Introduction

Vitrification of blastocysts: the evolving "state of the art" freezing technique

The proportion of births following transfer of cryopreserved blastocysts has increased dramatically during the last 10 years due to a remarkable improvement in the efficiency of vitrification techniques as alternatives to the classical slow freezing procedure (Vanderzwalmen *et al.*, 2002, 2003, 2010; Mukaida *et al.*, 2003a; Stehlik *et al.*, 2005; Liebermann and Tucker, 2006; Stachecki *et al.*, 2008; Van Landuyt *et al.*, 2011; Panagiotidis *et al.*, 2013). With the policy of single blastocyst transfer an increasing proportion of supernumerary blastocysts is vitrified either on day 5 or 6 (Liebermann and Tucker, 2006). Also, there is an increasing tendency to shift from fresh blastocyst transfer to vitrified blastocyst transfer to circumvent an inadequate uterine environment due to risk of ovarian hyperstimulation syndrome, inappropriate endometrium build up, endometrial polyps or uterine myomas. Moreover, vitrification of blastocysts is a valuable option when they originate from in vitro maturation cycles (Vanderzwalmen *et al.*, 2010; Ortega-Hrepich *et al.*, 2013).

Furthermore, several studies have reported that the safety of vitrified blastocysts in terms of obstetric outcome did not differ either from those of fresh blastocysts or blastocysts cryopreserved by slow-freeze methods (Takahashi *et al.*, 2005; Liebermann, 2009; Wikland *et al.*, 2010).

Principle and description of the different phases of a vitrification procedure

It is entirely the skill of being able to prevent ice crystals forming inside the cell (which can happen during the cooling as well as during the warming process) that will determine the viability of the embryos (Quinn, 2010). When the temperature decreases, liquid water can be converted either to a solid crystal or to a solid amorphous glass when the supercooled water is dropped instantaneously below the glass transition temperature. The physical process by which a viscous solution supercools to very low temperatures and finally solidifies into a meta-stable glass, without undergoing crystallization at a practical cooling rate, is called vitrification (Rall and Fahy, 1985). According to this definition, with the application of vitrification, formation of ice crystals is not theoretically possible in the intra-cellular or extra-cellular spaces.

The fundamental issue in all vitrification methods is to achieve and maintain conditions within the cells which guarantee an amorphous state throughout the cooling as well as during the warming process. Independent of the carrier device that determines the cooling and/or the warming rate, the key of success in order to achieve a "glass-like" state depends on an optimal balance between the speed of cooling/rewarming (time and temperature) and the optimal cell dehydration and penetration of cryoprotectant (CP) when cells are exposed to concentrated hypertonic solutions (Leibo and Pool, 2011).

Vitrification and warming of blastocysts consists of several steps irrespective of whether an open or a closed system is used:

(i) selection of blastocysts before vitrification;
(ii) exposure of blastocysts to the CP solutions;
(iii) loading on the carrier device and plunging into liquid nitrogen (LN_2);
(iv) storage in LN_2 containers;
(v) the warming process;
(vi) dilution of the CP;
(vii) selection of warmed blastocysts before embryo transfer (ET).

Step one: selection of blastocysts before vitrification

It should be obligatory to cryopreserve supernumerary blastocysts of moderate to good quality in order to increase cumulative pregnancy rates. With regard to this, it is very important to achieve a reproducible

outcome, especially in terms of survival after warming independently of the quality, to allow high success rates after vitrified/warmed blastocyst transfer.

Grading of blastocyst morphology incorporates assessment of degree of expansion of the blastocyst and the quality of the inner cell mass and the trophectoderm (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011; Ebner *et al.*, 2011). Several studies have shown that trophectoderm was statistically significantly related to the rate of ongoing pregnancy and miscarriage. By contrast, neither inner cell mass nor blastocyst expansion was statistically significantly related (Ahlström *et al.*, 2011; Honnma *et al.*, 2012). In a more recent article, Ahlström *et al.* (2013) analysed which pre-freeze morphological parameters can be used to predict live birth outcomes after vitrified/warmed blastocyst transfer cycles. They stated that blastocoel expansion and trophectoderm grade were identified as the most significant pre-freeze morphological predictors of live birth.

Acceptable results were also obtained after vitrification of blastocysts that originated from embryos that were not selected either for fresh ET or cryopreservation on day 3 because of their poor quality. Fifty blastocyst warming cycles resulted in a 76% survival rate, 44% clinical pregnancy rate, and 39% implantation rate (Shaw-Jackson *et al.*, 2013).

With respect to blastocyst quality and survival, the question arises whether artificial shrinkage or collapsing is mandatory.

Since the size of the blastocoel corresponds to the amount of water in this cavity, larger blastocysts might show reduced cryopreservation potential due to ice crystal formation during the cooling step and devitrification during the warming step.

In order to reduce the likelihood of ice crystal formation artificial shrinkage or collapsing of expanded blastocysts has been suggested, using either micropipettes (Vanderzwalmen *et al.*, 2002) or laser pulses (Mukaida *et al.*, 2006).

However, with the use of a vitrification solution containing a higher sucrose concentration of 0.75 M instead of 0.5 M, we observe that artificial shrinkage of the blastocoelic cavity is not necessary for preventing injury from intracellular ice because there is sufficient dehydration during the exposure to the vitrification solution.

In addition, Zech *et al.* (2005) showed the benefit of opening the zona pellucida some hours before the vitrification process. They observed that blastocysts with a larger blastocoelic cavity survived vitrification better when they partially or completely hatched even with short exposure to cryoprotectant solutions.

Step two: exposure of blastocysts to the CP solutions

Before the blastocysts are immersed in LN_2, they are exposed to a CP solution in order to increase the intra- and extracellular viscosity to a level that the liquid water molecules will solidify so quickly that they will not have time to rearrange themselves into a crystalline structure. To achieve this objective, in nearly all vitrification methods the blastocysts are exposed to a minimum of two steps of gradually increasing concentrations of non-vitrifying solution (nVS) and vitrifying solution (VS) (Vanderzwalmen *et al.*, 2013a).

In a single or sequential steps the blastocysts are first exposed to nVS. During this step, a certain amount of CP enters the cells. It may take 3–15 minutes according to the type of CP and the cooling rate which in turn depends on the carrier device. The length of time of exposure to the nVS at a defined temperature (T°) is of utmost importance and determines the amount of intracellular CP. The duration of exposure to the permeable CPs is determined by several biophysical factors such as the membrane properties (cellular permeability to water and CP), the type and concentration of CP, the surface/volume ratio of the cells, and the rate of cooling and warming (Leibo, 1980; Kasai and Edashige, 2010; Vanderzwalmen *et al.*, 2013a). The nVS is exclusively composed of permeable CP (e.g., DMSO, ethylene glycol, 1,2-propanediol, glycerol).

In the final step, the biological material is exposed for a shorter time to the VS (30–90 seconds). An intracellular vitrifying state is obtained due to the dehydration of the inner cell mass and trophectoderm cells in contact with the VS that concentrates the intracellular solutions of salts, proteins, and CP that have penetrated the cell in the course of exposure to nVS. This strategy will generate an intracellular environment that is compatible with a vitreous state when cells are directly plunged into LN_2. The extracellular vitrifying state is obtained by the high concentration of CP in the VS that encapsulates the embryo in a vitrifying sheath. Additionally,

non-permeable CPs with low (sucrose, trehalose) or high molecular weight (Ficoll) are present in the VS.

Although the solutions used to vitrify blastocysts contain high concentrations of permeable cryoprotectants as compared with the conventional slow freezing procedure, it has been observed that, contrary to common belief, the intracellular concentration of cryoprotectant is almost one-third of the concentration of the VS and even lower than after a slow freezing procedure (Vanderzwalmen *et al.*, 2013b). We may therefore state that although slow freezing has been the standard cryopreservation method for more than 25 years, few were aware that cell survival is the consequence of the presence of an intracellular vitrified state. This vitrified state is a result of a long-term effect of solution during cooling and is a reflection of a very high concentration of CP. We may therefore suggest that a drop in survival after slow freezing is probably more related to osmotic shock after warming than to mechanical injuries due to the formation of intracellular ice crystals. Moreover, in order to avoid the use of highly concentrated CP solution, it is not justified to continue with slow freezing procedures.

To conclude we may advise that slow freezing is ultimately another way to do vitrification.

Step three: loading on a carrier device and plunging in LN_2

Open system

It was postulated that ultra-rapid cooling and warming rates (as high as 20,000–30,000°C/minute) are mandatory during the vitrification process to reduce the risk of intracellular crystal formation and the concomitant damage to the cell structures (Lane *et al.*, 1999). To achieve ultra-rapid cooling rates, a very small volume of VS of less than 1 µl is deposited on an open carrier device (e.g., Cryotop, Vitriplug, Cryoloop, copper electron microscopical grids), which is directly plunged into LN_2 (Vanderzwalmen *et al.*, 2002; Mukaida *et al.*, 2003a, 2003b; Kuwayama, 2007).

The advantage of such an approach is that blastocysts are exposed in two steps to increasing concentrations of CP. However, this exposure is only for a short period of time – long enough to permit the extraction of the intracellular water while limiting the amount of CP permeating into cells, thus reducing osmotic stress.

One drawback of the "open" carrier devices is that the blastocysts are directly exposed to LN_2 during the cooling process as well as during the whole storage time. Although the theoretical risk of cross-contamination by bacteria, viruses, or fungi during cooling or storage in LN_2 has been widely debated (AbdelHafez *et al.*, 2011), the potential for contamination with reactive chemical compounds raises safety concerns (Bielanski, 2009).

Various methods for sterilizing LN_2 have been proposed or are under development, including ceramic filters (Cobo *et al.*, 2011) or ultraviolet light with subsequent hermetic cryostorage (Parmegiani *et al.*, 2010, 2011), or using LN_2 vapor for storage (Grout and Morris, 2009). Although the probability of an impairment of cellular structures by contact with LN_2 is still being discussed, this risk is important and the ongoing discussion indicates that the storage system, especially in the long term, should be revised. Even the standard storage conditions and refilling of the tanks pose a hazard when oxygen from surrounding air condenses and mixes with LN_2 during the regular opening of the nitrogen tank for routine refilling or whenever straws are added or withdrawn. Although it is generally assumed that thermally driven reactions do not occur in cells at −196° C, it has been reported that in the case of radiation of an LN_2/oxygen mixture a synthesis of oxygen radicals resulting from ozone formation and decomposition cannot be excluded and is even enhanced by the catalytic effect of nitrogen. Mouse oocytes show impaired survival, fertilization rates, and embryonic development after prolonged contact with LN_2 (Yan *et al.*, 2011).

Closed system

The European Directives (2004/23/EC) as well as the FDA directives on tissue and cell storage dictate the adherence to certain safety regulations, ensuring that gametes and embryos are protected from any possible contamination with pathogens and to prevent them from any harmful physical conditions during storage. To achieve the EU directive, a valuable option consists of switching from an open vitrification carrier device to a protocol that entails complete isolation of the biological samples from LN_2 during both the cooling process as well as storage by hermetically isolating the embryos from LN_2 in the tanks.

A huge difference exists in the cooling rate is the subject of an ongoing debate as the cooling rate is widely believed to be an important factor for success of the

vitrification protocols. Several studies have shown that vitrification of oocytes (Papatheodorou *et al.*, 2013), zygotes (Vanderzwalmen *et al.*, 2012), and blastocysts in closed carriers achieves good results in clinical studies (Kuwayama *et al.*, 2005; Liebermann and Tucker, 2006; Stachecki *et al.*, 2008; Vanderzwalmen *et al.*, 2010; Panagiotidis *et al.*, 2013).

In a recent study, Chatzimeletiou *et al.* (2012) investigated the effects of aseptic vitrification on the cytoskeleton and development of human blastocysts, by analyzing survival rates and spindle and chromosome configurations by fluorescence and confocal laser scanning microscopy. Although there was a significantly higher incidence of abnormal spindles in the vitrified group compared with the fresh group, the high survival rate following warming and the large proportion of normal spindle/chromosome configurations suggest that aseptic vitrification at the blastocyst stage on day 5 does not adversely affect the development of human embryos and the ability of spindles to form and continue normal cell divisions.

Step four: storage in LN_2 containers

Little is known about the risks of prolonged storage of cryopreserved cells as vitrification is the solidification of a fluid without formation of crystalline structures – a physically disorganized unstable system. This raises the question as to whether this state changes over time, thus impairing survival and implantation potential of vitrified gametes and embryos. Subsequently, any potential impact on the health of the newborn is unknown. Results after vitrification of blastocysts in a closed system show no alterations with respect to survival and live birth rates irrespective whether storage lasted for 1, 2, 3, 4, 5, or 6 years. A mean pregnancy rate (PR) of 43.6%; an ongoing PR of 35.8%, and a live birth rate of 29.0% were observed. More interestingly, no malformations were reported (Wirleitner *et al.*, 2013). From these observations covering a storage period of 6 years it may be concluded that vitrification of blastocysts is a safe technique.

Step five: the warming process

Since the rate of cooling engendered a hot debate, it is surprising that little emphasis is put on the warming procedure. However, it has become obvious that the warming rate might play a more essential role in modulating survival after vitrification than the cooling rate.

A fast warming rate prevents the vitreous water from re-crystallizing during the warming phase (Seki and Mazur, 2008, 2009). In fact, during the process of warming, cells first devitrify when they are warmed above the glass transition temperature. If the warming rate is not fast enough the supercooled liquid is transformed with high speed into small ice crystals. If the appropriate timing is not used or if warming rates are too slow, small ice crystals are subjected to the phenomenon referred to as re-crystallization, which may have lethal consequences.

It is well known that for any given concentration of CP the critical warming rate is much higher than the critical cooling rate (Fahy *et al.*, 1987). Consequently, the minimum concentration of CP to prevent crystallization during warming must be higher than during cooling. This means that it might be easier to maintain a vitrified state during the cooling than during the warming process for the same concentration of CP. If the warming rate is reduced by using devices isolating the drop containing the embryos, higher intracellular concentrations of CP are needed in order to reduce the likelihood of re-crystallization. However, these higher concentrations of CP might be toxic to the cells. Hence, in order not to increase the concentration of CP above the toxic level the biological material has to be warmed extremely rapidly.

Step six: dilution of the CP

During warming water re-enters the cells and the CP is washed out. This has to be performed in a controlled way in order to avoid cellular damage. A too rapid influx of water is circumvented by a stepwise exposure to solutions containing reducing sucrose concentrations.

Step seven: selection of warmed blastocysts before ET

ALPHA Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology (2011) published key performance indicators for all steps of cryopreservation procedures including minimum performance and aspirational benchmark values. Morphological parameters, however, were not in the scope of this workshop.

Though some post-thaw morphological predictors have been investigated in slow freezing of blastocysts,

e.g., immediate re-expansion (Van den Abbeel *et al.*, 2005; Shu *et al.*, 2008) or 24-hour survival, no such data have yet been published for vitrified blastocysts. It has been suggested that as the result of the presence and size of the blastocoelic cavity, vitrified/warmed blastocysts experience several morphologic changes and become collapsed during cryopreservation. Thus, it is more difficult to score a vitrified blastocyst after warming than a fresh one (Shu *et al.*, 2008).

Several factors (unrelated to vitrification method) are known to directly influence the fate of a cryopreserved blastocyst after thawing/warming and transfer. It is important to realize that survival rates in the literature are hardly comparable due to the fact that some embryologists focus on immediate survival while others suggest an additional waiting period of 24 hours to facilitate control of growth (Vanderzwalmen *et al.*, 2003). Differences in implantation rates may also be attributed to the fact that not all working groups apply assisted hatching to the thawed blastocysts (whilst shrunk), though this was found to improve outcome (Vanderzwalmen *et al.*, 2003).

In detail, re-expansion of the blastocoel (and consequently the blastocyst) after thawing is expected within 24 hours after thawing (Vanderzwalmen *et al.*, 2003; Van den Abbeel *et al.*, 2005). However, immediate re-expansion, e.g., within the first 2 hours after warming, has not been used for prognostic purposes in vitrified blastocysts. Since in slow freezing approximately half of the frozen blastocysts turned out to re-expand immediately after 2–4 hours in culture (Shu *et al.*, 2008), it is indicated that using vitrification a higher rate of re-expansion might be observed (Ebner *et al.*, 2009).

Even if it can be assumed that all viable blastocysts will re-expand after several more hours, any delay in this process could be the manifestation of altered osmotic and/or metabolic conditions (comparable to the situation found during blastocoel development when water enters the blastocoelic cavity via tight junctions, either diffusing passively or being pumped actively).

A recent publication (Ebner *et al.*, 2009) introduced a grading system based on re-expansion, hatching (out of the artificial gap in the ZP), cytoplasmic granulation, and presence of necrotic foci.

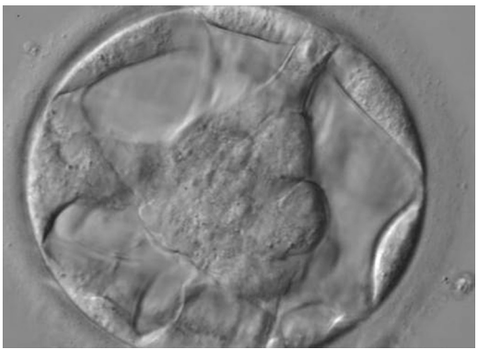
Vitrification in an open system (Linz, cases 1–70)

Vitrification

Routine in vitro culture for cases 1–70 was either performed in sequential media (EmbryoAssist and BlastAssist, Origio, Denmark) or global media (GM501 Cult, Gynemed, Lensahn, Germany). Irrespective of the type of medium, culture was performed in 30 µl drops under sterile mineral oil (GM501 Mineral Oil, Gynemed). Both approaches had the medium changed on day 3. All embryos that were not transferred in a fresh cycle were cultured up to day 5, the day vitrification was performed routinely. It should be noted that some patients needed special media to get blastocysts at all. In detail, those patients (cases 43, 65, 67) who suffered from azoospermia had their testicular tissue treated with collagenase (GM501 Collagenase) in order to facilitate collection of testicular spermatozoa. In these cases TESE sperm were further treated with theophylline (GM501 SpermMobil) to improve/restore motility. Others (cases 12, 27, 47, 53) had their oocytes cultured in an ionophore (GM508 Cult-Active) bath immediately after ICSI in order to overcome low fertilization (Ebner and Montag, 2014) or severe male factor infertility (Ebner *et al.*, 2012).

Prior to vitrification all blastocysts were scored according to the guidelines of Gardner and Schoolcraft (1999) focusing on expansion, inner cell mass, and trophectoderm. Only morulae and early blastocysts without fragments or blastocysts with both adequate ICM and trophectoderm were considered for cryostorage (Figure 1). According to in-house definitions, an adequate quality was reached if early blastocysts had only minor fragmentation and full to expanded blastocysts showed either a perfect ICM and/or TE (with none of these cell types allowed to be of worst quality according to the Gardner score).

In all cases, vitrification was done utilizing a commercially available kit (GM501 VitriStore Freeze, Gynemed) at room temperature (RT). All morulae/blastocysts were pre-incubated in a medium containing PBS and HSA for 1 minute (Figure 2). This step was followed by two media, a nVS and a classical VS with different composition. The time blastocysts were kept in the nVS (PBS, HSA, ethylene glycol, and DMSO) depended on the individual degree of expansion. In detail, morulae and early blastocysts were incubated for 1 minute, full blastocysts for 2 minutes and expanded or hatching blastocysts for 3 minutes (Figure 3). Partial shrinkage of the blastocysts was controlled under a microscope. Since in the VS (PBS, HSA, ethylene glycol, DMSO, and sucrose) CPs were at a higher concentration, as far as possible the exposure time was kept to a minimum (30 seconds) (Figure 4).



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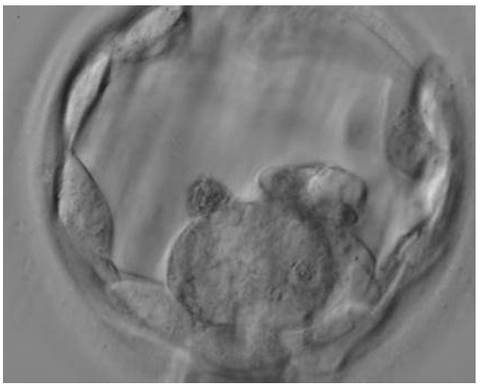

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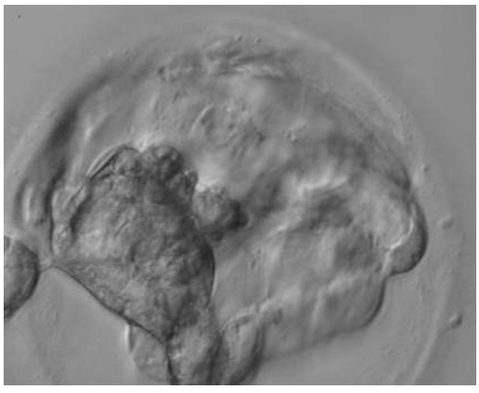

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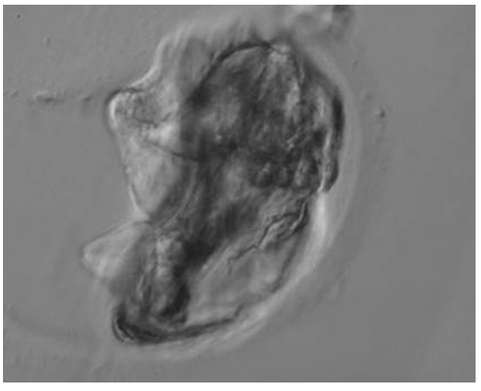

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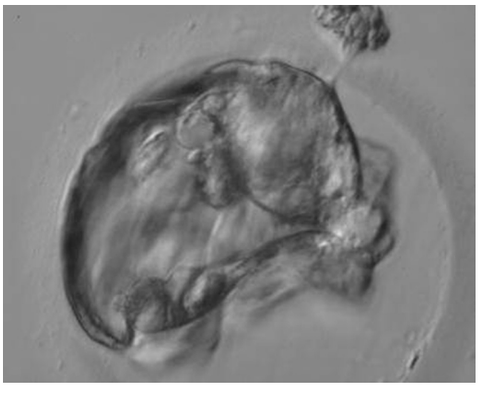

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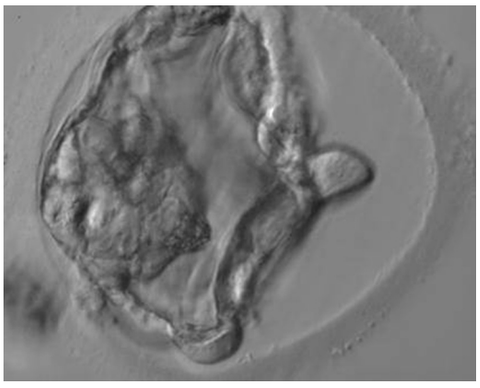
It should be noted that some expanded or hatching blastocysts had their blastocoelic cavity artificially reduced prior to vitrification (Vanderzwalmen *et al.*, 2002) using Microtech pipettes (Gynemed).

Within this last crucial period a maximum of two shrunk morulae/blastocysts were placed on the tip of a Hemi-straw (VitroPlug, Astro Med Tec, Salzburg, Austria) in a very small volume of vitrification medium 2 (<0.5 µl) followed by direct plunging into LN_2. Before storage in a special container (Arpege 170, Air Liquid, Vienna, Austria) all semi-straws were sealed with high security straws (Cryo Bio System, L´Aigle, France).

Warming

Special care was taken to ensure very fast warming rates which required rapid separation of the semi- and the protective high security straw followed by immediate plunging into the first warming solution at 37°C (Figure 5).

The other three media of the GM501 VitriStore Thaw kit (Gynemed) were kept at RT (Figures 6–8). All four warming media were based on PBS and HSA but had descending concentrations of sucrose (0.5 M,



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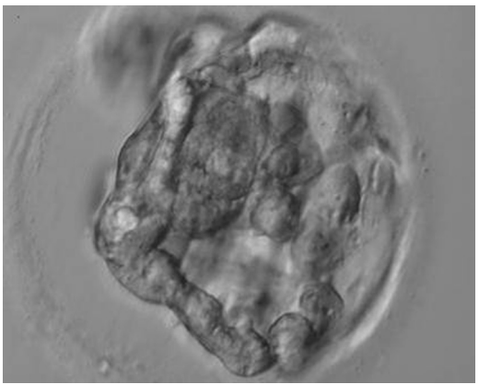

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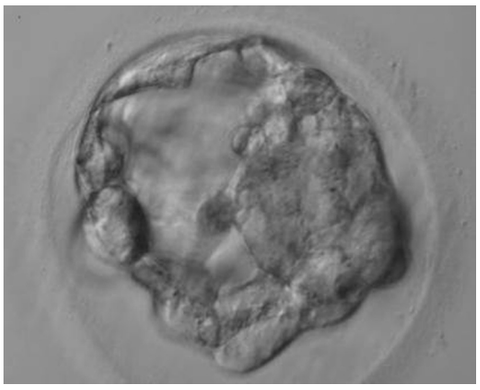

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0.25 M, 0.125 M, 0 M). After this warming process direct transfer into a pre-warmed culture medium, either GM501 (Gynemed) or BlastAssist (Origio, Måløv, Denmark), was performed.

All warmed blastocysts (except those that had started to leave the zona before vitrification) were hatched by means of a laser in order to minimize any theoretical impact of cryopreservation on ZP constitution (Vanderzwalmen *et al.*, 2003; Liebermann and Tucker, 2006).

All blastocysts considered to be viable after warming were scored according to a previously published grading system based on re-expansion, hatching out of the artificial gap in the ZP, cytoplasmic granulation, and presence of necrotic foci (Ebner *et al.*, 2009). Routinely warmed blastocysts were kept in culture for at least 2 hours prior to scoring and transfer.

Vitrification in a closed system (Bregenz, cases 71–100)

Exposure to the cryoprotectant

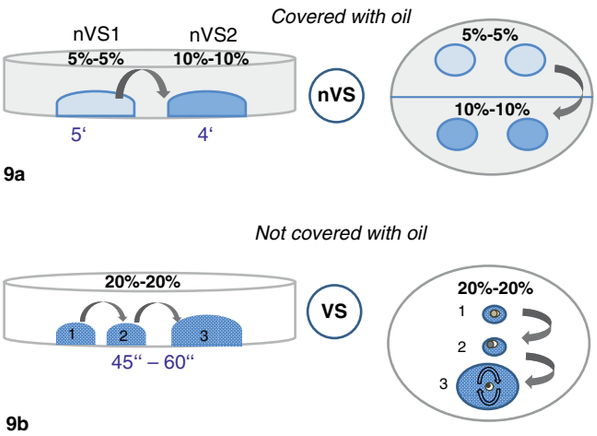
All the procedures occurred at RT utilizing a commercially available kit (VitriFreeze ES: Fertipro, Bernem, Belgium). The blastocysts were exposed to two nVS. The nVS1 was composed of 5% EG and 5% DMSO in PBS–HSA, whereas the nVS2 had double concentration. The VS was composed of 20% EG (v/v), 20% DMSO (v/v), 25 µmol/l (10 mg/mL) Ficoll (70,000 MW), and 0.75 mol/l sucrose.

Drops of 50 µl of nVS1 and nVS2 were deposited in a Petri dish and covered with oil. A maximum of two blastocysts were exposed for 5 and 4 minutes in nVS1 and nVS2 respectively. Except for expanded blastocysts the time in the nVS1 solution may be prolonged up to 7 min (Figure 9a).

The blastocysts were placed in the nVS2 drop and three elongated drops of VS (50 µl, 50 µl, 100 µl) were deposited in a Petri dish without oil overlay (Figure 9b). To avoid an osmotic shock, blastocysts were then transferred to the first VS drop with a small volume of nVS2 and left for 10 s before placing them in the second drop for 10 s more. Before transfer of the blastocysts in the second and third VS drops, the pipette was washed with pure VS solution. Finally the blastocysts were placed in the third drop and by gentle washing and rotating for almost 10 to 15 s encapsulation of the blastocyst in the VS was guaranteed. The VS induces a pronounced collapsing of the blastocysts, strong enough to permit an acceptable survival rate without previous artificial shrinkage of the blastocoel cavity.

Loading on the closed carrier device "VitriSafe" and plunging in LN_2

After incubating the biological material in the VS, a maximum of two blastocysts was deposited in the gutter of the Vitrisafe (Figure 10b, c) (IVF Distribution, Bregenz, Austria). When expanded blastocysts and blastocysts exhibited an inner cell mass and trophectoderm of quality A or B, vitrification of only one at a time was done.



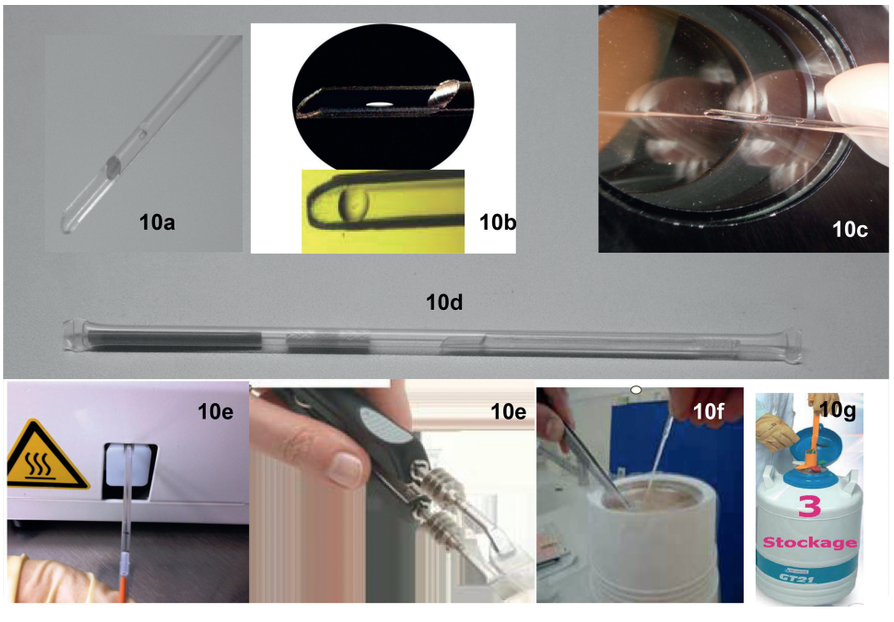
9 General procedure for vitrification of blastocysts

The Vitrisafe consists of a large gutter (Figure 10a) that is inserted into a protective outer straw of 0.3 mL (CBS, Cryo Bio System, France) (Figure 10d). The edges of the protective 0.5 mL straw are welded (Figure 10e) ensuring a complete isolation of the biological sample from LN_2. The hermetically closed straw is then directly plunged into LN_2 (Figure 10f). A cooling rate of 1300°C/min is achieved. The total time necessary to place and mix the blastocysts into the VS, load them on the carrier device and finally plunge them into LN_2 after welding the protective straw is 50 to 70 s. Afterwards the cooled straws were placed in a long-term LN2 tank (Figure 10g).

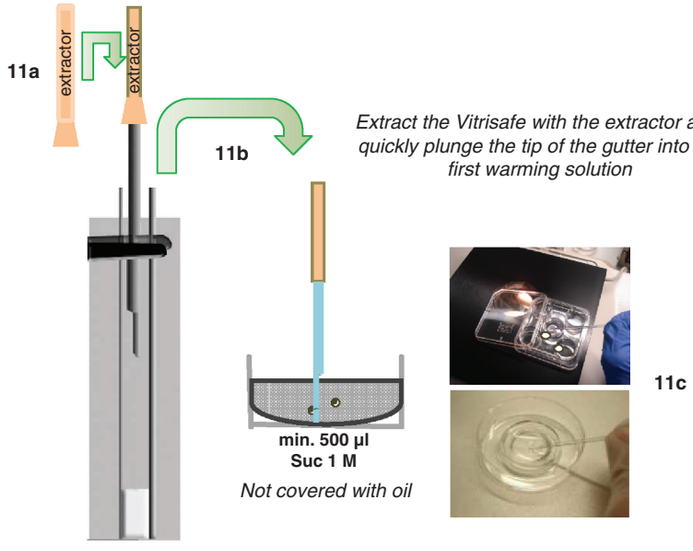
Warming steps and dilution of the CP

The solutions for warming were made of 1, 0.5, 0.25, and 0.125 M sucrose with 20% HSA (VitriThaw ES: Fertipro, Bernem, Belgium). An extra 0.75 M sucrose solution was made by mixing 1 M and 0.5 M sucrose V/V.

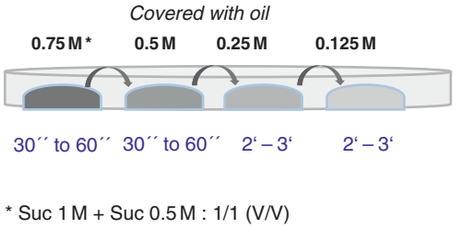
The Vitrisafe is designed to guarantee high warming rates of >25,000°C/min. The Dewar vessel with LN_2 containing the straw was placed close to the stereomicroscope. Avoiding contact with LN_2, the outer straw was cut with scissors and an extractor (a small straw with a conical end that entraps the Vitrisafe plug (Figure 11a)) was inserted to fix the Vitrisafe plug (Figure 11b). The plug was pulled out of the protective



10 Loading on the closed carrier device VitriSafe and plunging in LN_2



11 Loading on the closed carrier device VitriSafe and plunging in LN_2



12 General procedure of warming of blastocysts

larger straw and the tip of the gutter holding the blastocysts immediately immersed in a 4-well or a 1-well dish containing a minimum of 500 µl of 1 M sucrose (Figure 11 c). After 1 minute the blastocysts were transferred to a Petri dish containing drops of 50 µl of 0.75 M, then 0.5 M sucrose, for 30–60 s. Subsequently they were transferred for 2–3 min in 0.25 M, then 0.125 M sucrose (Figure 12). All procedures were performed at room temperature. The embryos were then washed several times in the culture medium. Transfer of a maximum of two blastocysts was scheduled after an additional culture period of 4–24 hours. All blastocysts considered to be viable after warming were scored according to a previously published grading system based on re-expansion, hatching out of the artificial gap in the ZP, cytoplasmic granulation, and presence of necrotic foci (Ebner *et al.*, 2009).

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