Experimental contamination assessment of a novel closed ultravitrification device

Enrique Criado, B.Sc.,* Federica Moalli, Ph.D.,† Nadia Polentarutti, B.Sc.,* Elena Albani, B.Sc.,* Giovanna Morreale, B.Sc.,* Francesca Menduni, B.Sc.,* and Paolo Emanuele Levi-Setti, M.D.†

*Department of Gynecology and Reproductive Medicine, Istituto Clinico Humanitas, Rozzano, Milan; and †Department of Immunology and Inflammation, Istituto di Ricoercere a Cur a Carattere Scientifico, Istituto Clinico Humanitas, Rozzano, Milan, Italy

Objective: To evaluate the safety of a new ultravitrification closed device.

Design: Ultravitrification research.

Setting: Private assisted reproduction center.

Animal(s): Microorganisms (bacteria).

Intervention(s): A styrofoam container holding 1,000 mL of liquid nitrogen (LN2) was contaminated with *Pseudomonas aeruginosa* and *Escherichia coli*. Forty closed devices (Ultravit) and 20 open devices (Cryotop) loading approximately 0.5 μL of antibiotic-free medium were plunged into this contaminated LN2 for 5–10 seconds and then inoculated into selective culture dishes. Colony-forming units were analyzed and counted after an overnight incubation at 37°C.

Main Outcome Measure(s): Detection of micro-organisms in different devices after incubation.

Result(s): There was no contamination in any of the closed devices, whereas in 45% of open devices these bacteria were present.

Conclusion(s): With this study we demonstrated, in an experimental model using contaminated LN2, that this new closed device is a safe system that does not allow cell contact with LN2, avoiding cell contamination. (Fertil Steril® 2011;95:1777–9. ©2011 by American Society for Reproductive Medicine.)

Key Words: Contamination, cross-contamination, risk, Ultravit, bacteria, oocyte ultravitrification, cryopreservation

The potential for disease transmission through contaminated liquid nitrogen (LN2) has been proposed by many authors (1–3), and the evidence of contamination in human patients has been described for different pathogens (4–10). It has to be stated that none of the reported infections after insemination or ET in humans and domestic animals can be clearly attributed to the applied cryopreservation and storage procedure (11, 12), but the use of safe vitrification devices is very important to avoid human cells contamination or cross-contamination in common LN2 tanks.

According to data from Bielanski et al. (11), contamination by direct contact with LN2 depends on the surface of cryoprotectant solution in contact with the coolant solution. We think that the exposure time of the sample to the infected coolant solution is determinant if the cryopreservation straw has a small diameter. Therefore, a cryovial with a diameter of 5 mm has more contamination risk than a straw or a microcapillary tube with a diameter of 0.300 mm with the same exposure time. At the same time, long direct contact (hours, days, or years) between the sample and the possibly infected coolant solution has more risk than contact of a few seconds.

Traditional slow-freezing techniques are associated with low contamination risk, cross-contamination, and often requiring a high concentration of CPA (4–6 M) that is toxic to most mammalian cells (13–21). A unique method for human oocytes ultravitrification was developed by Criado et al. (22); with this technique it is possible to obtain a vitrified state for human oocytes in the absence of toxic levels of CPA. Increasing the cooling rate by an order of magnitude makes it possible to ultravitrify human oocytes with 2 M of intracellular CPA + 0.5 M of extracellular CPA, with a 92% survival rate. According to Lee et al. (23), decreasing CPA toxicity improves survival, fertilization, and development rates, reaching 90% survival, 75% fertilization, and 59.1% blastulation with murine oocytes.

A new closed device (Ultravit, Criado, Spain) using this new technique allows ultravitrification with just 5 seconds’ contact of the microcapillary tube with the cooling solution. Cell position inside the microcapillary tube (Fig. 1) in Ultravit avoids direct contact of cells and of the cryoprotective medium with the cooling solution. After these 5 seconds the microcapillary tube is passed to the protective sheath and enclosed with an ultrasound sealer, enabling storage in common tanks without cross-contamination risk. To check Ultravit safety, we designed an experimental asset using contaminated LN2 comparing Ultravit’s microcapillary tubes and other different open vitrification devices in the same exposure timeframe.

MATERIALS AND METHODS

Ultravast Closed Ultravitrification Device: Ultravit

Ultravit is composed of a 0.3-mm internal diameter microcapillary tube and a flexible, transparent inert sheath that has been designed...
to protect and prevent its floating in the LN2. Loading the internal microcapillary tube and removing the cells from the device is very simple and easy using a Hamilton syringe. Before warming, the protective sheath is cut and the internal microcapillary tube is placed in a sterile medium at 37°C after the thawing protocol. The cooling rate obtained with Ultravit was 250,000°C/min (24), allowing ultra-vitrification with low concentration of CPA. The system is very resistant to low temperatures and pressures caused by the expansion of the liquids and the LN2. The open end of the sheath can be sealed ultrasonically in milliseconds without affecting the temperature inside the microcapillary tube, closing the system and ensuring a hermetic seal, thus preventing entry of LN2. Ultravit is designed for easy patient identification, with sufficient area to write necessary information and biocompatible materials to make safe its use with human cells.

Open Device: Cryotop

Kuwayama and Kato (25) developed the Cryotop method (Kitazato Supply, Fujinomiya, Japan) using a fine polypropylene strip attached to a hard plastic handle. Oocytes or embryos are suspended on this polypropylene strip and then plunged into LN2, where cells have direct contact with LN2. After vitrification a hard plastic cover is attached non-hermetically to the protective strip during storage in LN2, making possible its entry into the device when stored in LN2 tanks.

LN2 Contamination

For this study we used Pseudomonas aeruginosa (PAO1; kindly provided by Alessandra Bragonzi, San Raffaele Institute, Milan, Italy) and Escherichia coli (JM109; Takara Bio, Shiga, Japan). These microorganisms were previously used in another study on experimental contamination of LN2 vapor shipper dewars (26). The same microorganisms were detected in LN2 and in cryostored semen and embryos during long-term storage (12).

A sterile styrofoam container holding 1,000 mL of LN2 was contaminated with 3 mL of 3.6 × 10⁹ PAO1 and 3 mL of 1.8 × 10⁹ JM109 and then was agitated and left for 15 minutes before the experiment.

Experiment

We loaded approximately 0.5 μL of antibiotic-free sterile phosphate-buffered saline (PBS) in Ultravit’s internal microcapillary tube and the same volume on Cryotop’s strip and then plunged them into the contaminated LN2 for 5–10 seconds. After that, samples were plated on McConkey agar dishes (BD Biosciences, Franklin Lakes, NJ) for the selective isolation of enterobacteria, or on pseudomonas isolation agar dishes (Pseudomonas Isolation, BD Biosciences) for the selective isolation of P. aeruginosa.

Positive and negative controls were performed for both species of bacteria in Ultravit and Cryotop devices. For negative control approximately 0.5 μL of antibiotic-free sterile PBS was loaded into Ultravit’s microcapillary tubes and on Cryotop’s strips and then plated on selective agar dishes. For positive control approximately 0.5 μL of bacterial solution was loaded into Ultravit’s microcapillary tubes and on Cryotop’s strips and then plated on the selective agar dishes for both bacteria. To be sure that bacteria survived in LN2 we did a positive control in contaminated LN2 taking 50 mL of LN2 after the experiment, and when N2 evaporated 10 mL of sterile PBS was resuspended and then plated on selective agar dishes for both bacteria. Colony-forming units were counted after 20 hours of growth at 37°C (Fig. 2).

RESULTS

None of Ultravit’s internal microcapillary tubes (0 of 40) were contaminated with any pathogen, and 45% of Cryotop’s strips (9 of 20) were contaminated with some pathogen (Table 1). None of the negative controls (0 of 32) were positive for any pathogen in both devices, and 100% of the positive controls (32 of 32) were positive for both pathogens in both devices. One hundred percent of the positive controls in LN2 after the experiment (30 of 30) were positive for both pathogens, with a final concentration of 1.74 × 10⁹ for PAO1 and 1.17 × 10⁹ JM109.

DISCUSSION

These results using artificial contamination demonstrate that the Ultravit device is a safe vitrification system that can preserve human...
sections under 0.3 mm and the loading device is important in avoiding contamination with other cells.

Our data (Table 1) demonstrate that the lack of contamination is due to the short time of cell exposure to the coolant solution (5–10 seconds) and the microcapillary tube’s diameter (0.300 mm), and that the load device is important in avoiding contact of the medium containing cells with the LN2.

The complete absence of contamination in negative controls indicates that everything was sterile when we did the experiment, and the 100% contamination in positive controls demonstrates that a small volume of bacterial solution (approximately 0.5 μL) is enough to detect contamination in vitrification devices. The similar bacterial concentration we found in LN2 after the experiment shows that bacteria can survive storage at extremely low temperatures, with P. aeruginosa being more resistant than E. coli.

The new human oocyte ultravitrification protocol recently developed by Criado et al. (22) can be applied safely only through the use of the Ultravit device. This protocol allows ultravitrification of human oocytes with 2 M of intracellular CPA instead of the high CPA concentration generally used in vitrification (4–6 M). The survival rate with this technique in human mature oocytes is 92% (22) with a concentration of CPA typical of slow freezing. Using this new device, there is the possibility of storage in common tanks while avoiding contamination or cross-contamination.

Some authors describe protocols to sterilize LN2 by ultraviolet light (27) or by filtering (Cobo, personal communication, CRYO Congress, Valencia, Spain, 2010). Both systems would avoid cellular contamination at the time that the cells come in contact with LN2, but after vitrification these samples would be switched into general containers where the LN2 is not sterile, with risk of cross-contamination with other cells.

Oocyte cryopreservation has gained much attention in recent years. Protocols have improved and resulted in a much higher efficiency in outcomes. However, it remains important to always seek for amelioration in vitrification protocols and new vitrification devices to ensure a major benefit and patient safety during procedures.

**REFERENCES**