Efficiency and safety of human reproductive cell/ tissue vitrification

L. Parmegiani[1], R. Maggiulli[2], M. Menegazzo[3], T. Ebner[4]

[1] Reproductive Medicine Unit - GynePro Medical Centers, Bologna, Italy; [2]G.E.N.E.R.A. Centre for Reproductive Medicine, Clinica Valle Giulia, Rome, Italy; [3] Pathology of Human Reproduction Unit, Section of Clinical Pathology, Department of Molecular Medicine, University of Padova, Padova, Italy; [4] Kinderwunsch Zentrum Linz, Austria

Abstract

Vitrification is a cryopreservation technique increasingly applied in clinical practice for cells and tissue. This review article focuses mainly on the efficiency of vitrification of human reproductive cells and tissue, by analysing the clinical results reported in the literature. The second aspect discussed is safety of vitrification procedure. Different procedures and different types of carriers can be used, and in some cases vitrification requires a direct contact between cell/tissue/carrier and liquid nitrogen; this causes 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.

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Correspondence: Lodovico Parmegiani M.Sc., Reproductive Medicine Unit- GynePro Medical Centers, GynePro Medical, Via T. Cremona, 8 - 40137 Bologna, ITALY, Telephone: +39 347 472 5674; Fax: +39 051 441 135; Email: l.parmegiani@gynepro.it

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Introduction

Vitrification is a cryopreservation technique increasingly applied in clinical practice for cells and tissue. Other review articles in this journal have already described the technical details of vitrification technique and some of these have compared reproductive cell/tissue vitrification with slow freezing (Elnahas et al., 2010; Rodriguez-Wallberg et al., 2010; Javed et al., 2011; Isachenko et al., 2011).

However, the main focus of this review article is the efficiency of vitrification of human reproductive cells and tissue, by analysing the clinical results reported in the literature specifically for gametes, embryos and gonadal tissue. As a second focus, our paper analyzes the risks related to this cryopreservation technique, without overlapping the other articles regarding the aspects of different cryoprotectant solutions, protocols and carriers. 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 discussed by the authors.

Key performance indicators

Since the introduction of vitrification as a routine technic in Assisted Reproductive Technology (ART) laboratories worldwide, a long debate has been generated on websites, journals, newsletters, dedicated social forums regarding the efficiency of reproductive cell vitrification (e.g. embryomail.net, EM Digest, Vol 161). This debate basically concerns clinical outcomes when using vitrified cells or tissue.

Nowadays, all practicing embryologists have perceptions of how well reproductive cells and tissue should survive vitrification/warming, but "well-defined" indicators are needed. Almost all the indicators commonly used in reproductive cell/tissue vitrification relate to cryosurvival. These indicators are measured post-warming, evaluating the return of apparently normal function or morphological development; these can be defined "Vitrification Key Performance Indicators" (KPIs). KPIs are generally required

for evaluating the introduction of a technique or process, as minimum standards for efficiency.

Vitrification KPIs require precise definitions, objective and standardised methods for their determination. The vitrification KPIs should be compared with the performance obtainable with fresh cells and should be used in any ART laboratory for monitoring ongoing performance as part of a quality management system (both internal quality control or external quality assurance), and for benchmarking and quality improvement (The Alpha Consensus meeting on Cryopreservation Key Performance Indicators (KPIs) and Benchmarks, 2012).

Embryo vitrification

Cleavage-stage embryos

The first births from vitrified of human embryos were obtained in 1990s (Mukaida et al. 1998; Hsieh et al., 1999), and the following decade was characterized by various reports regarding the efficiency of vitrified cleavage-stage embryos using any type of open or closed devices (Kuwayama et al., 2005 b, Vajta and Nagy 2006). At first, vitrification was shown to be efficient in preserving intactness of the majority of blastomers but the early studies were not completely satisfactory in term of survival rate: survival of 79% was reported by Mukaida et al. in 1998, then 62.5% by Hsieh et al. in 1999 and 49.3% by El-Danasouri and Selman in 2001.

During the new century, the increasing skill of the pratictioners in this very operator-dependant cryopreservation technique combined with the use of different cryoprotectant cocktails and the introduction of new devices, allowed them to obtain higher survival rates (up to 90-95%) and implantation rates comparable to that of fresh embryos (Liebermann and Tucker, 2002; Rama Raju et al., 2005; 2009; Desai et al., 2007; Nakashima et al., 2010; Gvakharia and Adamson, 2011). In the light of recent studies, human cleavage-stage embryo vitrification is increasingly used in ART laboratories.

Blastocysts

Since vitrification offers some obvious benefits versus slow freezing, reports favoring this particular rapid freezing technique have become more frequent in the literature (Lane et al., 1999; Yokota et al., 2001; Mukaida et al., 2001; 2003; Vanderzwalmen et al., 2003; Van Landuyt et al., 2011) indicating that for day 5 embryos it is equivalent (Liebermann and Tucker, 2006) or even better (Stehlik et al., 2004; Youssry et al., 2008) than slow freezing. Several factors are thought to influence survival rate after vitrification of blastocysts (and slow freezing as well). Most importantly, blastocyst morphology will be associated with post-warm survival; therefore, only blastocysts with good to moderate cell lineages will usually be considered for cryostorage (Ebner et al., 2009). With respect to this, cryosurvival of morphologically inconspicuous blastocysts may fail if they stem from a cohort of bad day 3 quality embryos (Vanderzwalmen et al., 2003). It also seems to make a difference whether blastocysts are vitrified on day 5 or 6 (Mukaida et al., 2003). Breaking down their 80% survival rate to the actual day of cryopreservation, day 5 (87%) was superior to day 6 (55%). However, it should not be forgotten that as late as day 7 a 36% ongoing pregnancy rate can be expected (Hiraoka et al., 2008).

The most severe impact has been associated with the degree of expansion of the blastocoele (Vanderzwalmen et al. 2002; Zech et al., 2005). It is probable that inadequate permeation of the cryoprotectants or a very slow cooling rate leads to intra-blastocoelic ice formation during freezing, damaging the blastocyst. Several authors have suggested artificially shrinking this fluid-filled cavity, thus minimizing the chances of ice crystal formation. Vanderzwalmen et al. (2002) achieved artificial shrinkage of the blastocyst after pushing a needle into the blastocoele cavity until it contracted. By doing so these embryologists noted survival rates of up to 71%. The corresponding implantation rate after shrinkage of the blastocoelic cavity was significantly improved (12% versus 1%). At least in mice this technique did not negatively influence DNA integrity (Kader et al., 2010a). Other authors (Hiraoka et al., 2004) helped the shrinkage with mechanical pipetting using a hand-drawn glass pipette slightly smaller in diameter than the blastocyst. Alternatively, artificial holes in the blastocoele (causing shrinkage) could be produced by means of laser pulses (Mukaida et al., 2006). However, it remains to be discussed, whether these laser-generated holes are equivalent to those openings recommended (Vanderzwalmen et al., 2003; Zech et al., 2005) for assisting hatching out of the presumably hardened zona pellucida.

Although some post-thaw morphological predictors of pregnancy have been investigated in slow freezing of blastocysts (Van den Abbeel et al., 2005; Shu et al., 2008), no such data were published for vitrified and warmed blastocysts before the publication of

the paper of Ebner et al. (2009). These authors applied a four-part score to vitrified/warmed blastocysts to evaluate if certain morphological parameters could serve as predictors of implantation, pregnancy, and life birth. Apart from a more or less immediate re-expansion, a morphological parameter that had been widely used before, hatching out of the artificial gap in the zona pellucida, cytoplasmic granulation, and presence of necrotic foci were controlled and correlated to outcome (Ebner et al., 2009). The first two parameters were found to be positive predictors of life birth, while extensive cytoplasmic granulation was identified as a negative one.

More morphological details of warmed blastocysts were provided by Chatzimeletiou et al. (2012) who investigated the effect of aseptic vitrification - when using "straw in straw" closed carriers (see "vitrification carriers" section) - on the cytoskeleton of vitrified blastocyst. These authors observed that, despite the high survival rate, vitrified/warmed blastocysts revealed significantly more abnormally shaped spindles than the fresh ones (22% vs. 4%) and they concluded that further investigation is needed to elucidate any potential effects that may be reflected on the chromosomal constitution of the developing blastocysts. It is questionable whether the same observations would have been made using an open vitrification system, since ultrastructural data from metaphase II oocytes support that open systems may cause less damage at certain developmental stages (Bonetti et al., 2011). However, pretreatment with cytoskeletal stabilizers in order to improve blastocyst survival after warming obviously does not work (Chen et al., 2005).

Changes in equilibration time might have a negative impact on DNA integrity as could be shown in a mouse model (Kader et al., 2010b). However, gene expression was found not to be statistically altered in warmed blastocysts treated with different vitrification protocols (but in slow freezing some 100 genes were up- or downregulated) (Larman et al., 2011).

To summarize, vitrification clearly outruns slow freezing at blastocyst stage in terms of safety and efficacy. Not only that vitrification seems to work in "extreme" cases, such as vitrification after in vitro maturation of oocytes (Lee et al., 2007), biopsy (Zhang et al., 2009), zona-free (Hiraoka et al., 2007; Shu et al., 2010), or previously vitrified blastocysts (Peng et al., 2011), this method also perfectly works under aseptic conditions (Escribá et al., 2008; Vanderzwalmen et al., 2009; Kamath et al., 2011; Van Landuyt et al., 2011a; 2011b). Kuwayama et al (2005) compared an open (Cryotop) and a "single-straw" closed (CryoTip) system in terms of blastocyst survival after vitrification and they could not find any difference in survival (97% vs. 93%), pregnancy (59% vs. 51%), and deliveries (51% vs. 48%). The reported neonatal outcome after blastocyst vitrification further emphasizes the outstanding performance of the vitrification technique. A recent review (Wennerholm et al., 2009) reported the healthy birth of 399 healthy children born after various vitrification approaches. There were no statistical differences in the mean gestational age, birth weight, preterm delivery, or congenital birth defects as compared with fresh blastocyst transfer. The same group also focussed on the obstetric outcome (Wikland et al., 2010). Interestingly, singletons after transfer of vitrified/warmed blastocysts had a significantly higher birth weight (3560g) as compared to their fresh counterparts (3510g). In addition, more singletons born after transfer of fresh blastocysts were small for gestational age compared with those after vitrified blastocyst transfer. In contrast, a higher rate of post-partum haemorrhage was documented in the vitrified group. In addition, Lin et al. (2009) also found a shift in sex ratio toward female sex. In detail, approximately 66% of the babies were female after transfer of vitrified blastocysts. This percentage was significantly different from the 45% after fresh blastocyst transfer.

Ultimately, it has been postulated that vitrified-warmed blastocyst transfer cycles may indeed yield higher rates of implantation and pregnancy compared with fresh day 5 transfers (Zhu et al., 2011). This is a unique phenomenon for a developmental stage and if it holds true it would definitely change our embryo transfer strategies.

Zygote

Several authors have emphasized that zygote stage is an optimal developmental phase for cryopreserving human embryos (Senn et al., 2000; Salumets et al., 2003; Surrey et al., 2010). In detail, Salumets et al. (2003) had a double survival rate freezing zygotes (87%) compared to day 3 embryos (43%). The same, though on a less dramatic scale, also holds true for the blastocyst stage (Surrey et al., 2010). A Swiss group (Senn et al. 2000) found a significantly higher cumulative pregnancy rate if they transferred fresh embryos and cryopreserved all supernumerary zygotes as compared to freezing at a later stage (47% vs.

28%). Although the three above mentioned papers dealt with slow freezing protocols it became evident that this particular single-cell stage offered a great potential for cryopreservation (Keynezhad et al., 2004). With respect to this a zygote is even superior to an oocyte, e.g., because of the rather sensitive meiotic spindle on day 0. Ghetler and co-workers (2005) measured the lipid phase transition temperature of oocytes and zygotes and based on their data they concluded that zygotes showed a higher resistance to chilling injury. Apart from the single-cell stage another potential benefit involves the fact that zygotes will show no partial survival once vitrified and warmed. Either the cell is damaged after warming or it has survived, as can easily be controlled by the first mitotic division (Isachenko et al., 2004a; 2008a).

Unlike slow freezing (Senn et al., 2006) vitrification seems to change ultrastructure of the zygote rather than morphological appearance (Isachenko et al., 2004a; 2008a). This is especially the case if vitrified zygotes are rehydrated directly, e.g. if the warmed zygotes were directly expelled to culture medium (Isachenko et al., 2004a). Not even a single zygote cleaved to 2-cell stage if careful rehydration using a graded series of sucrose solutions was avoided (compared to >80% if this was done). Obviously, the harsh process of direct rehydration has a lethal osmotic effect with all affected zygotes showing signs of disruption of pronuclear membranes as well as intracellular organelles. The same authors (Isachenko et al, 2008a) subsequently examined whether the integrity rate of pronuclei after vitrification of zygotes might influence future embryo development and implantation. The integrity rate after warming was grouped into high and low. If pronuclear membranes were seen after 10 minutes of culture and at least half of the nucleoli could be observed, the integrity score was considered as being high. Indeed, growth to blastocyst stage was significantly increased if pronuclear integrity was high (40% vs. 4%). The same holds true with respect to outcome, since a 43% pregnancy rate was observed in terms of good prognosis integrity as compared to low scores (20%).

As it is valid for most of the rather new technologies, case reports on vitrification of zygotes were published in series (Jelinkova et al., 2002; Selman and El-Danasouri, 2002; Kumasako et al. 2005). Even under aggravated conditions, such as the presence of an artificial gap in the zona pellucida after polar body biopsy, unsuspicious development to blastocyst stage and live births were reported for mice and humans (Isachenko et al., 2005a, Naether et al., 2008; Macas et al., 2008, 2011; Bagis et al., 2010; Vanderzvalmen et al. 2012). Interestingly, re-vitrification of previously vitrified and warmed 2PN-stages turned out to be a feasible option (Yokota et al., 2001; Kumasako et al., 2009) although not always resulting in pregnancy and live birth (Hashimoto et al., 2007).

However, larger studies dealing with vitrification of day 1 embryos are scarce. Al-Hasani et al. (2007) introduced a routine vitrification protocol using ethylene glycol and DMSO (15% both) in their lab and achieved satisfying rates of survival (89%) and pregnancy (37% as compared to 10% with slow freezing). Variations in method and carriers followed in literature. Kuwayama et al (2005) demonstrated impressively that applying vitrification on day 1 of preimplantation development using the Cryotop carrier can result in a 100% survival rate and a 56% blastulation rate. Similarly efficient results were published from the United States using the Flexipet denuding pipette as a carrier (Liebermann et al., 2002). Utilizing open-pulled straws human pronuclear oocytes were successfully vitrified in aseptic manner (Isachenko et al., 2005b). In this paper similar rates of survival and further cleavage were observed in an open as well as a closed system. A similar approach using sealed pulled straws obtained even higher cooling rates when the authors worked with liquid nitrogen slush (Yavin et al., 2009).

Today, vitrification at zygote stage has passed its experimental status and has become a routine method in IVF labs (Al-Hasani et al., 2007). Vitrification of pronuclear oocytes seems to be a reliable, safe, and efficient technology. Nevertheless, in terms of survival, zygote stage seems to be the only developmental stage in which vitrification is not superior to slow freezing.

Gamete vitrification

Oocytes

Although the first success in freezing human oocytes was reported by Chen (1986), for approximately twenty-five years the overall efficiency of oocyte cryopreservation remained low, thus hampering widespread application. Oocyte cryopreservation may have several clinical, logistic and social indications including fertility preservation before chemotherapy, ovary removal or premature menopause;

storage in cases of difficulty with sperm collection or inadequate seminal samples; cryobanking oocytes for oocyte donation or to delay motherhood; and ethical concerns and legal restrictions related to embryo cryopreservation (Nagy et al., 2009)

In general, during cryopreservation, cells are exposed to mechanical, thermal and chemical stresses that can cause both transient and permanent alterations of the homeostatic state.

Because of its large volume that interferes with even distribution of cryoprotective additives (CPA) and the sensitive nature of the metaphase nucleus, oocyte in the second phase of meiotic division (MII oocyte) is more sensitive to cryodamage than later embryonic stages (Sathananthan 1987, 1988; Friedler et al., 1988).

However, in the past decade the efficiency of cryopreservation of MII oocytes has encountered a significant improvement and the vitrification approach has been suggested as a realistic and effective solution to the limitations related to standard freezing protocols.

Vitrification is an ice-free cryopreservation method that induces a glass-like solidification with rapid cooling of cells or tissues by exposure to high cryoprotectant concentrations (40% or more w/w) followed by a single-step ultra-rapid cooling to -196°C (Vajta et al., 2009). The physical properties of glass formation and the chemical aspects of solutes required for the design of vitrification of biological specimens have been reviewed by others (MacFarlane, 1987; Fahy etal., 1987).

A key point for a successful application of the vitrification approach concerns the choice of an appropriate support device. During the years different devices have been proposed and used, such as traditional plastic 0.25ml insemination straws, glass vials, Open-pulled straws, Hemi-straws, Cryotops, Cryoloops, Cryoleafs and Flexipets. Since a reduced volume of solution contributes to complete glass formation and enables faster cooling and warming rates, vitrification devices have been designed in order to carry a minimal volume of medium and to maximize surface area for rapid heat exchange.

To date, the most popular method for vitrification appears to be that one proposed by Kuwayama (2005, 2007). The procedure is performed at room temperature. The equilibration solution is composed by 7.5% EG and 7.5% DMSO, whereas the vitrification solution contains 15% EG, 15% DMSO and 0.5M sucrose. Oocytes are gradually equilibrated through the exposure to slightly increasing CPA concentrations for 10-12 min. They are transferred to the vitrification solution and incubated for 1 min, then loaded on the support (Cryotop) paying attention to minimizing the volume of the solution, and finally plunged in liquid nitrogen.

While the employment of open systems allows for the achievement of extremely high cooling and warming rates, it involves the hypothetical risk of pathogen contamination due to direct contact with non-sterile liquid nitrogen. Closed systems are safer for ensuring sterility than open system methods, but thermal isolation of the samples in closed systems may influence negatively the cooling and importantly, warming rates, potentially interfering with the stability of vitrification. In the ‘open systems’, aseptic vitrification can be performed by sterilizing liquid nitrogen for the vitrification procedure and subsequently cryostoring the carriers inside hermetical containers in a sterile nitrogen-vapour-phase environment (Vajta et al., 1998; Parmegiani et al., 2009). Recently, it has been demonstrated that UV-LN2 sterilization combined with hermetical nitrogen vapour cryostorage does not adversely affect the developmental competence of vitrified oocytes (Parmegiani et al., 2011a).

A significant number of studies have been published comparing laboratory and clinical outcomes of oocyte vitrification to standard freezing by slow cooling. Some authors showed rather little differences (Grifo & Noyes, 2010), but there are several data indicating a strong benefit for the vitrification approach (Fadini et al., 2009; Cao et al., 2009; Smith et al, 2010). The difference in efficacy between these procedures may be related to the lower impact of vitrification on oocyte physiology as compared to slow freezing with an improved maintenance of ultrastructure features and calcium function in vitrified oocytes (Gualtieri et al., 2011).

Moreover, convincing data demonstrated that vitrification has rather slight effects on human oocytes viability and developmental potential and the reliability of this approach has also been assured by a direct comparison with fresh controls. Rienzi et al. (2010) showed that there were no significant differences in the in vitro performance of sibling human oocytes that were vitrified or untreated. The overall

efficiency of this strategy has been confirmed by clinical results, namely pregnancy and implantation rates. High cumulative pregnancy rates were achieved with transfers of embryos derived from fresh and subsequently vitrified oocytes in a standard infertility program (Ubaldi et al., 2010).

Likewise in oocyte donation programs, controlled-randomized clinical trials confirmed the effectiveness of oocyte cryo-storage, failing to demonstrate the superiority of using fresh oocytes with respect to the use of vitrified egg-banked ones (Cobo et al., 2008, 2010) and some studies have underlined the potential application of oocyte vitrification in standard infertility programs to replace embryo cryopreservation (Schoolcraft et al., 2009; Rienzi et al., 2010; Ubaldi et al., 2010).

On the basis of the evidence provided by the randomized studies available, vitrification may be considered a safe and efficient method to cryopreserve oocytes (Cobo & Diaz, 2011). An observational longitudinal cohort multicentre study, involving 2721 warmed human MII oocytes, confirmed the safety and efficiency of vitrification approach with consistent results between centres and predictable delivery rate (Rienzi et al., 2102).

Therefore, in light of these scientific published evidences, the designation of oocyte vitrification as “experimental procedure” should be revisited and its application could be extended also to wider applications such as fertility preservation for both medical and social reasons.

Spermatozoa

Although vitrification is not routinely applied to spermatozoa and more evidence is needed in this field, this procedure of cryopreservation is considered one of the more promising emerging technologies in reproductive cryobiology. The method is based on the cooling of cells by direct immersion in LN2, thereby avoiding the formation of large intracellular ice crystals (Luyet, 1937).

Since the successful vitrification of frog spermatozoa by Luyet and Hodapp in 1938, and of fowl spermatozoa 4 years later by Shaffner (1942), the vitrification of spermatozoa has been considered an attractive alternative to conventional slow freezing.

However, early attempts at vitrifying mammalian spermatozoa using this approach resulted in low or null survival (Hoagland and Pincus, 1942; Smith, 1961) mostly because, as later shown, critical speeds of freezing and warming required by the low concentration of cryoprotective agent (CPA) tolerated by spermatozoa were unachievable at that time. In spite of this, in the early 1980s, Rall and Fahy (1985) managed to successfully vitrify embryos using high CPA concentrations and a relatively low speed of cooling and warming, and since then, this approach has also been applied to spermatozoa (Rall, 1991). At the beginning of the century, Isachenko and Nawroth both demonstrated the possibility for successful vitrification of human spermatozoa without permeable cryoprotectants, but using 1% of human serum albumin as a non-permeable cryoprotectant (Nawroth et al, 2002; Isachenko et al, 2003, 2004b,c, 2005c, 2008b).

Cryoprotectant-free vitrification

Isachenko et al (2005c) compared three different systems to vitrify human sperm and also evaluated the motility and viability. They evaluated cryoloops, droplets and open pulled straws and found that all methods were suitable for use in ART. However, they suggested that the open-pulled-straw system was the better method due to the low potential risk of microbiological contamination.

The same group also tested standard plastic capillaries which can be supplied by industrial manufacturers. The outcome indicated that vitrification in capillaries better preserved the motility of spermatozoa and the plasma membrane integrity compared to conventional freezing (Isachenko et al., 2011c, 2012).

Vitrification with cryoprotectant

Desai et al (2004) reported successful cryopreservation of individual human spermatozoa with a vitrification cryoloop by directly plunging a copper cryoloop loaded with sperm suspension into LN2. Microquantities of spermatozoa cooled in cryoloops exhibited overall motility and viability characteristics similar to those of control samples frozen in cryovials. Furthermore, individually selected spermatozoa which were cryopreserved in loops were easily warmed, and post-thaw motility was generally good.

Successively, Satirapod et al (2011) used “solid surface vitrification” (SSV) and evaluated the motility, morphology and DNA integrity of sperm. The SSV method is based on a direct exposure of spermatozoa mixed with cryoprotective agents to a cold metal surface, for rapid cooling. The cryo-containers used

were cryovials. They concluded that SSV was feasible and that the efficiency of the SSV method was largely comparable to the standard freezing method, with a slight advantage in DNA integrity and quality of sperm tail.

Endo et al (2011; 2012) compared Cell-Sleeper and Cryotop carriers to vitrify human sperm and evaluated the motility and vitality of the recovered sperm after vitrification. Both the carriers gave satisfactory results. The main difference was that, since Cryotop is an open cell-cryopreservation system, the gametes were directly exposed to LN2, unlike Cell-Sleeper, which is a closed system.

Although to date reports dedicated to sperm vitrification are rare, some evidence is starting to appear. It has been shown that permeable-cryoprotectant-free vitrification performed only with proteins (Nawroth et al, 2002; Isachenko et al, 2003, 2004b, c, 2005c) or in combination with sucrose (Isachenko et al, 2008, 2011a,b,c,d; Sanchez et al., 2011) as non-permeable cryoprotectant, provides a high recovery rate of motile cells and effectively protects the mitochondrial membrane and the DNA integrity of spermatozoa after warming (Isachenko et al., 2004b, c; 2008b). In contrast to slow conventional freezing, vitrification renders redundant the need for special cooling programs in addition to permeable cryoprotectants. Moreover, it is much faster, simpler and more cost-effective, while still effectively protecting spermatozoa from cryo-injuries (Nawroth et al, 2002; Isachenko et al, 2003, 2004b, c, 2005c, 2008b) and it does not require expensive equipment or special cooling procedures.

As successful demonstration of this vitrification technology, the first case was recently reported of a healthy baby born after intrauterine insemination of vitrified spermatozoa obtained after swim-up from an oligo-astheno-zoospermic patient (Sanchez et al, 2011). Successively, Isachenko and co-authors (2011b) reported two cases of healthy babies, born after intracytoplasmic sperm injection (ICSI) using motile spermatozoa vitrified without permeable cryoprotectants.

To date, there have been few published studies on vitrification and the current evidence is not enough to support the use of one type of vitrification over the other. Isachenko's data are the most important and indicate that vitrification with mixture of non-permeable cryoprotectants, human serum albumin (HSA) and sucrose could significantly enhance mitochondrial integrity and prevent capacitation and acrosome reaction in cryopreserved spermatozoa. Vitrification without permeable cryoprotectants is possible and effectively protects the crucial physiological parameters of the spermatozoa.

Reproductive tissue vitrification

Ovarian tissue

While the high effectiveness of vitrification of human oocytes has been widely demonstrated, few data are still available on the cryopreservation of human ovarian tissue. Theoretically there are several situations where ovarian tissue cryopreservation may offer significant advantages compared to other strategies of fertility preservation: no ovarian stimulation is needed and ovarian tissue can be harvested laparoscopically at short notice. It does not require the male involvement and represents the unique option of fertility preservation for pediatric patients.

Despite the promising results, however, cryopreservation and auto-transplantation of ovarian cortex remains a complex procedure, which requires great expertise and specialized centers to be performed.
Before clinical application, the impact of different protocols and carrier systems for cryopreservation on ovarian tissues preservation, mostly adapted from embryo and oocyte cryopreservation, has been investigated (Hovatta et al., 1996; Newton et al., 1996, Rahimi et al., 2003; Li et al., 2007; Isachenko et al., 2009; Keros et al., 2009;).

Both slow freezing and vitrification have been applied to ovarian tissue cryopreservation, but data from comparative studies led to diverging conclusions, and underlined the need to establish a standardized protocol. Some results have indicated (Gandolfi et al. 2006; Isachenko et al., 2009), that slow freezing is more promising than vitrification. However, animal and human studies showed several additional advantages of vitrification technique over slow freezing (Kuleshova and Lopata, 2002; Courbiere et al., 2006) as it does not induce apoptosis after warming (Rahimi et al., 2003; Mazoochi et al., 2008) and allows for a better preservation of ovarian stromal cells (Wang et al., 2008; Xiao et al., 2010, Keros et al., 2009). Moreover, clinical grade closed system has recently been developed to cryopreserve ovarian tissues avoiding direct contact with liquid nitrogen (Sheikhi et al., 2011). This system was able to efficiently preserve the ultrastructure of oocytes, since no

differences were observed between non-vitrified and warmed-vitrified tissue.

In the last decade, clinical application of ovarian tissue cryopreservation provided encouraging results. Following the first birth after autotransplantation of ovarian cortex in 2004, a total of 40 autotransplantations have been reported worldwide and 12 of them resulted in births (Donnez et al., 2004; 2011). Both spontaneous and assisted conceptions have been demonstrated after orthotopic re-implantation; however, so far no pregnancies have been reported after heterotopic transplantation of the cryopreserved tissue (Oktay et al. 2004).

The hypothesis of malignant contamination of the ovarian tissue must also be considered, mainly in case of hematological malignancies. In vitro oocyte maturation has been proposed as an alternative to limit the potential risk to reintroduce the original malignant cells. The dissection of cortical tissue and vitrification of denuded germinal vesicle oocytes and cumulus cells has been recommended in order to improve the efficiency of the cryopreservation technique and to preserve enough cumulus cells to allow for later in vitro maturation (Silber. 2012). However this procedure is still experimental and so far did not advance suitably to be considered as a therapeutic option.

Testicular tissue

Cryopreservation of intact testicular tissue is difficult due to the loss of cell-cell adhesions, to the ice crystal formation caused by the water trapped in tubules, and to the different cryo-biological properties of various cell types (Wald et al, 2009).

Keros et al. (2007) identified in slow programmed freezing the gold standard for cryopreservation of testicular tissue from prepubertal boys, showing the best maintenance of tissue integrity. The authors suggested that the cryoprotectant Dimethylsulfoxide (DMSO) preserved tubular structure better than glycerol or 1,2-propanediol in humans (Keros et al., 2005).
Vitrification of testicular tissue has been the subject of a recent investigation by Shaw et al (2003). Although testicular tissue has traditionally been cryopreserved using slow-freezing protocols (Kvist et al, 2006; Wyns et al, 2008), preliminary data in mice showed good short-term survival and proliferation of pre-pubertal mouse testicular tissue in culture after vitrification (Curaba et al, 2011).

In 2011, Gouk et al showed in a mouse model that vitrification of testicular tissue is superior to conventional slow freezing for the preservation of post-warming cell viability. Curaba et al. (2011) reported a protocol for vitrification of pre-pubertal human testicular tissue showing survival and proliferation of spermatogonia as well as preserved histology of spermatogonia and Sertoli cells after thawing. Furthermore, Sà et al.(2012) observed better results with open-pulled-straw vitrification of diploid germ cell suspensions compared with slow freezing. These results opened new roads for the potential use of human testicular diploid germ cell suspensions for restoring fertilization. Using spermatogonia isolated from human testicular biopsies may also provide a new individual strategy for fertility restoration and preservation (Conrad et al, 2008; Kossack et al, 2009; He et al, 2010, Sà et al, 2012).

The cryopreservation of testicular tissue, both as fragments or as stem cell suspensions, should be offered and performed simultaneously for fertility restoration and preservation in cases of threatened fertility. Optimal amounts of collected material and the survival of the diploid germ cells after isolation from the testicular tissue are key factors in guaranteeing the development of successful fertility restoration and preservation techniques (Ehmcke and Schlatt, 2008; Schlatt et al, 2009). Cell sorting is not yet efficient for either stem cell enrichment (Gassei et al, 2010) or for the depletion of contaminating cells in humans (Fujita et al., 2005; Geens et al., 2007).

The group of Sousa have demonstrated the first reliable and efficient method of testicular diploid germ cell isolation (Sousa et al, 2002; Sa´ et al, 2012), which allowed to obtain diploid germ cells from small fragments of testicular tissue with a mean viability of 72%. Moreover, they also evaluated four different cryopreservation protocols (programmed slow freezing, method of cryopreservation for somatic cell/tissue freezing, conventional semen cryopreservation method and vitrification using method based on open pulled straw) using methodologies available in any reproductive center.

The open pulled straws (OPS) vitrification protocol was applied for the first time to human testicular diploid germ cells suspensions by Cremades et al in 2004, this method gave the highest recovery (65%) and viability (56% per total recovered cells after thawing and 35% per total amount of frozen

cells) never reported before. The OPS vitrification method can be considered a promising approach for vitrification of human testicular cell suspensions (Brockbank et al., 2000, Wyns et al., 2010; Sa et al., 2012).

To date, there are few published studies on vitrification for human testicular tissue or cell suspensions. Further studies are required on cryopreservation of immature testicular cells suspensions, in order to determine their functional potential and their self-renewal and differentiation abilities. Finally, it is important to avoid the potential risk of neoplastic cell contamination in the testicular grafts, by developing new techniques which allow the sorting of putative malignant cells in testicular germ cell suspensions via specific markers (He et al, 2010).

Safety of vitrification

Vitrification carriers

Vitrification is increasingly applied in clinical practice for human reproductive cells. During vitrification, the cells/tissue need to be cooled and warmed at an extremely rapid rate (Vajta et al., 2009). This can be achieved by using specific “open carriers” such as Open Pulled Straw (Vajta et al.,1998), Cryoloop (Lane et al., 1999), Hemi-Straw (Vandervoost et al., 2001), Cryotop (Kuwayama et al., 2005 a), Cryoleaf (Chian et al., 2009), Cryolock (Bernal et al., 2009), Vitri-inga (Almodin et al., 2010), etc; these “open carriers” are generally preferred for oocytes (Antinori et al., 2007; Cobo et al., 2008; Rienzi et al., 2009; Ubaldi et al., 2010; Garcia et al., 2011). However, these systems cannot avoid the hypothetical risk of microorganism contamination during the vitrification procedure, if the LN2 is accidentally contaminated (Bielanski et al., 2000, 2003).

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 (Kuleshova and Shaw, 2000; Isachenko et al., 2005a). 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 (Liebermann, 2010; Isachenko et al., 2005a, 2010). However, the “straw-in straw” system causes a reduction in the rate of cooling and is not routinely used in clinical oocyte cryopreservation.

As an alternative to straw-in-straw, other types of closed systems allow faster rates of cooling, such as CryoTip (Kuwayama et al., 2005 b) or Cryopette (Keskintepe et al., 2009). 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 LN2 is avoided. Unfortunately, because of their design, these systems cannot avoid the transmission of micro-organisms in the culture medium during the warming procedure, due to the previous direct contact during vitrification between the external surface of the carrier and the accidentally contaminated LN2. This is because the contamination of cells occurs at 37°C, when any cryopreserved micro-organism found in the LN2 reactivates after thawing in the culture medium. Even though IVF culture media are supplemented with antibiotics, some micro-organisms 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 (Bielanski et al., 2000; Tedder et al., 1995). Another procedure to decontaminate the straw is to quickly wipe the carriers with 70% ethanol for disinfection at warming (Kuwayama et al., 2005 b). However, the de-activation of all microorganisms can be obtained only by a 5-minute-contact between ethanol and carrier (Sopwith et al., 2002); this prolonged contact time can damage human cells, which remain inside the carrier in the warmed vitrification solution rich in potentially toxic cryoprotectants (Fahy et al., 1990). Recently, Parmegiani and co-authors have proposed a reliable procedure to decontaminate frozen human specimens before warming.

This procedure consists in washing the specimens with sterile LN2 and it 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 (Parmegiani et al, in press).

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Cryostorage

Nowadays, human cells and tissues are cryostored in LN2 or in nitrogen vapour (NV):

this cryostorage is potentially hazardous because many pathogens can survive at the low temperature of LN2/NV (Russell et al., 1997; Morris, 2005; Bielanski et al., 2000; 2003; Bielanski, 2005; 2012, Grout and Morris, 2009) and may contaminate the frozen cells or their carriers/container surface into the cryobanks (Tedder et al., 1995; Bielanski et al., 2000; 2003; Bielanski, 2005; 2012; Criado et al., 2011; Grout and Morris, 2009, Parmegiani et al., 2011a).

To date, there have been no cases of disease transmission by transferred cryopreserved human embryos (Pomeroy et al., 2010; Bielanski and Vajta, 2009; Bielanski, 2012; Cobo et al., 2012); despite this we have no specific studies regarding possible negative effects of LN2/NV infectious agent contamination on the final outcome of IVF frozen cycles; since we know that some of these microorganisms negatively affect gametes and embryonic development at warming (Bielanski et al., 2003; Foresta et al., 2011; Kastrop et al., 2007; Klein et al., 2009). In addition, vitrification is increasingly used in humans and this cryo-procedure appears to be riskier than slow freezing due to the direct contact between cells/tissue and LN2 required for “open systems”. The hypotetical risk of culture contamination at warming cannot be excluded even when using some “closed vitrification systems” (Parmegiani, 2011; Parmegiani and Vajta, 2011; Parmegiani et al., 2011 a).

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 (Vajta et al., 1998, Parmegiani et al., 2009; 2011a; Parmegiani and Rienzi, 2011; Bielanski et al., 2012). Periodic cleanings 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 (Parmegiani et al., 2009; 2011b).

Regulations and Quality Assurance

Hypothetical cell/tissue contamination by LN2/NV requires us to guarantee the sterility of vitrification procedure, 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 will probably be introduced by the Food and Drug Administration (FDA) for Assisted Reproductive Centres in the United States (Pomeroy et al., 2010). 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. (2010) considered the cross-contamination of infectious agents a negligible risk and the majority of cryobiologists and embryologists maintain that vitrification with open systems using non-sterile LN2 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 due to direct contact with accidentally contaminated LN2.

Discussion

Vitrification has moved from experimental stage to a routine practice and 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 LN2/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 LN2/NV; for this reason open-system vitrification can comply with any existing directive, as long as aseptic procedures during vitrification-cryostorage-warming are established (Parmegiani et al, 2011ab, Parmegiani and Rienzi 2011).

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.

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