Aseptic procedures for vitrification, warming and cryostorage

Lodovico Parmegiani
Enzo Troilo
Alessandra Arnone
Antonio Manuel Maccarini
Azzurra Rastellini
Sara Lanzilotti
Silvia Bernardi

Reproductive Medicine Unit, GynePro Medical Centers, Bologna, Italy

Address for correspondence:
Lodovico Parmegiani, M.Sc.
Reproductive Medicine Unit, GynePro Medical Centers
Via T. Cremona 8
40137 Bologna, Italy
Phone: +39 347 472 5674; Fax: +39 051 441 135
E-mail: l.parmegiani@gynepro.it

Summary

Vitrification is a cryopreservation technique increasingly applied in clinical practice for cells and tissue. Different procedures and different types of carriers can be used and, in some cases, vitrification requires direct contact between cell/tissue/carrier and liquid nitrogen; this may cause concern regarding the safety of this cryopreservation technique. Although the risk of contamination during cryopreservation remains negligible, this article explains how to overcome the hypothetical risk of contamination when using different types of vitrification carriers, in order to satisfy all existing directives.

KEY WORDS: vitrification, embryos, oocytes, sterile liquid nitrogen, hermetrical goblets.

Introduction

As vitrification is a cryopreservation technique increasingly applied in clinical practice for cells and tissue, the main focus of this review article is to analyze the risks related to this cryopreservation technique. Since there are concerns regarding the safety of vitrification procedures and cryostorage, due to the contact of cell/tissue/carrier with liquid nitrogen (LN₂), these aspects are comprehensively treated by the authors.

Vitrification carriers

Open carriers

Vitrification is widely applied in clinical practice for human reproductive cells. During vitrification cells and tissue need to be cooled and warmed at an extremely rapid rate (1). This can be achieved by using specific “open carriers” such as Open Pulled Straw (2), Cryoloop (3), Hemi-Straw (4), Cryotop (5), Cryoleaf (6), Cryolock (7), Vitri-inga (8), etc.; these “open carriers” are generally preferred for oocytes (9-13). However, these systems cannot avoid the hypothetical risk of microorganism contamination during the vitrification procedure, if the LN₂ is accidentally contaminated (14, 15). The sterilization of LN₂ before vitrification procedure is thus recommended to ensure the safety of clinical application in humans.

Straw-in-straw closed carriers

Another option for vitrification is the closed carrier based on the “straw-in-straw” mode (High Security Vitrification - HSV), designed to insulate the inner carrier containing the cells/tissue against LN₂ during vitrification by using a sealed external straw (16, 17). This system avoids the direct contact between specimens and LN₂ and also any hypothetical risk of contamination and it allows good results with zygotes, cleaved embryos, blastocyst and ovarian tissue (17-19). However, the “straw-in-straw” system causes a reduction in the rate of cooling and is not routinely used in clinical cryopreservation of oocytes.

Single straw closed carriers

As an alternative to straw-in-straw, other types of
closed systems such as CryoTip (20) or Cryopette (21) allow faster rates of cooling. These closed carriers consist of a very thin straw specifically designed to load cells with minimum volume of cryoprotectant solution and to be hermetically sealed (single-straw closed carriers); in this way, direct contact between cells and LN$_2$ is avoided. Unfortunately, because of their design, these systems would not avoid the transmission of microorganisms in the culture medium during the warming procedure, if the LN$_2$ was accidentally contaminated; this is due to the direct contact between the LN$_2$ and external surface of the carrier (22, 23). In practice, the contamination of cells occurs at 37°C, when any cryopreserved micro-organism found in the LN$_2$ reactivates after thawing in the culture medium. Even though IVF culture media are supplemented with antibiotics, some microorganisms may resist the antibiotic and infect the culture. In these circumstances, the bacterial or viral particles released in the culture medium may attach themselves to the oocyte/embryo zona pellucida if this is cracked (14, 24). Another procedure to decontaminate the straw is to quickly wipe the carriers with 70% ethanol for disinfection at warming (20). However, the de-activation of all microorganisms can be obtained only by a 5-minute-contact between ethanol and carrier (25); this prolonged contact time can damage human cells, which remain inside the carrier in the warmed vitrification solution rich in potentially toxic cryoprotectants (26). For these reasons, the sterilization of LN$_2$ before the vitrification procedure is recommended for safe clinical application of these systems in humans.

Nitrogen vapours/supercooled air vitrification

Some authors have demonstrated that it is possible to vitrify reproductive cells by exposure to nitrogen vapours (27, 28). It has also been proposed to insert the carrier containing the cells into the supercooled air of a straw for instantaneous vitrification and then to seal the open end of the straw (postsealing method) to avoid direct contact with LN$_2$ (28). It is important to point out that the supercooled air inside the straw is basically composed of nitrogen vapour owing to the nitrogen’s rapid evaporation and molecular weight (29). For this reason, any microorganism accidentally present in the LN$_2$ can also pass to the nitrogen vapour phase (30), and this may lead to the hypothetical contamination of the oocyte/embryo and the inner carrier. This means that contamination issues associated with LN$_2$ cannot be avoided by using supercooled air, and the sterilization of LN$_2$ before evaporation is recommended also with this method (29).

Solid surface vitrification

With these systems, vitrification is performed on the solid surface of a chilled metal block partially submerged in LN$_2$ (CryoLogic Vitrification Method - CVM - http://www.cryologic.com/cvm.htm). Following vitrification, specimens are inserted into a sleeve which is then fully heat sealed. Since the air around the block is composed of nitrogen vapour (31), these systems too require LN$_2$ sterilization.

Cryostorage

Nowadays, human cells and tissues are cryostored in LN$_2$ or in nitrogen vapour (NV): this cryostorage is potentially hazardous because many pathogens can survive at the low temperature of LN$_2$/NV (14, 15, 30-35) and may contaminate the frozen cells or their carriers/container surface inside the cryobanks (14, 15, 24, 30, 31, 34-36). To date, there have been no cases of disease transmission by transferred cryopreserved human embryos (35, 37-39); however, we have no specific studies regarding possible negative effects of LN$_2$/NV infectious agent contamination on the final outcome of IVF frozen cycles; although it is generally known that some of these microorganisms negatively affect gametes and embryonic development at warming (15, 40-42). In addition, vitrification is increasingly used for human cells and this cryo-procedure appears to be riskier than slow freezing due to the direct contact between cells/tissue and LN$_2$ required for “open systems”. The hypothetical risk of culture contamination at warming cannot be excluded even when using some “closed vitrification systems” (22, 29, 31).

Some precautions may be routinely used in IVF 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) is recommended for human specimens in both vitrification and slow freezing (2, 31, 35, 43, 44) (Figure 1).
Aseptic vitrification

Periodic cleaning and refilling of cryo-dewars with sterile liquid nitrogen (SLN₂) are additional precautions to minimize the potential risk of cross-contamination; nowadays, certified SLN₂ can be easily obtained through UV irradiation (43, 45) (Figure 2).

Three-wash procedure in sterile certified liquid nitrogen

Recently, Parmegiani et al. have proposed a reliable procedure to decontaminate frozen human specimens before warming. This procedure consists in washing the specimens with sterile LN₂, and this has been shown to efficiently decontaminate vitrification carriers in extreme experimental conditions. This procedure could be routinely performed in IVF laboratories for safe thawing of human specimens which are cryostored in “non-hermetical” cryo-containers, particularly in the case of “open” or “single-straw-closed” vitrification systems (46).

Regulations and Quality Assurance

Hypothetical cell/tissue contamination by LN₂/NV
requires us to guarantee the sterility of vitrification procedures, particularly in Europe due to the directives on tissue manipulation (European Union Tissues and Cells Directive EUTCD: 2004/23/EC, 2006/17/EC and 2006/86/EC). These directives have been issued by the European Parliament in order to increase the safety and quality of tissues – including reproductive cells – processed for human re-implantation, through the control of equipment, devices and environment. Similar regulations could be introduced in the future by the Food and Drug Administration (FDA) for Assisted Reproductive Centres in the United States (37). Thus, both in Europe and potentially in the United States, human reproductive cells are treated in the same way as other non-reproductive tissues. For this reason, even though Pomeroy et al. considered the cross-contamination of infectious agents a negligible risk (37) and the majority of cryobiologists and embryologists maintain that vitrification with open systems using non-sterile LN$_2$ is in practice safe, international regulations and Quality Assurance require specific procedures in embryo/oocyte/ovarian tissue cryopreservation in order to avoid any hypothetical contamination of human cells through direct contact with accidentally contaminated LN$_2$.

**Discussion**

Vitrification has moved from bench to bedside and it is emerging as the preferred cryopreservation method for human reproductive cells/tissue, especially for oocytes, zygotes, cleavage-stage embryos and blastocysts. Regarding the risk of cell/tissue contamination through direct contact with LN$_2$/NV, any technique preventing hypothetical contamination must be welcome. A vitrification system which avoids any risk of contamination may be useful not only for reproductive cells/tissue but, in the future, also for other human specimens even including whole organs. In current directives worldwide there are no specific indications against direct contact between human specimens and LN$_2$/NV; for this reason vitrification “open-systems” can comply with any existing directive, as long as aseptic procedures during vitrification-cryostorage-warming are established (31, 44, 45).

Although the risk of contamination during cryopreservation remains negligible we can confidently choose the type of carrier (open or closed) best suited to our purposes in the knowledge that, when aseptic procedures are followed, both systems conform in equal measure to any existing directive.

**Disclosure**

L.P. holds an international patent: “Device and method for sterilizing liquid nitrogen by ultraviolet radiation”. The other Authors have nothing to disclose.

**References**

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