos were thawed and randomized into three treatments [1] refreezing in the CBS straws, [2] refreezing in IMV 0.25-mL straws, and [3] control embryos remaining in culture without refreezing. Embryos were refrozen using identical cryoprotectants and identical programmed controlled-rate freezers. After cryopreservation, straws were held in liquid nitrogen for a brief period before thawing and continued culture.

Main Outcome Measure(s): Postthaw murine blastocyst development rate.

Result(s): When the manufacturer’s filling and loading protocol was used for the CBS straw there was no significant difference in the blastocyst development rate between CBS (75.0%) and IMV (76.4%) straws.

Conclusion(s): The CBS straw may be a viable and potentially safer alternative for cryopreservation of human embryos, particularly for patients with known infections. (Fertil Steril 2003;80(Suppl 2):743–6. ©2003 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, embryo, murine blastocyst, infectious disease

Requests for infertility treatment from patients with serious viral infections are becoming more common. Many of these patients have limited clinical options. In some cases, denial of care is policy, whereas other patients are offered only fresh embryo transfers with no opportunity to cryopreserve supernumerary embryos in the absence of safe cryostorage. Although universal precautions are practiced in the IVF laboratory, extra measures may be needed to prevent cross-contamination between infected and noninfected gametes and embryos, particularly during cryostorage in liquid nitrogen. Although no cases of viral cross-contamination between cryopreserved human gametes or embryos have been reported to date, the reported cross-contamination of hepatitis B virus from cryopreserved bone marrow stored in bags raises concerns about the possibility of viral transmission through liquid nitrogen (1–3).

Because we wish to offer all patients the same level of care, we have attempted to validate the High Security Cryobiostraw (Cryo Bio System, IMV Technologies Group, L’Aigle, France, distributed by Conception Technologies, Inc., San Diego, CA; hereafter CBS) to allow future long-term storage of human embryos from couples with known infections.

According to the manufacturer’s information these straws, when used correctly, withstand bioexposure to neighboring infected samples in storage and contaminants in the
environment. Both CBS and standard cryopreservation straws have been compared in side-by-side tests for storage integrity using six different strains of bacteria. Postthaw survival and development of rabbit embryos, and viability and pregnancy rates postthaw and transfer of sheep embryos were compared in these studies. These unpublished data showed no transmission of bacteria into or out of the CBS straw and no significant differences in rabbit embryo development or sheep embryo survival and pregnancy between the two straw types (4).

The Assisted Reproductive Technology (ART) programs at Mayo Clinic Rochester, Minnesota, and Mayo Clinic Scottsdale, Arizona, have successful frozen ET programs. Embryo cryopreservation is routinely performed on supernumerary pronuclear-stage embryos in approximately 75% of IVF patients (5, 6) and is used exclusively for all pronuclear-stage embryos generated through the anonymous oocyte donation program at Mayo Clinic Rochester (7). The goal of the present study was to validate the CBS straw in our current cryopreservation system before using it in clinical practice. Our current human embryo cryopreservation program was previously established by conducting validation studies with murine embryos. In this study, we wished to use similar methods to assess the CBS straw in terms of embryo viability by a prospective comparison of blastocyst development rates in 278 murine embryos after refreezing embryo viability by a prospective comparison of blastocyst development rates in 278 murine embryos after refreezing and thawing at the two-cell stage using the CBS straw vs. the standard Instruments-Medicine-Veterinarian (IMV) 0.25-mL straws.

**MATERIALS AND METHODS**

Groups of frozen two-cell murine embryos (EmbryoTech Laboratories, Wilmington, MA and Conception Technologies Inc., San Diego, CA) thawed according to the supplier’s instructions on five separate days, were randomized into three treatments: [1] refreezing in CBS straws (n = 91), [2] refreezing in IMV 0.25-mL straws (n = 91), and [3] control embryos remaining in culture without refreezing (n = 96). Surviving embryos were pooled, allowed to equilibrate 10 minutes at room temperature, and randomized for refreezing or cultured as controls in equilibrated human tubal fluid with 10% (v/v) serum substitute supplement (HTF-SSS; Irvine Scientific, Inc., Irvine, CA) in a Falcon 3037 organ culture dish without oil overlay at 37°C at pH 7.35. Developmental progress was recorded every 24 hours up to 96 hours after thaw. The JMP 4 Statistical Software (SAS Institute, Cary, NC) was used to compare treatment groups using the Pearson exact test. This study did not require Institutional Review Board approval, as no human subjects or human material was used.

The freezing protocol, cryoprotectant, and equipment were identical for both straw types using two identical programmable controlled-rate freezers (Cryomed, Forma Scientific, Inc., Marietta, OH) simultaneously for refreezing. The cryoprotectant and thawing solutions, modified from Testart et al. (8), were identical to those used in our program for human pronuclear stage embryo freezing (5–7). Briefly, freezing solutions consisted of phosphate-buffered saline (PBS; Sage BioPharma, Bedminster, NJ) supplemented with 20% SSS (PBS-SSS), 1.5 M propylene glycol (PG; Sigma Chemical Co., St. Louis, MO), and 1.5 M PG/0.1 M sucrose in PBS supplemented with 20% SSS. Thaw solutions included 1.0 M PG/0.2 M sucrose, 0.5 M PG/0.2 M sucrose, 0.2 M sucrose in PBS-SSS and PBS-SSS without PG or sucrose. Two or three embryos were loaded into either the IMV straws using our standard loading technique or into the CBS straws according to the manufacturer’s recommended loading technique.

**Standard IMV Straw Method**

After cutting off the plugged end of the IMV straw, approximately 0.125 mL of the final cryoprotectant was aspirated into the straw as a solid column using a silicone connector attached to a 1.0-mL syringe. Using a finely drawn glass pipette, embryos were carefully placed near one end of the column and both ends of the straw heat-sealed using a Nyclave Impulse Heat Sealer (The Lorvic Corp., St. Louis, MO). A straw for controlling the freezing rate within the Cryomed freezing chamber was made by securing the tip of a thermocouple within the fluid column.

**CBS Straw Method**

A 5-mm section was removed from each end of the CBS straw. Before cutting the straw for use in the study (allowing us to use our programmable freezer without modification) the manufacturer was contacted and assurance given that the straw cutting would not jeopardize the biosecurity of the straw if proper filling and sealing procedures were followed.

To ensure straw “biosecurity,” the manufacturer placed emphasis on the following procedures: [1] obtaining a square cut for a secure attachment of the filling nozzle to the modified straw, [2] the use of a new nozzle for each straw, [3] secure fixing of the nozzle to the straw, [4] use of the correct submersion depth of the nozzle into the liquid used to fill the straws, [5] ensuring that fluid is not expelled back through the nozzle or that no fluid exists on the extremity of the straw after removal of the nozzle before sealing, and [6] completing a visual inspection to ensure an appropriate seal on both ends of the straw. All of these procedures were rigorously adhered to in the present study.

A color-coded identification (ID) rod was inserted into one end of the straw that is separated from the embryo column by a hydrophobic safety plug. A 1.0-mL syringe with an attached connection nozzle was placed over the end of the straw containing the ID rod and a filling nozzle attached to the opposite end of the straw. Approximately 0.05 mL of cryoprotectant, 0.05 mL of air, and approximately 0.125 mL of cryoprotectant (containing the embryos)
were aspirated in succession. The tip was removed from the cryoprotectant and the fluid column aspirated until the fluid was 5–10 mm away from the hydrophobic plug. The straw’s tip and then its ID rod end were sealed with the System of Manual Sealing (SYMS) (IMV Technologies Group, L’Aigle, France) heat-impulse sealing unit. A control straw was prepared as for the IMV straw.

**Freezing and Rethawing**

The different straw types were positioned in separate freezing racks that were placed simultaneously into two identical controlled-rate freezers. When the sample temperatures reached −7°C, manual seeding was performed by placing a liquid nitrogen-cooled metal rod over the fluid column opposite the end containing the embryos in the IMV straw and over the first small fluid column next to the air bubble in the CBS straws. Crystal formation and heat of fusion were recorded as an increase in the sample temperature to between −4°C and −5°C.

After cryopreservation, straws were held in liquid nitrogen for between 30 and 240 minutes, then rethawed, and allowed to continue culture. Straws were removed from liquid nitrogen and held at room temperature for 40 seconds, then warmed in sterile PBS at 30°C until the last ice crystals disappeared. Embryos were maintained at room temperature in each of the thaw solutions, before culture to the blastocyst stage in equilibrated HTF-SSS at 37°C.

**RESULTS**

Our laboratory considers a 75% blastocyst development by 72 hours post-thaw the minimum pass rate for commercially derived frozen murine two-cell embryos. Control embryos routinely achieve a >95% blastocyst development rate. Lower development rates were expected in this study, however, as embryos were subjected to a second freeze and thaw process. Preliminary results for the CBS straw using our standard filling and loading method and 10 embryos per straw were discouraging compared with the IMV straw (55.6% vs. 83.3% blastocyst formation after 72 hours, respectively). When the manufacturer’s filling and loading protocol was used for the CBS straw and the embryo number per straw decreased to two to three embryos (in line with our current clinical practice), there was no difference in the blastocyst development rate at 72 hours between the CBS (75.0%) and IMV (76.4%) straws (Table 1). Control embryos were used to verify appropriate developmental potential from the commercially derived embryos and optimal culture conditions. The 96 embryos randomized as controls had a 99% blastocyst development rate at 72 hours and a 100% development rate at 96 hours. These rates were significantly (P < .0001) higher than either the CBS or IMV refrozen embryos, as expected.

**DISCUSSION**

Cryopreservation of supernumerary embryos after IVF is a standard of care worldwide, the aim of which is to maximize pregnancy rates for each oocyte retrieval. The IVF laboratory has the responsibility to maintain the safety and integrity of patient embryos in cryostorage. The potential for secure gamete and embryo storage may be achieved with the CBS High Security Straw, as the straw is guaranteed by the manufacturer to prevent viral cross-contamination (“biosecurity”), although this characteristic of the straw was not tested in the present study. The manufacturers guarantee the biosecurity of the CBS straw only if there is an autogenic weld or “hermetic seal” on both ends of the straw. Correct use of the nozzle ensures that no potentially contaminated fluid is on the outside of the straw or around the straw opening that would prevent a secure hermetic seal.

The manufacturer’s literature describes a study comparing “inside-toward-outside” and “outside-toward-inside” contamination in both CBS and conventional straws during a 6- and 9-month period. The results showed the absence of contamination using the CBS straws under highly contaminated conditions, whereas protection with the conventional

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total 2-cell</th>
<th>Embryo loss</th>
<th>2 cells intact</th>
<th>1 cell intact</th>
<th>0 cells intact</th>
<th>Total loss or damage</th>
<th>72 hour blastocyst rate</th>
<th>96 hour blastocyst rate</th>
<th>96 hour hatching blastocyst rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1</td>
<td>91</td>
<td>3.3%</td>
<td>81.8%</td>
<td>10.2%</td>
<td>8.0%</td>
<td>21.5%</td>
<td>75.0% (66/88)</td>
<td>29.5%</td>
<td>72.7%</td>
</tr>
<tr>
<td>B 2</td>
<td>91</td>
<td>2.2%</td>
<td>87.6%</td>
<td>10.1%</td>
<td>2.2%</td>
<td>14.5%</td>
<td>76.4% (68/89)</td>
<td>34.8%</td>
<td>75.3%</td>
</tr>
<tr>
<td>C 3</td>
<td>96</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>99.0%</td>
<td>74.0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

A = CBS high security straw; B = IVM standard straw; C = control, nonrefrozen.

*Includes embryos lost and those with one or zero cells intact post-thaw.

AC, BC, P < .0001.

straw was described as moderate under highly contaminated conditions (9).

We wished to verify embryo viability postthaw using the CBS straw while making only minimal changes to previously optimized embryo cryopreservation protocols. The limited numbers of normal human embryos available for research coupled with unreliable and variable development of abnormally fertilized oocytes prompted the selection of murine embryos for this study. Commercially available murine embryos provide high-quality test material that may be obtained in large quantities. Thaw synchronization allows for good experimental design and murine embryos show remarkably consistent and reliable development under conditions optimized for human embryo culture.

Initially, our attempts to replace the IMV straw with the CBS straw with no changes to our filling and loading protocol were discouraging, possibly due to differences in overall dimensions of the two straw types. After adopting the manufacturer’s filling and loading instructions for the CBS straws and freezing fewer embryos per straw (a protocol more closely associated with our clinical practice) the results achieved between the two straws were nearly identical.

The data presented here have led us to implement a policy of using CBS straws to cryopreserve embryos from patients with infectious disease. These embryos will be stored in separate tanks as a further precaution against cross-contamination between known potentially infected samples and known negative samples. An additional precaution for the future safe long-term storage of gametes and embryos is the use of liquid nitrogen vapor-phase storage systems. Such measures are thought to virtually eliminate the chance of cross-contamination between samples by removing the vector (nitrogen in liquid phase) for viral transmission while reducing exposure of stored specimens to potentially infected liquid and the contaminants that reside therein (1). Vapor-phase storage is possible by simply maintaining samples at a constant level above the liquid nitrogen. However, the potential temperature variability inherent in this arrangement makes the advent of a new generation of automated “isothermal” vapor-phase storage vessels a more attractive option (1). Assuming validation studies are successful in maintaining both specimen integrity and viability, vapor-phase storage vessels may soon be used routinely for human embryo storage, as vapor-phase has already been shown to be effective for semen storage (1, 10) and in a small series using mouse embryos (1).

In conclusion, the sensitivity, homogeneity, and availability of frozen murine embryos makes them a useful model for validating cryopreservation conditions, solutions, and equipment before clinical implementation.

Using the manufacturer’s recommended filling and embryo loading protocol, we achieved similar postthaw blastocyst development rates using murine embryos frozen in either CBS or IMV straws.

After optimization to compensate for differences in straw dimensions, the CBS straw may be a viable and potentially safer alternative for the cryopreservation of human embryos, particularly for patients with known infections.

References