ORIGINAL ARTICLE: ASSISTED REPRODUCTION

Bacterial and fungal contamination risks in human oocyte and embryo cryopreservation: open versus closed vitrification systems

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Objective: To study the contamination risk in open and closed vitrification devices for oocyte/embryo cryopreservation by evaluating the contaminants present (bacteria and fungi) in the thaw medium and in liquid nitrogen (LN) storage containers.
Design: Retrospective study.
Setting: Human reproduction unit.
Patient(s): None.
Intervention(s): Retrospective study of vitrification device safety and LN sterility performed from July to October 2014.
Main Outcome Measure(s): From each bank container, both open and closed vitrification devices, devitrification media and LN in the containers and as supplied by the company were evaluated for contaminants. An automated system and the corresponding susceptibility to antibiotics were used for bacteria identification. Fungus detection was performed by evaluating the colony morphology and their microscopic characteristics.
Result(s): No bacteria or fungi were observed in any of the devitrification media regardless of the type of device used, nor in the LN supplied by the company. No fungi were observed in any of the LN samples tested. *Stenotrophomonas maltophilia* and *Bacillus* spp. were found in all oocyte/embryo bank LN containers. There was no relationship between the number of samples or the time that each container had been used and the presence of microbiologic contaminants in the LN. At the container's bottom, *Acinetobacter lwoffii*, *Alcaligenes faecalis* ssp. faecalis, and *Sphingomonas paucimobilis* were found.
Conclusion(s): Bacteria cross-contamination may not occur in oocyte/embryo banking in either open or closed storage devices. However, microorganisms can survive in LN. The bacteria cross-contamination risk is no greater for open than for closed containers. Storage containers should be cleaned periodically owing to the risk of lost straws or small particles of contaminated material. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Cross-contamination, liquid nitrogen, bacteria, fungi, vitrification systems, open and closed devices

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The risk of disease transmission in humans during assisted reproductive technology (ART) procedures has been described. Viral infection after artificial insemination with donor semen has also been observed, including human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and human T-lymphotropic virus III (1–5). Most microorganisms can survive storage at liquid nitrogen (LN) temperatures, and the cryoprotectants used in embryo and oocyte cryopreservation also protect bacteria and viruses (6).

There are many factors that influence the contamination of embryos with pathogens before and during cryopreservation. These include the

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integrity of the embryonic zona pellucida (ZP), the cryopreservation method, the loading and sealing of the container, and the sterility of both the LN and the storage container. Unfortunately, commonly applied techniques in human embryology (embryo biopsy, intracytoplasmic sperm injection [ICSI]–assisted hatching, or cryopreservation of hatched embryos) result in irreversible damage or lack of ZP, and the embryonic cells of those embryos are more susceptible to contamination compared with ZP-intact embryos (7).

During assisted reproductive procedures, cryostorage is the only situation where large quantities of biologic materials of patients are kept together in a common liquid medium. Although the temperature of LN is −196°C, it may transmit infective agents from one sample to another if they are not sealed properly.

Some reports confirm the presence of microorganisms, such as selected bovine viruses and certain species of bacteria, which may cause nosocomial infections in LN tanks (8, 9).

Theoretically, once the LN is contaminated, stored samples could also become contaminated. Thus, the LN itself can be considered as a potential source of pathogens during cryopreservation and long-term storage. This issue has led to serious concerns about the use of specific cryopreservation technologies (e.g., vitrification) when using open systems involving direct contact with LN during vitrification and/or storage (10, 11).

The aim of the present study was to evaluate the risk of contamination in open and closed vitrification devices for oocyte and embryo cryopreservation by evaluating the sterility conditions of the LN in oocyte and embryo banks, as well as the presence of contaminants (bacteria and fungi) in the same medium in which they were thawed.

MATERIAL AND METHODS

This study was approved by the Institutional Review Board of La Fe University Hospital. All procedures were compliant with ethical guidelines, i.e., approved by the Ethical Committee.

Evaluation of Sterility Conditions in Oocyte and Embryo Banks

The LN sterility conditions in the oocyte and embryo banks of the Assisted Reproduction Unit in La Fe Hospital were evaluated.

This bank consists of a total of five cryopreservation containers (Air Liquide GT40). Each one can store about 600 embryos and/or oocytes from couples with negative serology for viral agents that cause serious diseases (HIV, HCV, and HBV).

The number of years in operation and the number of stored samples of oocytes and embryos were considered for each storage container.

The bank sterility conditions was evaluated for four consecutive months, samples of LN from each of the five containers were evaluated at two key moments: when the nitrogen level in the tank was lower (just before filling the container), so that the waste was more concentrated; and immediately after filling the containers, because this process causes strong turbulence, which can disperse the sediment and facilitate collection. A total of 40 LN samples were collected and evaluated from five containers.

In addition, LN samples from a reservoir bottle immediately after filling were used as negative control. A total of four LN samples were collected and evaluated from the LN supplied by the company at the reservoir bottle whose sole function was to supply the vitrification laboratory. This LN was provided by Air Liquide, coming from a sterile cryogenic distillation process from previously filtered air.

Obtaining Samples of Liquid Nitrogen

LN management is complicated owing to the low temperature, which requires the use of protective gloves and prevents samples from being taken by aspiration. A search through the literature did not reveal any clear methodologic descriptions of how it was done in earlier studies.

In the present study, the LN samples were obtained with the use of a closed 120-mL sterile bottle with a hole in the cap. Using 40-cm-long forceps, the bottle was placed upside down in the bottom of the cryostorage container for ~1 minute. When the air-filled bottle arrived at the bottom, the LN sediments were sucked through the hole and into the bottle. Once filled, the bottle was placed right-side up and opened. It was then kept inside a laminar flow cabinet until the LN had evaporated. This method allowed the collection of LN from the bottom of the container, where impurities are accumulated. Finally, the bottle was sealed with the use of Parafilm and packaged for later analysis. This procedure was performed for each container of the oocyte and embryo bank.

Several storage embryo devices accidentally fell to the bottom of container number 3. This container therefore had to be completely emptied, and this opportunity was used to collect all the sediment at the bottom of the container. The LN sediment was obtained by decanting the LN to a low volume, and then the errant devices were removed and the sediment swirled and collected into a sterile container. The container was cleaned with the use of Instrunet Lab (Inibsa) and tested again 1 month later for the presence of bacteria and fungi.

Microbiology Procedures

Isolation and identification of bacteria and fungi in LN samples was carried out in the Microbiology Department of Hospital Universitari i Politècnic La Fe. A sample of 120 mL LN was withdrawn aseptically from each LN tank into sterile flasks and evaporated at room temperature in a biosafety cabinet. To resuspend any dry contents present in the original LN sample, 5 mL thioglycolate broth was added to each flask and thoroughly shaken. One hundred microliters of suspension from each flask was inoculated onto blood agar, chocolate blood agar, and MacConkey agar (for bacteria detection), as well as Sabouraud dextrose agar with chloranphenicol (for fungal detection) with the use of disposable inoculating loops. For bacteria, all culture plates were incubated for 48–72 hours at 37°C, and each unique isolated colony type was subcultured to a new plate for purity. Pure cultures were Gram