

The Problem of Contamination: Open vs. Closed vs. Semi-Closed Vitrification Systems

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1. Introduction

The Development of cryopreservation techniques, the increase in demand for cryopreserved cells or tissues and the use of these techniques in cells or tissues from patients with infectious diseases, has forced us to reduce the risk of contamination during the freezing process and the risk of cross-contamination during the storage of this material. Recent publications that demonstrate the survival of pathogens at low temperatures and possible contamination of the cells or tissues stored have changed the laws of each country and the customs and protocols used so far in the cryopreservation.

To understand the problem of contamination in cryopreservation we need to have an overview of the current problem in which all researchers are concerned about, seeking a cryopreservation protocol with good results but without contamination problems. Discussing the cryopreservation's different techniques such as slow freezing, vitrification, kinetic vitrification (extra-, hyper-, super-, ultra-fast vitrification) and the various components that help us understand the difficult balance between technique, device used and the risk of contamination. We need to use new products and new protocols' to have good results ensuring biological samples and patient safety.

Dr. Katkov's idea of find the "*Universal cryopreservation protocol*" (see the other Chapter in the Book) by Katkov at al. that we can use worldwide with all the possible biological samples would lower considerably the price of cryopreservation process and we would have better results because everybody would work with the same protocol and the same results.

We have to comprehend the difference between open, closed or semi-closed devices and the importance of choosing one device or another both in morphological survival post thaw cell or tissue as on non-contamination of these samples. The device used, the protocol used and the cooling solution used can change the outcome of cryopreservation and therefore we have to find a protocol for cryopreservation with a cooling solution and a secure device to provide us good results free of contamination.

2. Contamination and cross-contamination

The first thing we must learn is to differentiate their respective importance are the concepts of contamination and cross contamination of samples. The first relates to the contamination

of the sample by freezing or by direct contact with the cooling solution and the second refers to the contamination of the sample within the common container which is in contact with all cryopreserved samples, some samples may be contaminated or the liquid nitrogen (LN2) might be contaminated producing a possible cross-contamination. The potential for disease transmission and pathogen survival through contaminated 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 but the use of safe cryopreservation protocol is very important to avoid human cell contamination or cross-contamination in common LN2 tanks.

Although cryopreservation had a boom in the mid 70's and early 80's with the opening of the first sperm banks in America and Europe, it was not until the mid-80's when we saw the need for biological samples cryopreserved in quarantine and the lack of screening leads to infection of several recipients that had been inseminated with semen samples from donors HIV+ those unaware of their disease (11). In these cases it was found that samples stored in the same containers with frozen HIV+ samples were not contaminated, otherwise in 1995, six patients undergoing cytotoxic treatments hermetic problems developed an outbreak of acute hepatitis B after undergoing an autologous cryopreserved material that had been stored in the same cryogenic container as other patients infected with hepatitis B (12).

2.1 Cells and tissue contamination

In the field of assisted reproduction, although it hasn't been detected any contamination in the cryopreservation of gametes and embryos, the probability and the occurrence is low, the risk is not zero so it is recommended to follow the rules in biosecurity manuals for both the physical and chemical risk as well as the risk of contamination and cross contamination of samples.

The case in 1985 where there was infection with hepatitis B in the cryopreserved samples (12) the infection was due to an error in packaging and storage of samples. With time a deterioration of the bags containing infectious material causing the infection of the LN2 and other samples was observed.

Further studies have shown that the storage of samples is decisive. There is evidence that frozen samples in hermetically sealed straws are not contaminated even if they are in contaminated containers with contaminated LN2 and LN2 does not contaminate infective biological samples that were frozen in a sealed container (13,14)

During the cryopreservation, biological samples go through many processes before being cryopreserved. In the case of IVF cells are subjected to a phase of procurement, fertilization, development, transfer and finally cryopreservation. This represents an approximate 6-day process in which many factors can affect the contamination of the sample at the end of the process. We can find contamination or cross-contamination in the following cases (15):

- Handling contaminated biological samples (semen, follicular fluid, tissue, etc.). Without precautions to avoid contamination outside the base plate to be used for conservation

(cryotube, straw, etc.). It is very important to disinfect and clean the container before filling it with LN₂ (16). In this regard to ensure an adequate level of biosafety a study is needed of infectious diseases transmissible from any patient or donor who wants to freeze any samples. According to Castilla (17) the clinic policy for a donor with infectious diseases is radically different to that of a patient with any of these diseases wanting to freeze biological material for autologous use. In the first situation, the biological material at hand will not freeze. In the second, the biological material should be frozen but with measures that we discuss later. Screenings for infectious diseases that normally must be submitted are: To analyse serological studies for syphilis, hepatitis and HIV. To analyse the clinical studies infective clinical phases: toxoplasmosis, rubella, herpes virus, cytomegalovirus (CMV), *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. These tests are required for donors of semen every 6 months. As the risk of disease transmission during storage in LN₂ is mainly viral. Interestingly, the American Society of Fertilisation (18), ESHRE (19), British Andrology Society (BSA) (20) and the Spanish Association of Tissue Banks (AEBT) (21) also recommend serologic screening for CMV, not just clinical. The presence of CMV in semen has been associated with active disease (anti-CMV IgM + or recent seroconversion anti-CMV IgG +). Similarly, these companies recommend performing serologic tests for HTLV-I and HTLV-II. But although it is clearly demonstrated the transmission of human papilloma virus by using LN₂ cryotherapy and has been shown IUI transmission of herpes simplex virus (HSV) (22), none of the scientific associations mentioned above recommend a culture for detection or serological studies of HPV donors or patients with infection who are going to freeze biological material because the analysis to detect these diseases are not very sensitive. As rubella serologic screening of donors, its low prevalence in this population means that serological tests have a low positive predictive value, making it unadvisable. Finally, we believe a patient who needs to freeze some reproductive biological material should have at least one serology for HIV, hepatitis B and C. This proposal is consistent with the recommendations of the AEBT for cryopreservation of semen (21).

- Use of contaminated culture media. In these cases the degree of cross-contamination would reach very high levels having an impact on many patients. Although the preparation of embryo culture media and sperm extenders from specific ingredients are avoided in human clinics, it continues to be a common practice in animal ART (23). Nevertheless, many ingredients of embryo culture media and sperm extenders act as stabilizers for many micro-organisms at freezing temperatures (milk, serum or serum albumin, sucrose, sorbitol and other sugars). Unfortunately, the most common cryoprotectors (CPs) in applied oocyte cryopreservation and embryo (glycerol, DMSO, ethylene glycol, propylene glycol, methanol etc.) are toxic for cells. Also bacteria and viruses efficiently protect from cryoinjuries, eg Concentrations of DMSO as low as 5% enveloped viruses defend against the trauma of freezing (24). The Fact That micro-organisms survive in association with germplasm is not only important from the potential of disease transmission by embryo transfer to recipients, but also in approaches to the storage of samples for testing and health certification of embryos for international movement. On the other hand we must also bear in mind that all culture media containing antibiotics to prevent or limit survival of microorganisms.

- Conservation of contaminated material or straws cryotubes closed or sealed badly flawed causing the breakdown of the frozen straw, leaving the contaminated sample directly exposed to the LN2 tank risking contaminating the other samples. Closed systems can be sealed in many ways (thermal sealer, ultrasound sealer, radiofrequency sealer, polyvinyl alcohol powders, and solid caps). Given the sealing time and the temperature reached does not affect the cryopreserved sample, we have to ensure that the seal is airtight and that the device is built of resistant material to low temperatures of LN2 (Ionomeric resins, quartz glass capillary, Polyvinyl chloride, Polyethylene glycol tetralato, etc).
- Using contaminated LN2 during the freezing process. In this case we have proposed some solutions that we will see later.
- Poor source management of LN2 from our supplier contaminating commercial LN2 that comes to our lab in the process of manufacture or transportation and filling our containers.
- For transportation of contaminated material in containers. Storage containers should be emptied and cleaned periodically due to the risk of lost straws or small particles of contaminated material that falls to the bottom of a large container (25,26). Most of the companies of LN2 containers provide cleaning protocols. The main problem is the cleaning of transport cylinders called "dry" because the material that absorbs the LN2 in these bottles is difficult to sterilize. Bielanski (27) describes a method of disinfection of commercial dry shippers with two different types of a LN absorbent. Based on the results presented, it appears that solutions of sodium hypochlorite and ethylene oxide are equally useful for the disinfection of dry shippers constructed with a hydrophobic LN absorbent. In contrast, for dry shippers without a hydrophobic LN absorbent it is advisable to use gas only for decontamination in order sterilization to avoid their damage by liquid disinfectants.
- The air in the room. If the air that reaches the lab comes from another area that could be contaminated and there isn't a good filter. Some laboratories do not have filtration systems or positive pressure to prevent air contamination.
- Operators. If they are infected then that can lead to contamination by contact or peeling during processing of samples or the handling of cryogenic tanks. Staff must meet certain health and hygiene conditions: negative serology for HIV, HCV, HBV and vaccination against hepatitis B and other viral diseases for which there is a vaccine available. We must also have a detailed description of their jobs, tasks and responsibilities. In addition the centre must provide the worker training in freezing techniques for updating and improving procedures.
- Use of open devices. In recent times there is much talk of closed or open system and the possibility of contamination, so many countries have banned open systems and the trend is to ban the high risk of sample contamination. In a closed or semi-closed device the nitrogen of common container is never in contact with biological material frozen on the inside so cross-contamination cannot produced. In the open system, the biological material is in contact with the common nitrogen so contamination from the sample is very easy if the LN2 is contaminated or contamination of LN2 if the sample is contaminated. The latest study done by Criado and his group (28) showed 45% of contamination in an open device (Cryotop) Vs 0% of contamination in a semi-close device (Ultravit) equal and using a contaminated laboratory LN2.

2.2 Cooling solution contamination

The cooling solution plays a significant role in avoiding contamination of biological samples. It means that we will freeze the sample and we will deposit it for a long storage until thawed and used. Normally the LN2 cooling solution is the most widely used in cryopreservation and survival of pathogens at high temperatures (-196 ° C) has already been proven by many studies (1-3,27,28) cases also involved in seeing cross-contamination of human papillomavirus (14,29).

The need for better cooling rates to avoid formation of crystals in cryopreservation has resulted in the discovery and use of new cooling solutions (slush, slurry, etc.). So there are more components to consider when contamination is to be avoided. Using these new cooling solutions gives a lower temperature than the LN2 temperature and much faster transmission. The Slush nitrogen is obtained by a vacuum pump (Telstar TOP-3; Telstar S.A., Terrassa, Spain) that solidifies part of the LN2 in a few minutes. On return to normal atmospheric pressure, the nitrogen collapses, and the subcooled LN2 has solid particles in it commonly referred to as "slush" (30). The advantage of Slush nitrogen lies not only in the temperature difference with respect to LN2 (-196°C Vs -210°C) but also in the reduction of the Leiden frost effect, which is the formation of a layer of vapor around the sample when immersed in the cryogenic liquid from room temperature decreasing the cooling rate (31,32). It has not yet been demonstrated the survival or non survival of pathogens in this cooling solution of 15-20 ° C difference in LN2, this is obtained by vacuum pressure, which can lead to rupture of the cell wall of pathogens to balance internal and external pressure of these in the process of forming Slush. The 'Slurry' nitrogen is a mix of LN2 with different particles for example copper powder. At present investigations are being carried out as an alternative to LN2 to increase the cooling rate because with this cooling solution the thermal conduction is increased. Likewise, experiments are ongoing with various solutions to increase the thermal conduction and the cooling rate.

These cooling solutions "alternatives" are only used at the time of freezing the sample and once frozen, it passes to the general container that is filled with LN2, although these solutions where they freeze cool samples have to be sterile we have to ensure that the general LN2 container does not have contact with the frozen sample in order to not contaminate the sample and the LN2 if the sample is positive for any pathogen.

Retrospective studies in which commercial LN2 cryotanks were examined after 35 continuous years of service revealed various bacterial and fungal contaminations in the LN2 detritus (23). Many of the identified bacteria isolated in these studies were ubiquitous environmental micro-organisms and were rare opportunistic pathogens of low significance in producing disease in humans or animals (Table I). It should be acknowledged that some of the isolates may have been derived from laboratory contamination during semen and embryo processing for cryopreservation rather than genuinely being present within the sample. In agreement with Bielansky and Vajta the risk of contamination by human pathogens seems to be rather low. Components of the standard LN2 production system comprise a compressor, a cryogenerator and containers. From a practical point of view, the complete sterilization and maintenance of sterility in such a robust system might be a very demanding task, if possible at all. Accordingly, some ubiquitous bacterial agents can be expected in any commercially produced LN2. Nevertheless, it is an 'in and out' system and only air-borne contaminants are supposed to enter it (LN2 compressor) via air used for LN2

production. As they are not air-borne, it is unlikely that viral agents of human concern such as HIV, hepatitis and herpes viruses would enter the LN2 production system.

Sample tank	Identified microbial contamination			Years of storage	Total no. of stored samples
	Liquid nitrogen	Semen	Embryos		
Research					
Laboratory tanks					
1	<i>Staphylococcus aureus</i>	Nd	Nd	20	580
2	<i>Bacillus licheniformis</i> , <i>Bacillus</i> spp.	<i>Stenotrophomonas maltophilia</i> , <i>Staphylococcus sciuri</i>	Nd	15	840
3	CDC group IVc2, <i>Alcaligenes faecalis</i>	<i>Proteus vulgaris</i>	Nd	8	460
4	<i>Brevandromonas vesiculata</i>	<i>E. coli</i>	Nd	15	1350
5	<i>Stenotrophomonas maltophilia</i>	—	Nd	12	650
6	<i>Staphylococcus capitis</i> , unidentified Gram-negative rod, <i>Comamonas acidovorans</i>	<i>Morganella morganii</i> , <i>Gemella morbillorum</i> , <i>Stenotrophomonas maltophilia</i> , <i>Citrobacter koseri</i>	<i>Bacillus subtilis</i> , <i>Ochrobactrum anthropi</i> , <i>Staphylococcus epidermidis</i>	15	1200
7	<i>Stenotrophomonas maltophilia</i> , <i>Comamonas testasteni</i>	<i>Stenotrophomonas maltophilia</i> , <i>Citrobacter koseri</i>	<i>Stenotrophomonas maltophilia</i> , <i>Bacillus</i> spp., <i>Pseudomonas fluorescens</i> , <i>Acinetobacter lawffii</i>	18	1480
8	<i>Bacillus pumilus</i> , <i>Eikenella corrodens</i>	—	<i>Stenotrophomonas maltophilia</i> , <i>Bacillus</i> spp., unidentified Gram-negative rod	10	960
Commercial tanks					
9	Nd	Nd	—	10	58 456
10	Nd	<i>Aspergillus</i> spp.	—	30	138 430
11	<i>Aspergillus</i> spp.	<i>Corynebacterium aerosol</i> , <i>Bacillus sphaericus</i>	—	30	34 962
12	Nd	<i>Stenotrophomonas maltophilia</i>	—	35	150 000
13	Nd	<i>Stenotrophomonas maltophilia</i>	—	15	280 864
14	<i>Aspergillus</i> spp.	<i>Photobacterium damela</i>	—	15	260 912
15	Nd	<i>Bacillus sphaericus</i> , <i>Corynebacterium</i> spp., <i>Staphylococcus sciuri</i>	—	12	262 642
16	<i>Stenotrophomonas maltophilia</i>	<i>Ralstonia pickettii</i>	—	12	404 955

Nd, not detected; —, not available for testing.

Table 1. Microbiological contamination of embryos and semen during storage in LN2 (23)

One of the biggest discussions recently in the world of cryopreservation focuses on the importance of the sterility of LN2. As shown in Table I and in total agreement with Bielansky and Vajta and many other authors the commercial LN2 reaching our lab is not contaminated enough to cause any infection to freeze biological material. The major problem is common containers where the samples are deposited with a LN2 stored for months, years or even decades in contact with many samples, which, many clinics do not empty and do not disinfect, so it is in common containers where we can find the highest risk of contamination and cross contamination.

As a possible solution to minimize the risk of freezing biological material some systems have been proposed where we sterilize the LN2 and where we ensure that the sample is not in contact with LN2 with the use of semi-close devices or devices that are the only ones that guarantee a hermetic sealing of the device and avoid any risk of breakage of the solder thus ensuring the aseptic samples. The fact that LN2 can be quickly and safely sterilized could encourage the clinical application of human cell/tissue vitrification, both with open carriers and with closed systems. The problem is that if this device is an open device and is passed to the general container where all the other cryopreserved samples there is a huge risk of cross contamination, so it has not helped.

- LN2 Filtration: One of the solutions that have been developed is the filtration of LN2. Air Liquid has marketed CERALIN a liquid filtration system through LN2 ceramic filters. The CERALIN ON LINE consists of two elements of liquid filtration connected in series and inserted into a section of vacuum transfer line. The ceramic membrane is made from multiple layers formed into a multi-channel element. It is housed in a vacuum insulated pipe, itself installed close to the end-use point. The filter minimizes

bacterium *Deinococcus radiodurans* is inactivated (> 4log) by administering 400.000 $\mu\text{Ws}/\text{cm}^2$ per each sterilization cycle. An adequate amount of UV radiation deactivates the growth of all kinds of micro-organisms, from viruses like Hepatitis (which require an 8.000 UV dose) to fungi like *Aspergillus Niger* (330.000 UV dose) (33). At CRYO 2011 Dr. Parmegiani spoke about a new device of UV sterilization of the common containers with cells or tissues inside but the scientific community thinks that is too dangerous biological samples exposed to UV rays without any protection. Although his group is proposing special canisters "not transparent" I think they have to do many more tests to rule out damage to the samples because the common view is confirmed that UV light is harmful, even if used just overnight decreased embryo developmental rates.

- LN2 Steam: As an alternative to hermetical storage in LN2, cryostorage contamination might be avoided by storing the carrier containing the vitrified oocytes in LN2 vapour (34, 35). However, Grout and Morris (36) maintain that storage in the vapour phase of LN2 still carries a risk of sample contamination. Storage of semen in LN2 vapours was discarded early in the development of sperm cryopreservation techniques and it was found that long-term viability of sperm was reduced compared with LN2 storage (37,38). However, recent experiments with new materials have succeeded in developing the technique with acceptable results for both semen and embryos (39,40) and in our last experiment we demonstrated 0% of contamination in vapor nitrogen in a experimental contaminated laboratory LN2 (non published). The drawback of the generalization of this form of storage is the need for careful monitoring of temperature in different parts of the container, which makes the marketing of these containers type (40) more difficult.
- Before entering discussions regarding the sterility in LN2 used for vitrification, we should debate the use of communal containers, which is where cross-contamination can be found, as there is a possibility that the "contaminated cells" could come into contact with each other, and where a number of viruses and bacteria are found, which would never be found in the commercial LN2.

2.3 Contamination in transport

To carry out a safe transportation of biological material we should clearly distinguish a number of concepts (17).

1. Infectious substances: those that contain viable microorganisms (bacteria, virus, prions, parasite, fungus) or bacterial toxins that are known or believed to cause disease in animals or humans.
2. Diagnostic specimens: human or animal materials (body fluids, blood, tissue, tissue fluids, etc.). Obtained for diagnostic or investigational (41).

Most often transported biological reproductive materials are cryopreserved semen donor and follicular fluid when the laboratory is separated from the follicular puncture site. In both cases, we consider the recommendations to follow are those of diagnostic specimens. There are several documents related to the transport of biological material, such as the Universal Postal Union (UPU), the International Aviation Organization (ICAO) and International Air Transport Association (IATA) (42-44).

At European level, all documents related to transport are based on the recommendations of the Committee of Experts of the United Nations Dangerous Goods (UN) (45). There is also a european agreement on international transport of dangerous goods by road (ADR), approved by RD 2115/9838 (46). We will describe some aspects of the mentioned regulations on the transport of diagnostic specimens. The basic system consists of packaging:

1. Primary container, watertight, leak proof, labeled and contains the sample. This container should be wrapped in absorbent material. In terms of labeling, according to AEBT, if it is a semen sample from a donor, must contain an alphanumeric code that identifies the donor and the sample number of the donor. On the other hand, if the sample is for autologous use may be noted also the surname of the patient (21).
2. Secondary container, sealed, leak-proof and protects the primary container. You can place multiple primary containers wrapped in a secondary container. This should be sufficient absorbent material used to protect all primary containers and avoid collisions between them.
3. Outer shipping container: the secondary container is placed in a shipping package that protects the secondary container and its contents from outside elements, such as physical damage and water.

The data forms, letters and other identifying information of the sample should be placed taped outside the secondary container. The label for submitted materials consists of:

1. Basic triple packaging.
2. Does not require signs from United Nations (UN).
3. No substances require pictogram or declaration from the sender.
4. "Biological material for clinical use" must be indicated.
5. Tag address:
 - Name, address of destination, as detailed as possible, and phone number.
 - Name, address, telephone number and contact person at the semen bank.
6. The documents included with the storage conditions and special instructions for shipping. One of the special considerations that we must have in mind when transporting a sample of semen is not breaking the cold chain, so you must use a container or LN2 as well as avoiding the possible use of dry ice.
7. Permission for import / export and declaration.
8. Label orientation.
9. Date and time of departure of Semen Bank (21).

The requirements to be met for local transport are as follows:

1. Sealed and resistant containers.
2. Threaded tubes upright (rack, tray ...).
3. Use of resistant boxes and perfect closure.
4. Secured box in the transport vehicle.
5. Appropriate Labeling.
6. Have the forms with necessary details.
7. Vehicle with kit (gloves, absorbent material, disinfectant, waste container, etc.).

You must ensure perfect coordination of transport between the sender, carrier and recipient to ensure delivery. Thus, each party involved should carry out its part perfectly and

appropriately. So stand out from other actions that the sender must ensure the proper identification, packaging, labeling and documentation according to established biosafety guidelines in the "Recommendations of the Committee of Experts of the United Nations Transport of Dangerous Goods" transporting must be kept in appropriate conditions (temperature, light ...) the material from which the sender receives it until it is delivered to your destination and have the appropriate licenses to perform this type of transport, and finally, the recipient must confirm with national authorities that the material can be legally imported.

According to AEBT (21), the possibility of returning a material that hasn't been used should be avoided, as a rule, the return of the semen that has been provided by the Bank, as it will only accept the return of the displayed when you meet the following 3 conditions:

1. The sample wasn't thawed.
2. You can demonstrate the integrity of the packaging (the seals are intact).
3. The temperature of the sample was maintained throughout the transport.

3. Open device

Following recent studies of cell and tissue contamination in freezing and the recent debates regarding the sterility of LN2 in vitrification processes the devices play an important role in the asepsis of the frozen sample. There are many different types of device and of various materials but from the point of view of sterility, devices can be divided into Open, Closed and Semi-closed devices.

There is a lot of controversy and confusion about the concept of Open device. For most cryobiologists Open devices are devices that allow direct contact of the biological sample to be frozen with the cooling solution but when there is contact with the interior of the device but not with the sample to be frozen it would not be considered an open device. Once inside the common cooling containers the cooling solution enters and leaves the device keeping all frozen samples in contact.

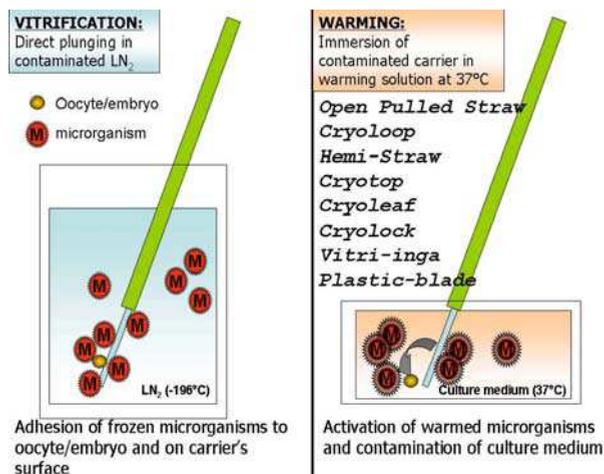


Fig. 2. Risk of Contamination with open devices (47)

Generally, using open devices the achieved cooling rates are approximately 20.000-30.000 °C / min which favor good vitrification of the sample. The problem is that being in direct contact with the cooling solution there is a risk of pathogen transmission to the biological sample at the time of freezing and a high risk of cross contamination in the common cooling containers. They have been prohibited in many countries for this high risk of contamination and the global trend is to ban them for use with human samples. Recent microbiological studies indicate a 45% pathogen contamination (*Pseudomonas* and *E-coli*) with a simple 10-second contact with the open device (Cryotop) with contaminated cooling solution (28).

The most known and used open devices are the following:

Open pulled straw

In the OPS method, 0.25 mm standard insemination plastic mini-straws were heat-softened over a hot plate and pulled manually, as originally described by Vajta et al. (48). The inner diameter and the wall thickness of the pulled part of the straw are approximately 0.8 and 0.07 mm respectively. Cells are load into the pulled straws by placing the narrow end of the pulled straw in the third droplet of medium and aspirating oocytes within a 2-3 mm long liquid column (1-1.5 µl) using capillarity. The straws are then cooled by being plunged directly into LN2 and stored briefly. For warming, the open end of the straw is immersed vertically into 4.5 ml of the warming solution at 37°C. The solidified vitrification solution became liquid within 1-2 s. A cooling rate of 16.700°C/min is obtained with this device (49).

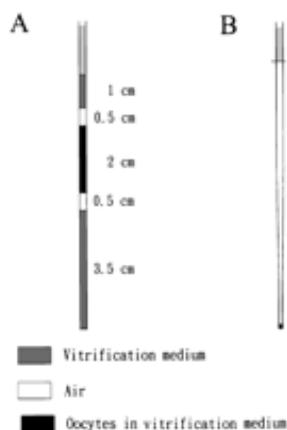


Fig. 3. A) The 0.25 ml conventional straw is loaded with 1 cm of vitrification medium, 0.5 cm of air, 2 cm of vitrification medium containing oocytes, 0.5 cm of air, and 3.5 cm of vitrification medium using a syringe. (B) The open pulled straw is loaded with vitrification solution (1-2 µl) containing oocytes by means of the capillary effect by a simple touch.

Cryoloop

The Cryoloop (Hampton Research, Aliso Viejo, CA, USA), used as a vessel in vitrification, is a thin nylon loop used to suspend a film of cryoprotectant containing the oocytes and directly immerse them in LN2. Vitrification of oocytes using the Cryoloop has advantages over conventional vitrification procedures in that the open system lacks a thermo insulating

layer, together with the small volume of $<1\mu\text{l}$, results in both rapid and uniform heat exchange during cooling. A cooling rate of $20000^\circ\text{C}/\text{min}$ is obtained with this device.



Fig. 4. Cryoloop

Hemi-straw

The Hemi-straw (Astro-Med-tec, Salzburg, Austria) is an embryo carrier that consists of a large gutter on which a small quantity of CPAs ($<1\mu\text{l}$) containing the cell is deposited. The Hemi-straw is subsequently inserted into a larger pre-cooled 0.5 ml straw (CBS, Cryo Bio System, Grenoble, France) under LN₂. Prior to the commencement of the warming process the Hemi-straw is pulled out of the larger straw under LN₂ and the tip of the Hemi-straw is immediately immersed into a petri dish containing a sucrose solution. A rapid cooling rate of $>20.000^\circ\text{C}/\text{min}$ is achieved by allowing direct contact of the biological material with LN₂. (50)

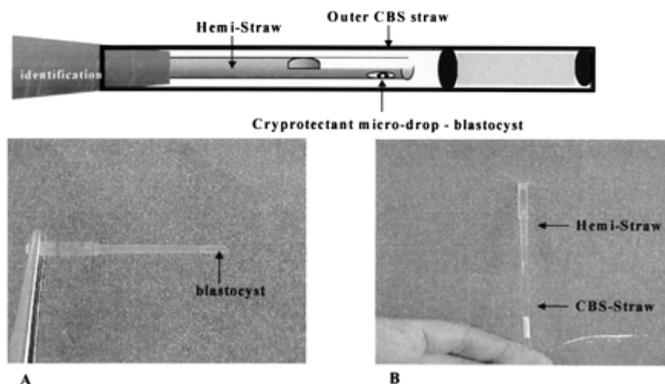


Fig. 5. Scheme of the Hemi-Straw: (A) loading the cell on the tip of the Hemi-Straw; (B) insertion of the Hemi-Straw into a larger 'CBS' straw. (51)

Cryotop

(Kitazato Supply Co, Fujinomiya, Japan) Individual oocytes were picked up in an extremely small volume ($<0.1\mu\text{l}$) of vitrification solution and placed on top of a very fine polypropylene strip (0.4 mm wide \times 20 mm long \times 0.1 mm thick) attached to a hard plastic

handle specially constructed according to specifications by Kitazato. The droplet volume was estimated from the length of the fluid column within the pipette tip. As soon as the oocyte was placed onto the thin polypropylene strip of the Cryotop, it was immediately submerged vertically into filtered LN₂. Then, the thin strip was covered with a hard plastic cover (3 cm long) on top of the Cryotop sheet to protect it during storage in LN₂ containers. For warming, the protective cover was removed from the Cryotop while it was still submerged in LN₂, and the polypropylene strip of the Cryotop was immersed directly into the solution at 37°C for 1 min. A cooling rate of 23,000°C/min is obtained with this device (49).

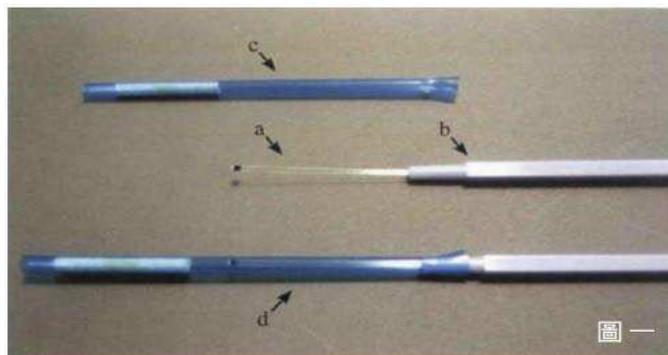


Fig. 6. Cryotop

Cryoleaf

The McGill Cryoleaf™ is very similar to Cryotop but with a number of features designed to improve the loading and storage of cells. Safety during storage has been improved, as the cells are double protected from stress and contamination through a closed cover system but not hermetically sealed leaving cells in direct contact with LN₂. The McGill Cryoleaf™ and the vitrification media have been developed by Dr. Chian and Prof. Tan at McGill University, Montreal.



Fig. 7. Cryoleaf

Cryolock

(Biodiseño, Colombia) With this device, cells are deposited near the black mark using the minimal amount possible of vitrification solution (2 μ l aprox.) The black mark eases the

cover up. The Cryolock® is immediately plunged into LN2, whilst holding the Cryolock®. After this the cap is grasped with forceps and plunged into LN2 until bubbling stops, be aware to not take the Cryolock® out of the LN2 whilst covered up, twist and lock gently. Finally, place the Cryolock® in the goblet with the cap downward facing and store for the desired time. For warming, remove the patients canister from the dewar and place in a styrofoam box completely cover the Cryolock® with LN2. Grasp the Cryolock® body at the indentation with forceps and remove from the goblet. Grasp the cap at the indentation with forceps, twist and pull down without taking the Cryolock® body out of the LN2, it must always remain in LN2. Finally remove the Cryolock® from the LN2 quickly and pass into thawing solution at 37°C and follow the protocol.

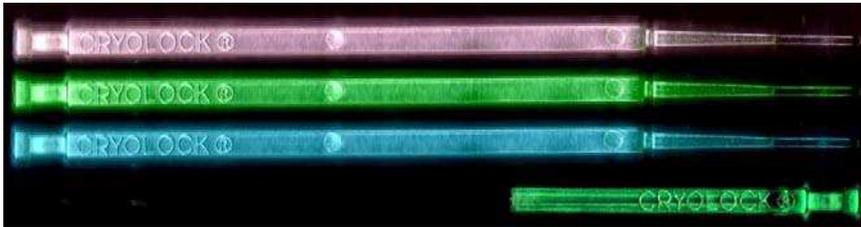


Fig. 8. Cryolock®

Vitri-Inga

The Vitri-Inga vitrification strip is an apparatus that consists of a fine, very thin polypropylene strip (0.7 mm thick) with a specially designed round tip, in which there is a minute hole to receive the cell; the strip is connected to a hard and thicker plastic handle. Vitri-Inga plastic sheaths are 0.5 ml semen straws with a cut in the middle. The total time from when the oocyte was placed into the vitrification solution till its immersion into LN2 is between 50 and 60 seconds. The plastic sheaths, which had been previously cooled for at least 2 min in LN2 vapor on the metal rack inside in the Vitri-Equip, are vertically immersed into LN2. The Vitri-Inga strip with the vitrified oocyte is then inserted into the plastic sheaths for storage, and transferred to a LN2 tank. (52)

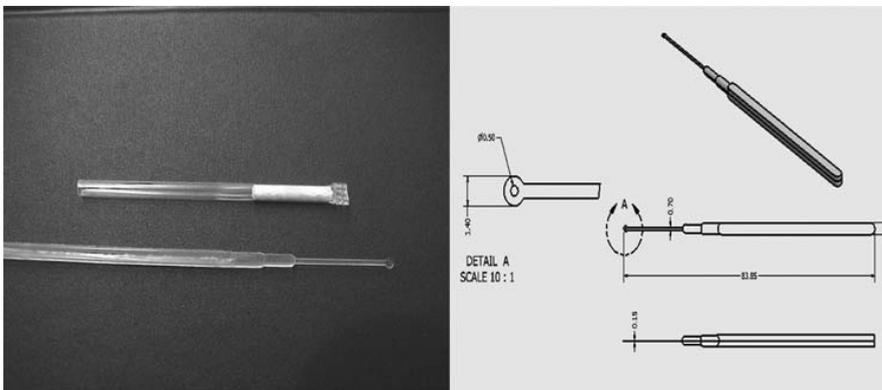


Fig. 9. Vitri-Inga

Plastic-blade

A serum Tube (Sumitomo Bakelite, Tokyo) was employed as a vessel for cryopreservation. A clear polyethylene terephthalate film (50 mm in thick) was cut into a T-shaped piece. As shown in Fig. 10, the horizontal arm of the "T" shape was rolled and fit securely to the inner wall of the cap. After equilibration with cryo-medium, the embryo for vitrification was placed at the center of a plastic blade, the vertical limb projected from the cap, and five embryos were the maximum allowed on one blade. The width of the plastic blade was significantly wider than that of the Cryotop which was commercially available tool for the storage of the vitrified human embryo. The blade was submerged directly into LN2 and inserted into the tube that was pre-cooled with LN2, then the cap was fastened on the serum tube, which accomplished preservation in the LN2 container. For warming, the serum tube that contained the plastic blade was submerged under LN2, and the serum tube was opened and the plastic blade containing vitrified cells was removed from the LN2 and placed directly into the well of the base medium at 37°C. (53)



Fig. 10. Plastic-Blade

4. Closed device

Closed systems were born as a need to solve this direct contact with the open devices. The pioneer and first major proponent of close systems for freezing embryos, oocytes or sperm was F. Ostashko in 1960 as an aseptic alternative to the Cassou method. In such systems the biological sample is not in contact with the cooling solution at the time of freezing or at the time of storage in the common containers. This prevents contamination by contact and cross contamination from shared containers. The main feature is that the cooling rate is much lower with these closed devices. By lowering the cooling rate most vitrification protocols with closed systems have a high concentration of cryoprotectants to prevent crystal formation making them "dangerous" protocols for the cell due to the cytotoxicity of the cryoprotective substances. Many comparative studies of open and closed devices listing very similar results.

In the market there are many closed systems to vitrify and more appear daily due to the emphasis that cryobiologists put into finding the perfect vitrification system that will prevent contamination of the sample and cross-contamination allowing a survival and cell viability with a protocol free or low of cryoprotective substances. The closed devices can be closed or sealed in many ways but most importantly a hermetic seal must be made, preventing entry to the inside and leakage of pathogens to the outside. Thermo seal, radiofrequency seal and ultrasound seal are some of the most used systems that ensure that the stalled sample remains suspended in time.

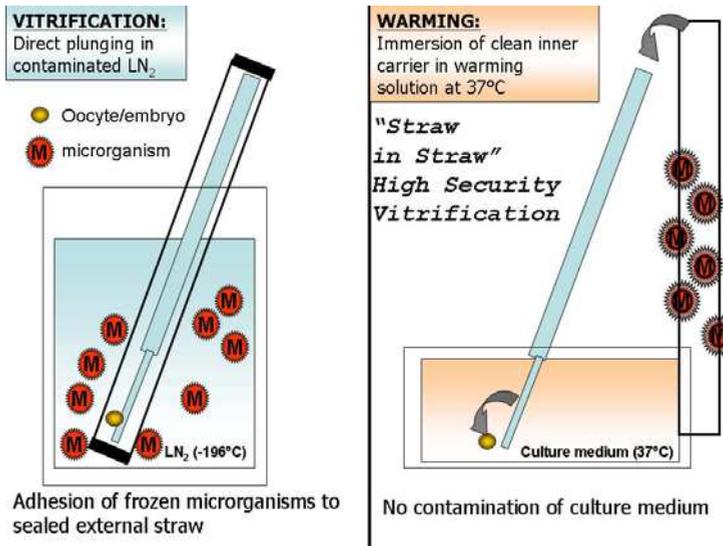


Fig. 11. Risk of Contamination with closed devices (47)

Amongst the most commonly used closed systems are the following:

25 to 0.5 ml Straw

This was one of the first devices used to freeze semen, oocytes, embryos or tissues. If the device is not hermetically sealed they are open devices, but if they are hermetically sealed they are closed systems, as the sample does not come into contact with the cooling solution. The main problem is the cooling rate, as the device is constructed from PVC or ionomeric resin and having a substantial wall thickness there is little temperature transmission (CBS). The cooling rate achieved by these straws is approximately $2.500\text{ }^{\circ}\text{C} / \text{min}$ (49)



Fig. 12. straw

CVM Ring Fibre Plug

CVM™ (Cryologic, Australia) involves the rapid cooling of specimens without their immersion in, or direct contact with the cooling solution. This reduces the risk of any potential contamination by pathogenic microorganism that may be present in the cooling solution. The specimens are put into a droplet which is transferred to the hook at the end of a custom designed fibre called a Fibreplug™. The Fibreplug™ is then transferred to the specially treated surface of a CVM™ Block that has been chilled to LN₂ temperature. The droplet vitrifies into a glassy bead and the Fibreplug™ is placed securely into a pre-cooled CVM™ sleeve. A cooling rate of $10.000^{\circ}\text{C}/\text{min}$ is obtained with this device. Besides the cooling rate, another main problem with this method is that to cool the CVM block the

surface makes contact with LN2 thus “contaminating” the surface, which could then provoke a contamination of the sample.

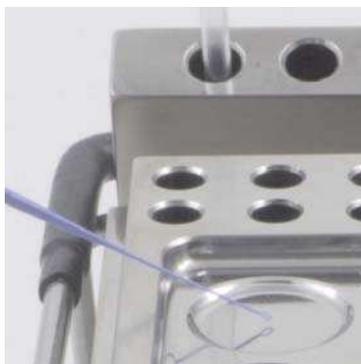


Fig. 13. CVM Ring Fibre Plug

Rapid-i™

Rapid-i (Vitrolife, Sweden) is based on the same principle as the open vitrification system of the Cryoloop meaning that the embryos are placed in a minute volume of vitrification solution in a hole and held there by surface tension. The Rapid-i™ holding the embryos is in turn placed in a pre-cooled RapidStraw™ sitting in the container filled with LN2. This unique feature of the Rapid-i™ vitrification System means that vitrification actually takes place in super-cooled air reducing contamination risks. The straw is sealed after vitrification making the critical time frames of the dehydration steps easier to keep and creating an aseptic vitrification system without any contact between vitrified material and LN2. The main problem is that a cooling rate of 1.200°C/min is achieved with this device (54).



Fig. 14. Rapid-i

Vitrisafe

Is a modification of the previous Hemi-straw vitrification plug that allows a complete insertion in high security 0.5 ml straw. The Vitrisafe consists of a large gutter that is totally inserted into a larger pre-cooled 0.5 ml straw (CBS, Cryo Bio System, Grenoble, France). Only after welding both ends of the 0.5 ml straw to ensure the complete isolation of the biological sample is the complete straw plunged into LN2. For warming, the gutter is removed from the outer straw without contact with LN2 and the tip containing the biological material is directly plunged into the dilution solution in order to archive a rapid warming. A cooling rate of 1.300°C/min is archived in the vitrification process (50).

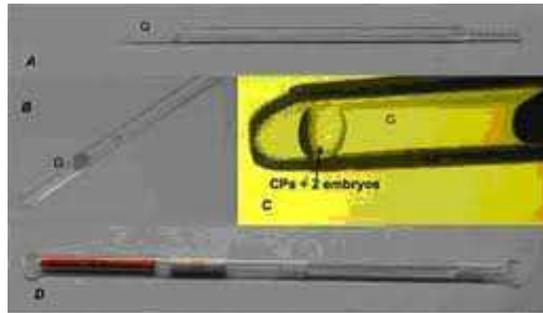


Fig. 15. Vitrisafe

High security vitrification straw

The CBS™ High Security vitrification straws are made from an ionomeric resin that is chemically inert, biocompatible and has physical characteristics resistant to ultra low temperatures and pressures created by expanding liquids and LN₂. Sealed straws are resistance tested to 150 kg/cm² (2133 lb/sq.inch), both the seals and the material should resist in order to have the batch approved. The HSV (High Security Vitrification) kit is composed of a High Security ionomeric resin straw, a capillary tube with a pre-formed gutter attached to a colored handling rod and a blue plastic insertion device. For freezing the sample is deposited into the gutter a few millimeters from the end using a micropipette. The drop holding the sample must be under 0.5 μL. immediately place the capillary rod and handler into the straw and push until the rectangular portion of the handler comes in contact with the flared end of the straw. While still holding the straw in place, seal the open end, hold the straw using tweezers in the area of the handling rod and quickly plunge the entire straw into LN₂ vertically. For thawing lift the straw enough to expose the colored handling rod. Make sure the end with the sample remains immersed in the LN₂. Holding the straw, use the opening device for HSV kit to section the straw and immediately (within 2 seconds), plunge the gutter into the first dilution media. A cooling rate of 2.000°C/min is archived in the vitrification process (55).

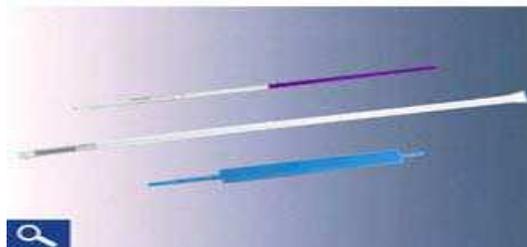


Fig. 16. High security vitrification straw

CryoTip

(Irvine Scientific) A plastic straw container which can be sealed as a closed device to hold gametes or embryos in a specialized medium during cryopreservation procedures and subsequent long term storage in a LN₂ tanks. CryoTip consist of a drawn plastic straw with

an ultra fine tip and a protective metal cover sleeve. This device has been optimized as a closed system for cryopreservation procedures. For freezing aseptically remove one CryoTip when ready to use. Aseptically attach the wide end of the CryoTip to an aspiration tool, such as a luer tip syringe, using the Connector. When specimens are ready to load into the CryoTip, aseptically slide the metal cover sleeve carefully along the straw to expose the fine tip end. Gently load the specimens into the CryoTip by aspiration using the plunger on the syringe to control the uptake of medium and specimens. Heat seal the fine tip below the 1st mark, then slide the metal cover sleeve down over the fine tip to protect it and plunge the sealed CryoTip into the LN₂ reservoir. A cooling rate of 12.000°C/min is obtained with this device (49)

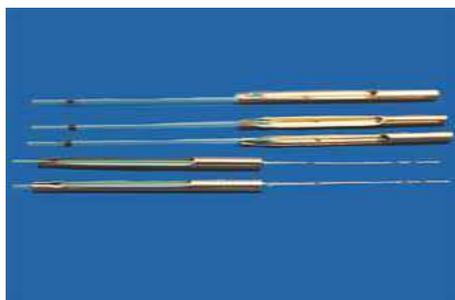


Fig. 17. Cryotip

Cryopette®

(Origio) It is derived from the original STRIPPER® family of denudation tools. It includes a sterile STRIPPER® tip with an integrated bulb to facilitate loading. This eliminates awkward external handles, rods, and pick-up tools required with other devices and guarantees simplicity, speed and ease of use. The bulb is designed to deliver the sample to the desired location every time. The maximum load volume is 1.2 µl, producing a cooling rate of approximately 23.700°C/min.



Fig. 18. Cryopette

These last two devices have been criticized by Parmegiani and his group's latest articles and in the last CRYO congress 2011, for the "potential danger" they have in his opinion: In the thawing process the external part of the device is in direct contact with the warming solution and any pathogen that could be on the exterior could pass onto the sample. In my opinion it is a very remote hypothesis and the probability that this could occur is minimal in

comparison with potential contamination of an open device, a bacteriological study would be necessary that could demonstrate that with a high % of contamination, a simple contact of the end of the device with the warming solution is sufficient to contaminate the sample.

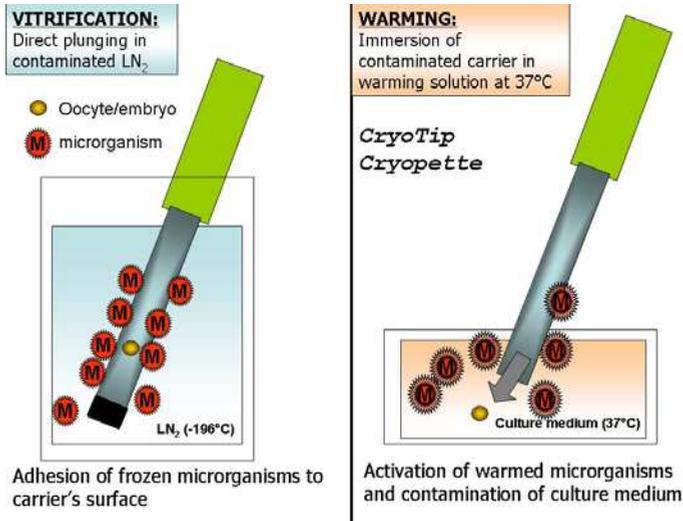


Fig. 19. Risk of Contamination with some closed devices (47)

Ultravit

Ultravit is a novel device composed of a 0.3 mm internal diameter quartz glass microcapillary tube and a flexible, transparent inert sheath that has been designed to protect and prevent it floating in the LN₂. Loading the internal microcapillary tube and removing the cells from the device is very simple and easy using a 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 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 cross-contamination. The last microbiological control of Ultravit showed that the 5-10 seconds contact of Ultravit's internal part with contaminated LN₂ (*E. coli* and *Pseudomonas*) is not sufficient to produce direct contact of cells with the cooling solution and does not result in contamination (0% of Ultravit Vs 45% of Cryotop) (28). In this study we didn't find contamination in the microdrops into which we emptied the contents of the microcapillary, also submerging the end of this (0.2mm diameter, 0.01mm of wall thickness and 1 mm in contact with the warming drop). There is a great difference between thawing with an open device (in which the entire strip is submerged in the warming solution with a surface of 42-50 mm²) and thawing with Ultravit with 95.5% less of surface in contact with the warming solution at 37°C (1.7-1.9 mm²). With Ultravit protocol, only the end of the microcapillary touches the base of the dish used to thaw, but at no time does the external part touch the warming drop (56). The following diagram shows the use of Ultravit, presuming that the cooling solution is contaminated:

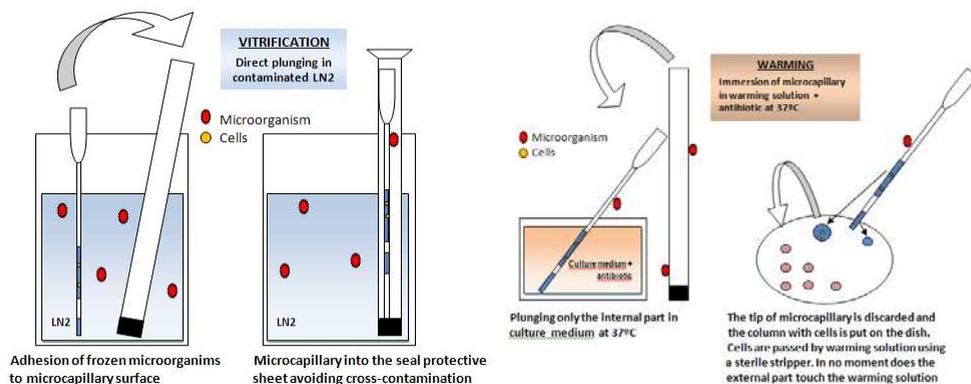


Fig. 20. Risk of Contamination with Ultravit

Our work has demonstrated that the microorganisms that may be in the cooling solution, on the outside of the microcapillary, cannot come into contact with ultra-vitrified cells inside due to the loading procedure, the contact time with the cooling solution and the diameter and surface of Ultravit making it a secure device and is enough to exclude the theoretical danger of contamination. The cooling rate obtained with Ultravit was 250.000 C°/min (57) with Slush nitrogen allowing ultra-vitrification with low concentration of CPA (1.5-2 M) and a morphological survival rate of 92% of human mature oocytes and 59.1% of blastulation rate in mouse embryos.

5. Semi-closed device

As a consequence of the necessity of a device which avoids contact of the biological sample with the cooling solution but that would achieve cooling rates high enough to ensure a high rate of vitrification, semi-closed devices were designed. Gabor Vajta was one of the first to hypothesize the enclosure of open carriers (after direct contact of cells/LN2) in pre cooled hermetical containers (48). There are systems in which there is direct contact of the biological sample with the cooling solution only at the time of vitrification. Once the sample is vitrified, the device is placed in a protective sheath which is hermetically sealed before being passed to the communal container. This ensures no cross-contamination in the tanks. As stated earlier in this chapter, microbiological studies performed (23) showed that many of the Identified bacteria isolated in the commercial tanks are ubiquitous environmental micro-organisms and are rare opportunistic pathogens of low significance in producing disease in humans or animals so these devices are an important tool for high survival of biological samples to avoid cross-contamination. Theoretically all open systems can become semi-closed systems to protect the biological sample from cross-contamination with a high cooling rate but the most important is:

OPS safe method

Vajta in 1997 devised a vitrifying system with OPS, but once submerged in LN2, the OPS straw is transferred to a 0.5ml CBS straw. Using this method a cooling rate of approximately 16.700 °C/min is achieved, but once passed into the 0.5ml CBS straw it is protected from cross-contamination in the common tanks.

A summary of the cooling rates obtained with each device is as follows:

DEVICE	VOLUME	COOLING RATE
CRYOLOOP	>1 µl	20.000 °C/min
HEMI-SATRAW	>1 µl	>20.000 °C/min
CRYOLEAF	>1 µl	23.000 °C/min
VITRI-INGA	1 µl	20.000 °C/min
CVM-RING	>1 µl	10.000 °C/min
VITRISAFE	>1 µl	1.300 °C/min
HSS	0.5 µl	2.000 °C/min
0.25 ML STRAW	25 µl	2.500 °C/min
OPS	1 µl	16.700 °C/min
CRYOTOP	0.1 µl	23.000 °C/min
CRYOTIP	1 µl	12.000 °C/min
RAPID-I	0.5 µl	1.200 °C/min
CRYOPETTE	1.2µl	23.700 °C/min
ULTRAVIT	0.2 µl	250.000 °C/min

Table 2. Different cooling rates

6. Cooling rate Vs closed systems

Today the differences between Slow freezing and Vitrification are known worldwide. We all know that slow freezing is characterized by a prolonged cooling curve and the use of low concentrations of cryo-protectors generally “non-toxic” for the cells (1-2 M) with cell injury due to ice formation (58) and that Vitrification is characterized by the rapid procedure and the use of a high concentration of cryo-protectors (4-6M) to prevent cell damage that is toxic to most mammalian cells (59-67). Thus, vitrification with a semi-close devices have a better cooling rate without cross-contamination or novel cyopreservation techniques are needed that allow rapid cooling to achieve vitrification in the absence of high concentration of CPA or if is possible without CPA.

The requirements and relationships for conditions to achieve satisfactory vitrification in the area of mammalian ART are well displayed in the equation of Yavin and Arav (68)

$$\text{Probability of vitrification} = \frac{\text{Cooling and warming rates} \times \text{Viscosity (CPA concentration)}}{\text{Volume}}$$

Fig. 21. Probability of vitrification by Yavin and Arav (68)

The main points to be gathered from this relationship are that the smaller volume of the vitrification solution in which the cellular material is placed for the vitrification process, the faster cooling and warming rate that can be achieved and the lower concentration of CPAs

needed reducing the detrimental effect of the inherent toxicity of CPAs and increasing the overall success of the procedure (50).

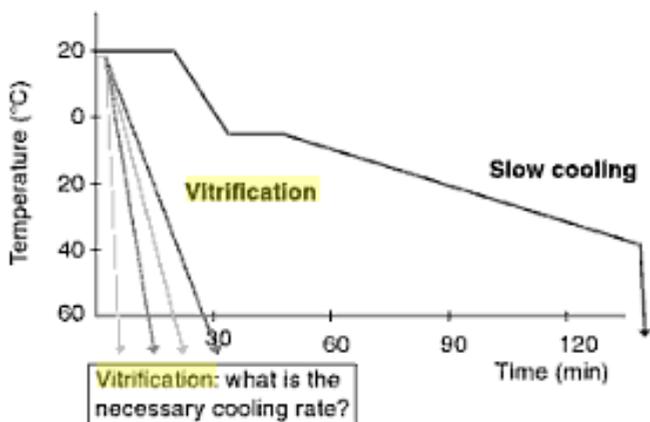


Fig. 22. necessary cooling rate to have a good probability of vitrification

What would happen if We could vitrify without CPA's or with a low concentration of CPA's? What would happen if We could combine the advantages of Slow freezing and Vitrification and vitrify with low concentrations of CPA's with a secure and free contamination device? That is Kinetic vitrification (Ultra-vitrification). Perfecting the techniques of Vitrification has been achieved a morphological survival rate comparable to normal Vitrification protocol (30) or a 59.1% of blastulation rate in mouse embryo (69) with Kinetic vitrification and concentrations of CPA's typical of Slow freezing.

Previous studies have tried to achieve high cooling rates for cell vitrification. However, none of them utilized low CPA concentrations (1.5-2 M). In 1985, Rall and Fahy successfully vitrified mouse embryos in 6.5 M cryoprotectant cocktail solution (70). In that case the method consisted in a 0.25 ml straw container plunged into LN₂; the cooling rate was 2.500 °C/min. When this container was plunged into Slush nitrogen, the cooling rate increased up to 4000 °C/min (71). The use of OPS (instead of the 0.25 ml straw) in LN₂ increases this cooling rate up to 5.300 °C/min (71) and to 10.000-20.000 °C/min if plunged in Slush nitrogen (71,72). Similar cooling rates were achieved in the case of a Cryoloop quenched in Slush nitrogen (73). The use of electron microscope copper grids has also been investigated, but the cooling rates were in the same order of magnitude that the afore mentioned works: 11.000-14.000 °C/min in the case of plunging the grid in LN₂ (74) and 24.000-30.000 °C/min if plunged in Slush nitrogen (74,75). From Boutron's theory, none of these approaches reaches the critical cooling rate to achieve vitrification with low concentration of CPA (1.5-2M). It's impossible to use open devices with Slush nitrogen as the cell is on the outside and there is a possibility of detaching from the device.

Adjusting to Yavin and Arav formula the Ultra-vitrification technique arose achieving a cooling rate above 250.000 °C/min and of 90.000 °C/min in thawing. This rate is one order of magnitude higher than the highest cooling rate achieved in different strategies (electron microscope copper grids in Slush nitrogen (74,75), whilst keeping all the advantages of a

straw-like form for the container and being in the range of the necessary cooling rate to achieve vitrification. To have this increase in the cooling rate a few changes were made to the normal vitrification process:

Slush Nitrogen

As a cooling agent this technique uses Slush nitrogen, much colder than LN2 (-196°C Vs -210°C) and with the property of avoiding the Leidenfrost Effect. When something is submerged in LN2, bubbles rise to the surface through the device, varying the thermal conductivity from the outside into the inside of the device. This does not happen with Slush nitrogen. Slush nitrogen is achieved with a vacuum pump in 5 to 10 minutes and it remains slush for a further 5 - 10 minutes before returning to liquid.

It was shown for oocytes and embryos that increasing the cooling rate would improve survival rates by up to 37% (76)

Model	Survival slush (%)	Survival LN (%)	Sig.	Publication
Bovine MI	48	28	$P < 0.05$	Arav & Zeron (1997)
Ovine GV	25	5	$P < 0.05$	Isachenko <i>et al.</i> (2001)
Porcine blastocysts	83	62	$P < 0.05$	Beebe <i>et al.</i> (2005)
Bovine MI	48	39	$P < 0.05$	Santos <i>et al.</i> (2006)
Mouse four-cell embryos with biopsied blastomere	87	50	$P < 0.05$	Lee <i>et al.</i> (2007)
Rabbit embryos	92	83	NS	Papis <i>et al.</i> (2009)
Porcine blastocysts	89	93	NS	Cuello <i>et al.</i> (2004)
Mouse MI	> 80	> 80	NS	Seki & Mazur (2009)
Rabbit oocytes	82	83	NS	Cai <i>et al.</i> (2005)

LN, liquid nitrogen; GV, germinal vesicle; Sig., statistical significance; NS, not significant.

Table 3. Effect of cooling rate on survival; comparison between LN2 and Slush nitrogen (75)

Quartz Micro-capillary

Another determining factor to achieve a high cooling rate is the device used. To increase the thermal conductivity and minimize the volume, this technique has used a quartz micro-capillary. This has a 0.2-0.3 diameter allowing to ultra-vitrify 0.1-0.2 μ l with a 0.01mm wall, a lot thinner than any other device (0.075 mm in OPS). Another important characteristic is the material it is made from: Quartz. The thermal conductivity of quartz glass is a lot higher than that of plastic of which other devices are made of. This converts it in one of the materials that best conducts the temperature (77)

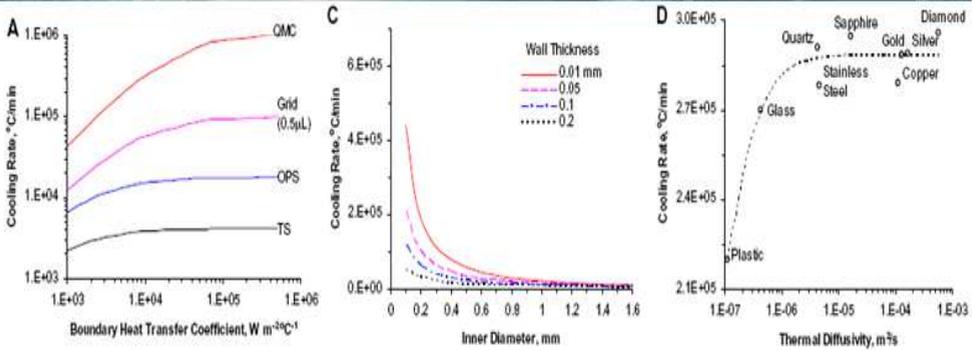


Fig. 23. Xiaoming He et al (77)

In a thermal performance of quartz capillaries for vitrification done by Risco and his group (57) a commercially available version of the OPS (MTG Medical Technological Vertriebs, GmbH) was used. The thermal conductivity of these PVC straws was $0.19 \text{ W m}^{-1} \text{ K}^{-1}$. The inner diameter is 0.800 mm and the thickness of its wall is 0.075 mm (Fig. 1a). The QC used (The Charles Supper Company, Inc.) have an inner diameter of 0.180 mm and a wall thickness of 0.010 mm. These geometrical improvements (4.44 times smaller in diameter and 7.50 times thinner) translate not only into a faster heat transfer, but also into a 20 times reduction in volume of the contained solution (for a given height). This is beneficial because the thermal conductivity of the quartz glass is $1.3 \text{ W m}^{-1} \text{ K}^{-1}$, that is almost one order of magnitude higher than that of PVC.

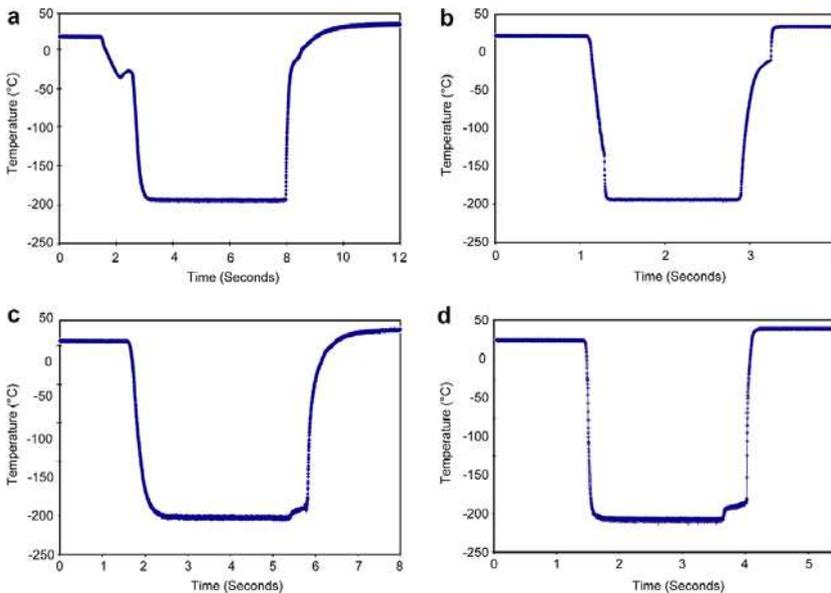


Fig. 24. (a) Thermal history for the OPS when filled with a 1.5M propane-1,2-diol and 0.3M sucrose cryoprotectant solution quenched in LN2 and then thawed in a water bath at 37°C.

A clear heat release peak is present during cooling as well as melting during rewarming. (b) Thermal history for QC when filled with a 1.5M propane-1,2-diol and 0.3M sucrose cryoprotectant solution quenched in LN₂ and then thawed in a water bath at 37°C. Crystallization of water is not obvious during cooling, but melting is shown during rewarming. (c) Thermal history for OPS when filled with a 1.5M propane-1,2-diol and 0.3 M sucrose cryoprotectant solution quenched in Slush nitrogen and then thawed in a water bath at 37°C. In this case, crystallization during cooling and melting during rewarming was not recorded. However, visual inspection reveals the presence of ice. (d) Thermal history for QC when filled with a 1.5Mpropane-1,2-diol and 0.3 Msucrose cryoprotectant solution quenched in Slush nitrogen and then thawed in a water bath at 37°C. The sample keeps its transparency over all the cooling–rewarming cycle, an indication of the capability of this approach to vitrify the studied solution.

All these changes have allowed us to maintain a concentration of cryoprotectors typical of slow freezing, 2 M PrOH+0.5 M sucrose, obtaining a morphological survival rate of 92 % in human oocytes (31). Dr. Ho-Joon Lee et al (69) tested this new technique on mouse oocytes and they saw that using Ultra-vitrification with low concentrations of cryoprotectors improved the fertilization rate and above the blastulation rate. Only the use of Ultravit device in this technique ensures the non contamination of the sample or cross-contamination in communal containers.

%	Slow Freezing (78)	Vitrification (78)	Ultra-vitrification murine oocytes (69)	Ultra-vitrification human oocytes (31)
Surv. rate	61	91,8	92.5	92
Fert. rate	61,3	67,9	75	?
Blast. rate	12	33.1	59.1	?

Table 4. Comparison between slow Freezing, Vitrification and Ultravitrification (78, 69, 31)

This comparison demonstrates the use of low concentration of cryoprotectant in the Ultra-vitrification protocol favours the morphological survival (92%) and increases the blastulation rate (59.1%). Thus confirming the hypothesis that cryoprotectants are toxic to the biological sample and if we could find a vitrification protocol that would allow us to vitrify without cryoprotectant, we would achieve a better embryo development and a greater chance of pregnancy in the case of freezing eggs or embryos. A lot more studying is needed regarding this new technique but a priori the results indicate that we can hopefully lower the concentration of the cryoprotectants decreasing the toxicity in cells.

7. Conclusion

Cryopreservation protocols have improved and resulted in a much higher efficiency in outcomes in the last years. However, it remains important to always seek for amelioration

on cryopreservation protocols and devices to ensure a major benefit and patients' safety during procedures. We need to find a method that combines a high cooling and warming rate, high survival and function of cells and tissues and is made in a way that ensures patient safety.

We must be clear that survival after a vitrification process is a "morphological survival" and that this cell has to fertilize perfectly and develop normally to rule out any damage in the process of cryopreservation and to consider a real survival.

With so much variety of devices and many different protocols, laboratories have to find the protocol and the device that best suits their skills, provided they ensure the sterility of vitrified samples and prevent cross-contamination in general containers. It has created an exaggerated paranoia about the vitrification cell and tissue contamination of the cooling solution that everything can contaminate our samples. In my opinion we must think scientifically, leaving aside the commercial interests of many of us and worrying about more logical things and research data: the probability of contamination must be demonstrated with % of contamination and leave a little aside science fiction and theoretical assumptions. For many cryobiologists avoiding cross-contamination of samples in the general containers is the most important matter as it has been proven that commercial LN2 does not have a high enough risk to have to sterilize it. The use of a closed or semi-closed device that would allow us a high cooling rate and a sealing prior to being deposited in the general container should be enough to ensure sterility and good survival and development results.

We must centre all of our interest in more practical things like finding a vitrification protocol that allows us to a vitrify without cryoprotectant, discovering new cooling solutions, discovering new materials, new devices, new procedures in which we can safely freeze samples without cryoprotectants toxicity problems, thus ensuring a good development cell after thawing, all in secure systems which ensure sterility of the sample and avoid cross-contamination. Finding an "Universal Protocol" risen several times by Dr. Katkov (79), allowing us to use the same protocol worldwide, as movement of frozen specimens around the world has increased dramatically and the lack of component preparation in the laboratory that is to thaw the frozen sample with a protocol and a device different to those they know, would not give us 100% guarantee of sample survival. Today freezing has become a luxury and not all patients can afford the excessive cost of the freezing products, if we could find a protocol without the need of cryoprotectants or a universal protocol that all could use, the price of products would decrease and the patients would benefit economically.

8. Acknowledgment

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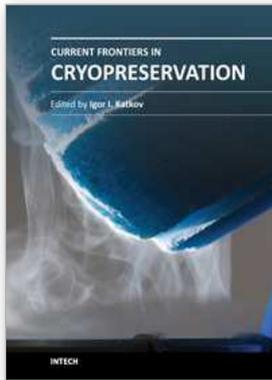
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Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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