

Closed versus open vitrification systems for human oocytes and embryos. A mini-review

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Summary

Vitrification is an established and successful technique for preserving human oocytes and embryos. It can be achieved either by direct (open systems) or indirect (closed systems) contact with liquid nitrogen and there is not a consensus on the optimum vitrification protocol. Scientific societies agree that there are no particular concerns regarding vitrification other than direct contact with a non sterile product. Moreover, European directives pose the need for aseptic procedures as a critical point. Therefore, several strategies have been developed in order to avoid the risk of contamination, including closed devices and liquid nitrogen sterilization. There have been concerns with closed vitrification devices that a reduction in cooling rate compared to open vitrification systems due to thermal insulation of samples would cause ice crystal formation resulting in impaired results. It has been proposed that a correct exposure to cryoprotective agents before closed vitrification and a high warming rate can adequately compensate the reduction in cooling rates. This reduction in cooling rate can also be

prevented with direct plunging of samples in sterile liquid nitrogen followed by hermetical cryostorage (semi-closed system). Studies comparing different protocols suggest that aseptic vitrification is an effective strategy both for embryos and oocytes.

KEY WORDS: vitrification, liquid nitrogen, hermetical cryostorage, closed carrier, oocyte cryopreservation.

Background

There are two methods of cryopreservation used for gametes and embryos; these are slow freezing and vitrification. Although procedures are deeply different, they aim of both strategies is to dehydrate cells and store them in liquid nitrogen with the minimal amount of intracellular ice. The following potential damaging factors are common between slow freezing and vitrification: toxicity of cryoprotective mixtures, chilling injury, osmotic stress, ice crystal formation and "solution effect". Comparative analyses of disadvantages and advantages of these methods have been widely discussed in the literature (1, 2) and will not be treated in this review which is focussed on vitrification.

Vitrification is a recently developed technique even if its first use in embryology harks back to the mid-eighties (3). Vitrified embryos and oocytes undergo a process that, in the absence of suboptimal conditions, produces a glass-like solidification that completely avoids the formation of ice crystals both during cooling and warming.

In recent years vitrification has become an established and successful technique for preserving human oocytes and embryos. It has been documented that, in specific groups of patients/clinical conditions, vitrified oocytes maintain the same developmental potential of their fresh counterparts (4). All of the available protocols share the relative high concentration of cryoprotective agents and are designed in order to enhance the rate of cooling/warming of the biological sample. This goal is achieved by two main strategies: using the smallest possible

volume of cryoprotectant mixture surrounding the oocyte/embryo (minimum volume cooling) and reducing any thermo-insulation between the sample and the liquid nitrogen. There is not a consensus on the optimum vitrification protocol but the direct exposure of oocytes/embryos to liquid nitrogen in a virtually absent volume of medium – the “open system” (5) – represents the ideal procedure, at least from a physical point of view. However, it has been suggested that open methods may increase the risk of contamination with pathogens during the vitrification procedure or cross-contamination during long term storage. To avoid the risk of contamination, several closed-systems have been developed, however, there have been concerns with closed vitrification devices that a reduction in cooling rate compared to open vitrification systems due to thermal insulation of samples would cause ice crystal formation resulting in cell death or reduced developmental capacity.

Position papers and legislation

Scientific Societies have discussed vitrification in order to inform members that there are no particular concerns regarding this procedure other than direct contact with a non sterile-product. In particular, the American Society for Reproductive Medicine (ASRM), in 2013 (6) stated that “there is good evidence that fertilization and pregnancy rates are similar to *in vitro* fertilization with fresh oocytes when vitrified/warmed oocytes are used as part of *in vitro* fertilization in young infertility patients and oocyte donors. This technique should no longer be considered experimental”.

The European Society of Human Reproduction and Embryology (ESHRE), through the task force for ethics and law, wrote that “the introduction of oocyte vitrification significantly advanced the outcome of oocyte cryopreservation resulting in outcomes comparable to those achieved with fresh oocytes”. On the other hand, the same paper specifies that centres offering this service must have the necessary expertise to employ oocyte cryopreservation efficiently with the so far non-standardized protocols (7).

The Human Fertilisation and Embryology Authority stated that when using vitrification methods open systems expose eggs/embryos to contamination and therefore closed methods of vitrification are preferable (8).

Recent suggested amendments to NICE guidelines regarding fertility treatment advise that clinics

should use vitrification instead of controlled-rate freezing if the necessary equipment and expertise is available (9).

From a legal point of view, the European directives on tissue manipulation force laboratories to minimise the risk of contamination of tissues and cells, including cross-contamination between donations, with validated and monitored procedures (10).

Briefly, the requirement of training and proper protocols for vitrification is stressed by the scientific community and, although a closed system is not established as compulsory, the need for avoiding a direct contact between cells and unsterile liquid nitrogen is felt as a critical issue.

Risk of contamination

Even though there is not a direct evidence of disease transmission by transferred cryopreserved human and animal embryos, it was experimentally demonstrated that cross-contamination between liquid nitrogen and embryos may occur if cells are not protected by a sealed container and infectious agents are present in the liquid nitrogen (11, 12). Most microorganisms can survive at low temperatures and cryoprotectants or ingredients of culture media can protect them from freezing injuries (13, 14). Infectious agents can be introduced in the cryopreservation system from different sources: infected donors, unsterile media/cryoprotectants or devices, contaminated liquid nitrogen, air or operators. For this reason, several strategies have been developed to prevent direct contact of cells both during vitrification and banking. These includes the usage of hermetically sealed containers, the application of a double bagging and liquid nitrogen sterilization.

A possible strategy to minimize the risk of contamination during vitrification is the sterilization of liquid nitrogen used for cooling; although this method does not prevent successive contamination in common containers for long term storage when open systems are used, it represents a safe and efficient procedure for semi-closed systems (15). In this case samples are exposed to sterile liquid nitrogen during vitrification with the advantage of achieving a high cooling rate but are subsequently sealed before storage in order to avoid any risks of contamination. The fact that liquid nitrogen can be easily sterilized (16) could encourage the clinical application of human cells vitrification, both with open and semi-closed systems. As an alternative to hermetical sealing in order to reduce the risk of

contamination it has been proposed the use of nitrogen vapours instead of liquid during storage (17, 18). According to Cobo's data, the survival rate of vitrified oocytes stored in vapour is similar to that of oocytes stored in liquid nitrogen. However, it has been recognized from authors that vapours of nitrogen are not necessarily free from infectious agents and vapour storage is linked to a higher risk of accidental and lethal warming of cells.

Open, closed and semi-closed vitrification systems

As mentioned earlier, vitrification is not represented by a single established protocol but can vary greatly in itself with particular regards to type of cryoprotectants, cooling-warming procedures and devices. Of particular interest is the possibility of using three main strategies for vitrification, namely open, semi-closed and closed systems. In the open systems cells are directly exposed to liquid nitrogen during cooling and storage; in the closed systems cells are never in direct contact with liquid nitrogen or vapours thanks to a hermetic seal; in the semi-closed systems open carriers are enclosed in pre-cooled hermetical containers after cells have being in direct contact with liquid nitrogen.

It is worth mentioning a study with mouse oocytes showing that very high rates of cooling are not mandatory during vitrification and that warming rate is of fundamental importance for survival: this suggests that closed devices, even though their insulation results in a lower cooling rate, can perform efficiently if warming rates are properly adjusted (19). There is a general consensus that the rapid warming is a crucial point for successful vitrification. Closed-vitrification protocols should guarantee warming rates similar to those used with open vitrification devices; this goal is generally achieved through removal of the sample from the insulating container whilst still submerged in liquid nitrogen and subsequent direct immersion of the cells into the warming solution.

Unfortunately, prospective randomized studies are scanty but several trials are ongoing and results will be available in the next future. By now, good results have been reported with numerous strategies and a direct comparison is practically impossible as methodology often varies deeply among groups. Under these limitations, some papers aimed to compare closed and open vitrification will be discussed briefly.

Embryos

According to Isachenko et al. (20), the deposition of human zygotes in open-pulled straws which are placed inside a hermetically-closed larger straw or container guarantees a complete isolation of samples from liquid nitrogen and is as efficient as conventional open vitrification. In fact, they found that thawed zygotes development to expanded blastocyst stage was similar when open-pulled straws were directly plunged into liquid nitrogen or were first located and sealed in a 0.5 ml straw. Other papers confirmed that also human blastocysts can be successfully vitrified in a closed system without varying exposure time to cryoprotectants compared to the open method. This is the case of the work by Kuwayama et al. (5) who compared the CryoTip® closed and Cryotop® open vitrification systems: no difference was found with regard to supporting blastocyst survival (93 and 97% for and respectively), pregnancies (51 *versus* 59% respectively) and deliveries (48 *versus* 51% respectively). Similarly, Isachenko et al. (21) found that survival and implantation rates of blastocysts vitrified with cut standard straws (open) or with "straw-in-straw" (closed) are comparable. Liebermann et al. (22) compared the Cryotop® and the High Security Vitrification® straw for blastocyst vitrification using the same exposure strategies for both methods and found similar survival, implantation, and pregnancy rates between open and closed systems. More recently, the Cryotop® open vitrification system was compared to the Rapid-i® closed system in a randomized study by Hashimoto et al. (23). They found that both human zygotes and blastocysts can be vitrified with the closed system without impairment of their developmental and implantation competence. Similar results were reported by Chen et al. (24): in their study a retrospective analysis of blastocysts vitrification cycles performed with Cryoleaf® (open) or High Security Vitrification® straws was made. Cryosurvival, clinical pregnancy and implantation rates were comparable; moreover, the neonatal outcome was reported to be similar between the two groups suggesting that the closed vitrification device is safe and effective as the open one. Panagiotidis et al. (25) have recently confirmed that the introduction of aseptic vitrification through a closed system does not affect clinical efficacy of blastocyst vitrification. They performed a prospective randomized clinical trial in an oocyte-donation programme comparing an open (Vitrisafe®) and a closed (VitriSafe® sealed in a high secu-

urity straw before vitrification) device. Embryo survival, clinical pregnancy, implantation and live birth rates were similar between groups. Of note, in this work, the exposure time of blastocysts to equilibration cryoprotective solution was increased in the closed system (9 minutes) compared to open vitrification conditions (4 minutes). According to the pilot study by the same group (26), authors suggest that the additional exposure of blastocysts to solution of lower concentration allows a higher intracellular amount of cryoprotectants that can successfully compensate the reduction in cooling rate caused by the insulation of the sample in the closed device.

Oocytes

A comparative morphological study of human oocytes after vitrification with closed (Cryotip®) and open (Cryotop®) devices concluded that ultrastructure of metaphase-II oocytes is less altered with an open system (27). The same devices were used in a prospective study comparing vitrified oocytes with sibling fresh ones. Fertilization, cleavage and good-quality rates were significantly reduced using the closed system while they were similar between fresh and sibling eggs vitrified when the Cryotop® method was used. The open system also resulted in a higher pregnancy rate compared to the closed system (28). Of note, in both studies the methodology between the groups was very different therefore results should be interpreted with caution and differences can not be ascribed exclusively to the direct exposure of oocytes to liquid nitrogen. Moreover, while these two studies suggest a lower efficacy of oocyte vitrification with the Cryotip® compared to Cryotop® method, the same devices gave similar results with blastocysts (5).

More recently, a prospective randomized study was performed in order to compare vitrification with Vitrisafe® and Vitrisafe® sealed in a high-security straw in an oocyte donation program (29). Sibling donated oocytes were randomly assigned to the open or the closed group; similarly, recipients were randomly allocated to receive vitrified oocytes either from the closed or open group. With the aim of compensating a diminished cooling rate, a longer and more gradual addition of equilibration solution and a higher final concentration of cryoprotectants were used in the closed system compared with the open one. The survival rate resulted significantly lower with the closed device;

however, no differences were recorded in terms of fertilization rate, developmental competence of resulting embryos, pregnancy, implantation and live birth rates.

Conclusions

Many methods are available for oocytes and embryos vitrification. More robust and comprehensive data are required to assess which method is optimal. However, studies comparing different carriers agree that aseptic vitrification is an effective strategy for human embryos and oocytes. An adequate exposure to cryoprotective agents before closed vitrification and a high warming rate can compensate the reduction in cooling rates caused by the thermal insulation of the sample in closed systems. This reduction in cooling rate can also be prevented aseptically with direct plunging of samples in sterile liquid nitrogen followed by hermetic cryostorage (semi-closed system).

Future controlled randomized clinical trials will shed more light on the comparison. When available results are confirmed on a larger scale, aseptic techniques will be used routinely, leaving behind any concerns about safety of vitrified human cells.

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