Cross-contamination risk management in biobanking. Lesson from the pandemic

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ABSTRACT

Millions of human gametes are stored worldwide. Cryostorage presents many potential risks to the cryopreserved cells/tissues, including loss of viability and most of all contamination. Assisted reproductive technology (ART) clinics need to develop effective strategies to manage these risks. Human ART is the only medical discipline where human gametes and embryos with the potential to produce live births are routinely stored in liquid nitrogen or nitrogen vapor (LN_2/NV).

The risk of infection is real, especially when new, highly infectious agents arise. Airborne contaminants come into contact with LN_2/NV and are cryopreserved. While some industrial sectors (drug manufacturing, food and beverage sterile packaging) carry out raw filtration of LN_2 before use, this precaution is not yet mainstream, although it is increasingly used in the field of human ART. During the COVID-19 pandemic, manufacturers of drugs/disposables/culture media tried to mitigate contamination risk by sourcing raw materials from low-risk regions, but this approach is not feasible in the case of LN_2/NV, which can thus become potential vectors of contamination. The risks associated with the use of contaminated LN_2/NV are virus awakening and contamination of thawing cells, the environment, and operators.

KEYWORDS

Cryopreservation, cryostorage, sterile liquid nitrogen, risk management, contamination, personalized virus-free vitrification.

Cryostorage-associated risks

Cryopreservation of gametes and embryos has now become mainstream in assisted reproduction (AR). The exponentially growing inventory of cryopreserved cells around the world has many implications. On the practical side, the primary concerns are related to the safety and longevity of the cells while in storage.

Duration of storage

Recent studies, including those focusing on oocytes subjected to slow freezing, support the conclusion that long-term cryostorage is generally safe [1-4]. However, the shift in the past decade from slow freezing to vitrification necessitates studies focusing on vitrified cells specifically. Vitrified cells are suspended in minute volumes of cryoprotectant (less than 1 microliter) and for this reason they are more sensitive to temperature shocks because they are not protected by the ice layer typical of slow freezing. Furthermore, the majority of oocytes and embryos are vitrified with open carriers, which exposes the cells to potential contamination. It has been demonstrated that cryostorage of vitrified oocytes for up to 3.5 years has no effect on the chromosomal integrity of the resulting embryos [4] and that cryostorage of vitrified blastocysts in open devices (Cryotop) for up to 8 years does not impact clinical and neonatal outcomes [5]. Furthermore, cryostorage duration has no impact on the viability of biopsied blastocysts [6]. Thus, current evidence does not suggest that duration of cryostorage, per se, threatens oocyte or embryo integrity or viability. However, the conditions under which the cells are stored and handled over time can undoubtedly influence these outcomes.

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Handling and transportation

Vitrified cells are highly sensitive to temperature shocks. Temperature during handling, shipping and storage should not rise above a safe threshold of –180°C even though glass transition temperature is approximately –130°C. Despite the predominant use of liquid nitrogen (LN_2) for storage, shipment of cells/tissue is generally performed with tanks or dry shippers that use nitrogen vapor (NV). Failure of these shippers is not uncommon according to formal and informal surveys [7]. However, risks associated with handling and transport of vitrified human eggs and embryos have scarcely been investigated through experimentation [8]. McDonald *et al*. [9] showed that the survival rate of vitrified shipped oocytes was lower than that of their non-shipped counterparts or shipped slow-frozen oocytes. They speculated that this effect was likely due to one of many abnormal conditions during shipping, including vessels being

exposed to elevated temperatures and air pressure, vibration/other physical shocks, and horizontal storage.

In a study by Parmegiani *et al*. [10], shipping temperature and type of transportation appeared to impact survival of vitrified oocytes transported from Spain to Italy. It was the experience of these investigators that road courier was the safest means of transportation; in their case, the shippers were filled with LN_2 rather than "charged" with NV, although this is not necessarily common practice globally. According to the data logger, shippers maintained the temperature below −196°C for the duration of the 24-hour journey. During transport by air (where LN_2 onboard is not permitted due to its hazardous material designation), despite the shipper temperature being recorded as below the safe threshold of −180°C in both cargo and cabin baggage, both locations seemed to negatively affect oocyte survival rate, which decreased by up to 20% compared with that of oocytes transported by road.

Shipment of cryopreserved samples is complex, comprising multiple critical steps. What can be expected is that during transfer between storage vessels, moving from liquid to vapor phase of nitrogen and vice versa, temperature and humidity can change in uncontrolled ways, and these fluctuations may be harmful to vitrified oocytes. Devitrification and recrystallization of intracellular water can occur and have been shown to lead to cryoinjury during warming of vitrified samples [11].

Multiple handling steps are involved in the shipment of samples from one laboratory to another. It is not yet known how temperature fluctuations during shipment and handling may affect cell survival and quality.

Cross-contamination

During both cryopreservation and storage, particular attention should be paid to avoidance of bacterial or viral contamination. Depending on the storage vessel type, both LN_2 and NV may be used for cryostorage of human gametes and embryos. Storage in either phase can be potentially hazardous because some pathogens can survive at cryogenic temperatures and may contaminate the frozen cells or surfaces of the cryostorage containers [12]. To date, there have been no cases of disease transmission via transferred cryopreserved human embryos [13], but microorganisms at any stage during culture and transfer of embryos can ultimately reduce the chances of a successful pregnancy [14]. Vitrification, in conjunction with so-called open devices, requires direct exposure of the sample to LN_2, and this exposure poses additional contamination risks [15]. However, the hypothetical risk of culture contamination at warming cannot be excluded even when using some "closed" vitrification systems [16].

Precautions

Some precautions can be routinely taken in AR laboratories to minimize the risk of cross-contamination during cryopreservation. For example, cryostorage in hermetically sealed containers and the use of a secondary sleeve (straw-in-straw) has been recommended for both vitrified and slow-frozen cells [17]. Another step that can be taken is sterilization of LN_2 to prevent contamination [18]. Sterile LN_2 can easily be obtained through UV irradiation. Periodic refilling of storage dewars with sterile LN_2 and annual decontamination of the cryotanks (which requires removal of the specimens) can help minimize the potential risk of cross-contamination. It is also possible to decontaminate frozen human specimens before warming [19]. This procedure consists of washing the specimens with sterile LN_2 and has also been shown to efficiently decontaminate vitrification cryo-devices even under extreme experimental conditions, in which the concentration of microorganisms was more than ten thousand times higher (108 to 109 CFU for bacteria and > 105 CFU for fungi) than any observed under ordinary conditions.

Regulations

There are currently no regulations regarding device type, nor any mandates for sterility of LN_2 /NV in the USA, although such regulations could be considered by the US FDA, since it does regulate medical devices and is sensitive to the issue of infectious disease transmission. European authorities, in their effort to ensure safety and quality of tissues, may also consider regulating both the devices and the environment in which reproductive cells are stored. From the regulatory point of view, human reproductive cells are treated in the same manner as other non-reproductive tissues; for this reason, even though there is consensus among practitioners that vitrification with open devices using non-sterile LN_2 is safe, best practice guidelines, as well as current and future regulations, must make provision for maximum care and concrete steps to reduce the risk of contamination during or after cryopreservation and cryostorage. Indeed, some European countries have specific requirements geared at reducing the risk of contamination at cryopreservation [20,21].

Cryopreservation in the age of COVID-19

During the COVID-19 pandemic, scientific societies in the field of fertility released guidelines geared at minimizing the risk of contamination in IVF laboratories.

The Italian Society of Embryology, Reproduction and Research (SIERR) pointed out that LN_2/NV can be a source of infection, especially during a pandemic involving a respiratory virus. They highlighted the need for safer and more protective measures, including LN_2 sterilization, to minimize viral exposure during cryopreservation and cryostorage.

The Spanish society ASEBIR released recommendations to minimize risk to reproductive cells at cryopreservation during the COVID-19 pandemic. In this document ASEBIR suggested increasing the number of washes of oocytes and embryos with culture medium and making dilutions in LN_2 before passing the sample to the final tank (Recomendaciones para la seguridad y reducción de riesgos ante la infección por coronavirus [SARS-CoV-2] en las clínicas de reproducción asistida). The procedure for vitrification carrier washing with sterile LN_2 was originally described by Parmegiani *et al*. in 2012 [19]. This procedure is also

recommended by the Practice Committee of the American Society for Reproductive Medicine in its "Recommendations for reducing the risk of viral transmission during fertility treatment with the use of autologous gametes" [22].

Finally, the World Health Organization (WHO), in its 2021 "WHO laboratory manual for the examination and processing of human semen" (Sixth Edition), recommended some precautions to take to avoid or limit viral contamination:

- sterilization of LN_2 to prevent contamination – useful in case of vitrification where the sample is directly immersed in LN_2;
- periodic refilling of dewar storage flasks or tanks with sterile LN_2 and annual decontamination of the cryotanks;
- decontamination of frozen specimens with sterile LN_2 before warming.

Articles suggesting precautions for the pandemic

Recently many authors have suggested implementing "good manufacturing" practices in the field of ART to minimize the risk of LN_2-mediated contamination. Maggiulli *et al*. [23] suggested the use of single-personalized-disposable vitrification containers; Arav [24] and Alteri *et al*. [25] recommended the sterilization of LN_2 before use. Hickman *et al*. [26] and Shapiro *et al*. [27] advocated for the washing of cryopreserved specimens with sterile LN_2 before thawing/warming. Pomeroy and Schiewe [28], Scarica *et al*. [29], and Vajta *et al*. [30], in their recent articles, encourage using precautions for the safe use of LN_2, namely sterilizing it before use.

Risk management when using LN_2

De Santis *et al*., on behalf of SIERR, observed that the safety of cryopreservation in the context of reproductive medicine is a crucial topic to mitigate any risk of cross-infection. For this reason, they suggested changing perspectives with regard to the use of LN_2 in IVF labs, arguing that it is necessary to overcome the old diatribe between open and closed vitrification carriers, given that LN_2 and NV can themselves potentially be a source of infection. Since the use of contaminated LN_2/NV increases the risk of virus awakening, as well as that of contamination of warmed samples, the local environment and operators, LN_2/NV sterilization is recommended [31].

Adoption of single-use medical devices for personalized vitrification/warming

In a recently published detailed risk analysis Maggiulli *et al*. suggested using sterilized LN_2 and disposable LN_2 trays to mitigate risk [23]. Washing the polystyrene boxes used in IVF laboratories for vitrification and warming is difficult due to the porosity of the material; it is also time consuming and, most of all, difficult to verify. It therefore seems obvious that, in the future, vitrification and warming performed in single-use sterile containers will become the "best practice". Furthermore, these disposable containers should be certified medical devices, given that they are used to contain human reproductive cells to be reimplanted.

Specimen washes with sterile LN_2 before warming/thawing

LN_2 can be effectively sterilized with UV-C radiation [18], and "vitrification carrier washing" with UV-sterilized LN_2 before warming has been demonstrated to eliminate vitrification carrier contamination in extreme experimental conditions. In a 2021 study, Parmegiani *et al*. used a concentration of microorganisms over 10,000 times higher than any observed in LN_2 IVF storage vessels and the washing procedure fully removed the carrier contamination mediated by LN_2 [19]. Scientific society guidelines and various authors advise using this procedure to eliminate viral pathogens [22,26,27,32].

Setting up a virus-free vitrification program

In healthcare, blockchain technology can be used to address challenges around sensitive data sharing and traceability of medical and laboratory procedures. Recently, Parmegiani *et al*. described the first application of this technology in IVF for obtaining incorruptible traceability of a "virus-free" vitrification/warming procedure involving the combined use of UVC-sterilized LN_2 and CE medical devices (CE-MDs) [33]. They reported 2346 Ethereum blockchain data transactions for IVF laboratory procedures mined from 1st October 2019 to 31st December 2021. The procedures involved oocyte/embryo vitrification, warming and handling in LN_2 after cryopreservation. For each vitrification/warming procedure, a UVC-sterilized batch of LN_2 was associated with the code assigned to the vitrification/warming procedure and with the lot number of the single-use sterile vitrification box (N-Sleeve). The clinical results obtained from warmed oocytes/embryos were observed as the outcome of this process. A blockchain-trusted "virus-free" vitrification/warming program was set up using a specially designed CE-MD N-Bath-System (Nterilizer, Italy).

Each procedure was traced by the CE-MD software and a dedicated web application. Finally, data were made incorruptible using Ethereum blockchain transactions. Before oocyte/embryo warming, vitrification carrier washing with UVC-sterilized LN_2 was performed in accordance with recent international anti-COVID guidelines. Of the 2346 blockchain transactions, 1268 were vitrification and cryopreserved specimen handling procedures; 1078 transactions were frozen cell warmings (308 oocytes and 770 embryos) performed in 799 patients. To date, 445 pregnancies have been obtained (pregnancy rate: 41% per cycle; 56% per patient) and 219 babies have been born. This is the first evidence of the application of blockchain technology in IVF and many others will probably follow. Blockchain immutable records of LN_2 sterilization combined with procedure codes and disposable lots represent incorruptible traces of "virus-free" vitrification/warming. During the pandemic period (December 2019 to December 2021) 219 babies were born from blockchain-powered "virus-free" cryopreservation procedures.

Conclusion

The risk of using contaminated LN_2 has been underestimated for many years. However, in the wake of the SARS-CoV-2 pandemic, even authors who more than ten years ago considered the risk of cross-contamination in ART cryotanks negligible are now suggesting sterilizing LN_2 [28]. The solution to minimize LN_2 contamination risk is straightforward and easy to implement. It is hoped that LN_2 sterilization will become a mainstream practice before the first cases of specimen cross-contamination or operator infection occur in IVF. LN_2 sterilization and adopting single-use medical devices for personalized vitrification/warming are best practices suggested by many authors and scientific societies, after the recent SARS-CoV-2 pandemic.

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