

Journal für

Reproduktionsmedizin und Endokrinologie

– Journal of Reproductive Medicine and Endocrinology –

Andrologie • Embryologie & Biologie • Endokrinologie • Ethik & Recht • Genetik
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J. Reproduktionsmed. Endokrinol 2013; 10 (Sonderheft
1), 45-54

www.kup.at/repromedizin

Online-Datenbank mit Autoren- und Stichwortsuche

Offizielles Organ: AGRBM, BRZ, DVR, DGA, DGGEF, DGRM, D-I-R, EFA, OEGRM, SRBM/DGE

Indexed in EMBASE/Excerpta Medica/Scopus

Krause & Pachernegg GmbH, Verlag für Medizin und Wirtschaft, A-3003 Gablitz



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Cryopreservation of Human Unfertilized and Fertilized Oocytes

P. Vanderzwalmen^{1,2}, N. H. Zech¹, Z. P. Nagy³, A. Stecher¹, P. Papatheodorou⁴, M. Schuff¹, B. Wirleitner¹

Cryopreservation of human fertilized oocytes and embryos are nowadays well established in IVF practice with a wide range of clinical applications. However, freezing of unfertilized MII oocytes turned out to be one of the greatest challenges in the field of human reproductive cryobiology since the protocols remained ineffective for over 25 years. Only in the last 10 years the efficiency and safety of oocyte cryopreservation tremendously improved with the realization that zona hardening occurs in the process of cryopreservation and the introduction of vitrification. The possibility to cryopreserve unfertilized oocytes introduces new applications in the field of cryopreservation. We have now the opportunity to preserve fertility for cancer patients or to freeze oocytes for IVF patients when now sperm is available at the day of pick-up. This chapter will be dedicated to the vitrification of unfertilized MII oocytes and fertilized oocytes (pronuclear or zygote stage). An overview on the different indications for both stages of development will be described in the first part of this chapter. The second part will be about the technical and practical description of the vitrification process using hermetically closed and thus aseptic systems such as the Vitrisafe as carrier device. Finally, an overview on the clinical aspect, including our experience, will be given. **J Reproduktionsmed Endokrinol 2013; 10 (Special Issue 1): 45–54.**

Key words: cryopreservation, IVF, ART, vitrification, closed system, MII Oocyte, zygote

■ Introduction

The proportion of births following transfer of cryopreserved embryos and particularly oocytes has increased dramatically during the last 10 years. Freezing of human fertilized oocytes and embryos are today well established with a wide range of clinical applications. However, cryopreservation of unfertilized oocytes was already first reported in the late 1980s, but it turned out to be one of the greatest challenges in the field of human reproductive cryobiology since the protocols remained ineffective for over 25 years [1].

It was until recently that the efficiency and safety of oocyte preservation has tremendously improved. First it had to be recognized that zona hardening occurs in the process of cryopreservation and the intracytoplasmic sperm injection (ICSI) instead of in vitro fertilization by insemination (IVF) has to be applied. Another breakthrough was the application of vitrification instead of slow freezing protocols for cryopreservation of unfertilized oocytes. Most recently highly developed aseptic vitrification protocols are entering the IVF laboratories.

The possibility to cryopreserve unfertilized oocytes introduces new applica-

tions in the field of cryopreservation. We have now the opportunity to preserve fertility for cancer patients for example or to freeze oocytes for IVF patients when now sperm is available at the day of pick-up. This chapter will be dedicated to the vitrification of unfertilized MII oocytes and fertilized oocytes (pronuclear or zygote stage). An overview on the different indications for both stages of development will be described in the first part of this chapter. The second part will be about the technical and practical description of the vitrification process using hermetically closed and thus aseptic systems such as the Vitrisafe as carrier device. Finally, an overview on the clinical aspect, including our experience, will be given.

■ Indications

MII Oocyte Vitrification

With the introduction of highly secure vitrification protocols there is an emergent change in the general attitude towards oocyte cryopreservation. This offers solutions for example, in the field of fertility preservation or in cases of ovarian hyperstimulation (OHSS) syndrome. Egg accumulation cycles can be offered to poor responder patients and cryo-banking for eggs are applied in donation programs. Finally it allows over-

coming ethical concerns and legal restrictions to freeze fertilized oocytes or human embryos.

Fertility preservation

Oncological indications

Aspects of fertility preservation in women with cancer in their reproductive age are evolving due to changes in treatment options and prospects of egg freezing. In the western countries approximately 1 of 10.000 women in the reproductive age is affected by a cancerous disease. The most common forms are leukaemia, lymphomas, cancer of the central nervous system, breast cancer and cervical cancer. Young cancer patients nowadays have very good prognosis to survive the disease due to early diagnosis and improvement of treatment modalities. However, even due to the treatment (in most cases either chemotherapy and/or radiotherapy) the patients are often faced with non-reversible damage to their ovaries and oocytes. Thereby they are predisposed to menstrual disorders, premature ovarian failure (POF) with subsequent precocious menopause leading to high chance of sub- or infertility. Abdominal radiotherapy was found to induce ovarian damage in a dose-dependent way whereas total body irradiation was reported to cause

Received: July 11, 2012; accepted after revision: September 18, 2012

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premature ovarian insufficiency (POI) in 97% of all reported cases [2]. Administration of chemotherapy might cause loss of follicles within the ovary, lead to hormone deficiency and infertility [3].

Besides the possibility to cryopreserve embryos in order to preserve fertility in these patients, which is only applicable for couples, two main options exist. Ovarian cortex ablation and cryopreservation with subsequent autografting is the only strategy for pre-pubertal girls and is still considered as an experimental approach with limited results. Oocyte vitrification after minimal invasive ovarian stimulation has become an important tool for cancer patients. This technique does not run the risk of cancer cell transmission – as present in ovarian cortex re-transplantation and is therefore one major advantage of this method. More about fertility preservation in oncological patients will be presented in a later chapter of this issue.

Non-oncological indications

Besides cancer, other non-neoplastic diseases also imply treatment with chemo- or radiotherapy. This includes autoimmune diseases such as systemic autoimmune rheumatic diseases requiring cyclophosphamide therapy and benign haematological diseases [4]. Another non-oncological medical indication for egg freezing is severe and symptomatic endometriosis. In these patients the deleterious effect of the chronic inflammation processes on the ovarian reserve and oocyte quality during the years can be circumvented by this technique [5]. Genetic predispositions leading to risk of fertility loss at young age is another indication as reported for Turner syndrome mosaicism [6].

In the daily IVF routine, oocyte cryopreservation helps couples where no semen can be retrieved at the day of pick-up due to medical or psychological reasons. Formerly, the oocytes once gained after pick-up had to be discarded after about 8 hours due to the aging process. Now they can be thawed and fertilized when sperm samples are available. Egg freezing can also be considered an option in poor-responder patients where accumulation cycles are applied before continuing with ICSI [7]. Also for exaggeration of OHSS in a fresh cycle freezing of oocytes is regarded as an excellent

alternative to the cryopreservation of embryos with the new techniques and the promise of high survival rates after thawing.

Ethical Reasons for Oocyte Cryopreservation and “Social Freezing”

Apart from couples with moral or religious objections about cryopreservation of embryos also some countries have law restrictions regarding the cryopreservation of embryos. Here, surplus oocytes can be frozen [8]. A very young application of oocyte cryopreservation is the so called “social freezing” [9]. For many women it is not cancer but the passage of time that denies them a chance of motherhood. There are social, educational, and financial pressures that force them to delay starting a family until their late 30's. In this age their chance for a pregnancy is comprised by low fecundity rate and increased rates of spontaneous abortions. At the same time the risk of having a baby with chromosomal abnormalities such as Down's syndrome is significantly increased. By cryopreserving their eggs at young ages, women get the chance to postpone childbirth and at the same time circumvent the negative impact of age on oocyte quality.

Oocyte Vitrification in Donor Programs

Oocyte donation has become a frequently applied technique in assisted reproductive technology (ART). Main indications for donor oocyte cycles are POF, heritable maternal genetic disorders, early perimenopausal and/or menopausal women, poor responders and patients with recurrent implantation failures in IVF cycles. Recurrent implantation failures might be linked to genetic abnormalities, such as chromosomal translocations or dysfunctions of the spindle apparatus leading to a high incidence of aneuploidies in the oocytes. More and more women also enter an oocyte donation program due to advanced age. Ovum donation has consistently produced the highest pregnancy rates reported for any assisted reproduction methods, basically due to the selection of oocytes from young healthy donors.

Oocyte donation requires hormonal synchronization of the donor and recipient menstrual cycle. In order to meet the high demands for donor oocytes and the large logistical effort, vitrification offers

the best option for oocyte banking and has to be performed under the rules of the European Tissues and Cells Directive (Directive 2004/23/EC). With oocyte cryopreservation the need for donor/recipient synchronization is eliminated. Egg banks can be established facilitating the logistics of coordinating egg donations [10]. Temporary quarantine of donor eggs to test the donors for transmissible diseases becomes possible with application of freezing.

Vitrification of Fertilized Oocytes

In contrast to the freezing of unfertilized oocytes, the cryopreservation of fertilized oocytes at the 2 pronuclei (2PN) or zygote stage was successful quite early in the history of freezing and has become an essential part of assisted reproduction procedures. Cryopreservation of 2PN stage embryos shows high survival rates after thawing [11, 12]. The unicellular form and lack of spindle apparatus may account for its high post-thaw survival and implantation potential. Therefore this technique is nowadays widely applied not only for supernumerary embryos but also for a number of medical indications such as OHSS and inappropriate build-up of the endometrium and uterine environment [13, 14].

Zygote Freezing as Strategy to Circumvent Inadequate Endometrial Receptivity

Due to the high dosages of hormones prescribed in IVF cycles in order to stimulate multifollicular growth, it is difficult to achieve an optimal preparation of the endometrium at the same time. Under suboptimal environmental conditions the implantation chances for the embryos are reduced. Using highly efficient cryopreservation protocols, a reliable strategy to overcome the problem of an impaired uterine environment and to maximize the chances of pregnancy is to substitute embryo transfer (ET) in the same fresh cycle by transfer in a cryo-cycle. Thereby the embryo is replaced in an optimized, well-prepared luteal phase.

The cryopreservation of zygotes and the replacement of blastocysts in a cryo-ET after extended culture to day 5 have already been reported as a reliable strategy to circumvent inadequate endometrial receptivity [15, 16].

Medical Reasons to Cryopreserve Zygotes

Medical indications to vitrify 2PN embryos are, for example, risk of OHSS. Even mild hyperstimulation may be associated with decreased endometrial receptivity due to histologically advanced and dysregulated endometrial tissue [17, 18]. In IVF-patients inappropriate endometrium build-up, or the occurrence of endometrial polyps or uterine myomas during stimulation may decrease implantation chances. An ET after medical surgery or in another cycle often improves the outcome.

Fertility Preservation

Zygote vitrification represents an option for women faced to the diagnosis of cancer. Before undergoing radio- or chemotherapy, there is the opportunity to put the patients, if medically not contraindicated, through a stimulation cycle and freeze the obtained oocytes after fertilization with the partner sperm at the zygote state. However, a male partner has to be available, otherwise the patients have to accept donor sperm for fertilization or use egg freezing as a good alternative strategy.

Oocyte Donation Cycles

An option to achieve best synchronization of donor cycle and appropriate endometrial preparation in the recipient, zygote vitrification is applied. Thereby the administrative expense and costs of oocyte donation cycles are reduced. However, it is inappropriate for non-clarified serological status of the donor as it takes place after fertilization.

Ethical Reasons or Due to Legal Restrictions

Apart from the reasons mentioned before, in some countries such as Germany and Switzerland, legal restrictions make it mandatory to cryopreserve the fertilized oocytes at the zygote stage before the pronuclei fuse, as at this time point the oocyte is defined as an embryo. Many patients also decide for cryopreservation at the pronuclear stage due to ethical, moral, and religious reasons.

■ Vitrification: the Evolving "State of the Art" Freezing Technique

Prevention of Intracellular Ice Formation

Water, the solvent of salt and cryoprotectants is the major component of

the cell and is the main actor involved in the process of cryopreservation. According to cooling conditions, when the temperature decreases, liquid water can be converted either to a solid crystal or to a solid glass, an amorphous vitreous state. This amorphous state occurs when supercooled water is dropped instantaneously below the glass transition temperature. It is obvious that intracellular crystallisation of water is incompatible with any living organism. It is solely the skill of being able to prevent ice crystals to form inside the cell (which can happen during the cooling as well during the warming process) that will determine the viability of unfertilized or fertilized oocytes [19].

During the past few decades, two major methods differing in their principle methodology were developed to achieve this objective: the slow rate freezing [20] and the ultra-rapid cooling procedure termed vitrification [21]. Independently of the cryopreservation protocol, when oocytes or zygotes are dropped instantaneously below the glass transition temperature, the intracellular part of the cell will vitrify. Or, in other words, the superviscous, supercooled intracellular liquid converts into a "glassy-like" state without formation of ice crystals. In this context, vitrification consists in the whole solidification of a solution without ice crystal formation due to an increase of viscosity on cooling. Vitrification theoretically does not involve the formation of ice crystals, neither in the intracellular nor in the extracellular spaces because the whole sample turns directly to "glas" when plunged in liquid nitrogen (LN2).

The fundamental issue in all vitrification methods is to achieve and maintain conditions within the cells which guarantees an amorphous state throughout the cooling as well as during the warming process.

How to Vitrify? Outline of the Different Phases

The objective of this section is to describe the different phases and technical aspects that are implicated in a vitrification process [22, 23]. Vitrification of oocytes or embryos consists of 5 steps:

1. Exposure of gametes or embryos to the cryoprotectant solutions (CPs)
2. Loading on the carrier and plunging in LN2

3. Storage in LN2 containers
4. Extraction of the carrier from the protective straw and warming of the cells
5. Dilution of the intracellular cryoprotectant (CP) concentration

Step One: Exposure to Cryoprotectant Solutions

Required Conditions to Achieve a Vitrified State

The combination of high concentrations of CPs and extremely fast cooling/warming rates are two conditions that support the formation of a glass-like state. Independent of the carrier device, the key of success is to find the optimal balance between the speed of cooling - rewarming (time and T°) and the minimal and optimal conditions of exposure to high cryoprotectant (CP) concentrations needed to obtain and maintain an intracellular non-toxic vitrified microenvironment [24]. This means that the success of cryopreservation protocols depends on optimal cell dehydration and penetration of CP when they are exposed to hypertonic solutions.

The permeability and the way of entrance of water and CP through the cell membrane, changing during the different stages of development and the cell size and morphology are important aspects that have to be taken into account in the establishment of a vitrification protocol.

Practical Aspect: Exposure to Non-Vitrifying (NVS) and Vitrifying (VS) Solutions

Before plunging the biological material in LN2, cells are exposed to CPs with the aim to create an intracellular environment that remains vitrified in a defined cooling-warming rate. To achieve this objective, nearly all vitrification methods consist of exposing the biological material in a minimal of two steps to gradual increasing concentrations of (NVS) non-vitrification and vitrification solutions (VS). The NVS is exclusively composed of permeable CP. In contrast, in the VS additionally non-permeable CP with low and high molecular weight are present.

The oocytes or zygotes are first exposed once or several times to the NVS. During this step, a certain amount of CP enters the cells. The time of exposure to the

NVS at a defined temperature (T°) is of utmost importance and determines the amount of intracellular CP. It may range between 3 and 15 minutes according to the type of CP and the type and stage of the biological material. 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 [25–27].

In a last step, the biological material is exposed for a short time to the VS. An intra-cellular vitrifying state is obtained due to the dehydration of the embryos in 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 LN2. The extracellular vitrifying state is obtained by the high concentration of CP in the VS that encapsulate the embryo in a vitrifying sheath.

Practical aspect: Procedure of Exposure to the CPs

In Figure 1 the times and procedures to expose oocytes and zygotes to NVS and VS are explained in detail. The required time to place and mix the oocytes or embryos into the VS, loading them on the carrier device, and finally plunging the carrier into LN2 after welding the protective straw should take a maximum of 60 seconds. Warming of oocytes with the aseptic vitrification protocol is performed in sucrose solutions starting with 1M in five steps.

Step Two: Loading on a Carrier Device and Plunging in LN2

From Open to Closed Carrier Devices: Justifiable?

It was postulated that ultra-rapid cooling and warming rates (as high as 20.000°C–30.000°C/min) were mandatory during the vitrification process to reduce the risk of intracellular crystal formation and the concomitant damage to the cell structures. To achieve ultra-rapid cooling rates, embryos are placed in a very small amount of VS of less than 1 µl on open carrier devices (Cryotop, Vitriplug, Cryoloop, copper electron microscopy grids), which are directly plunged into

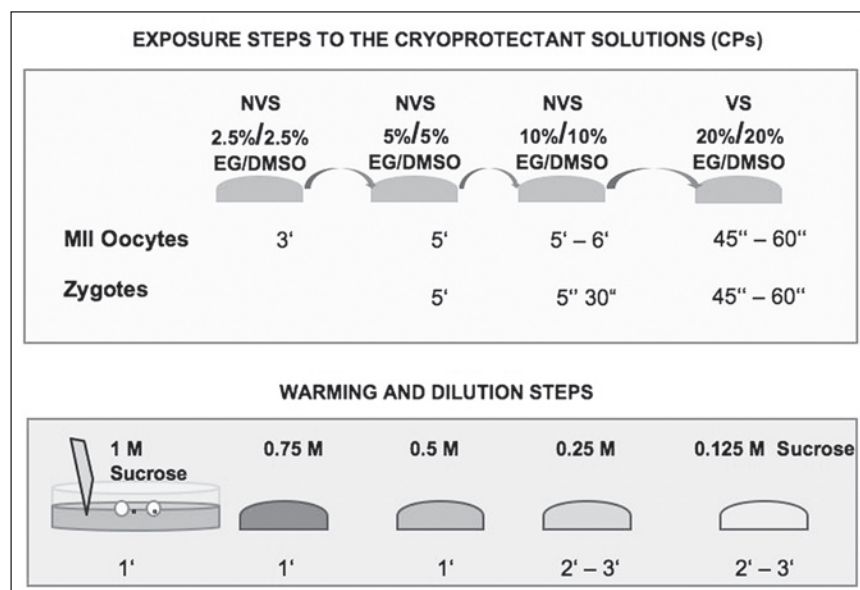


Figure 1. Exposure to the CPs for vitrification and to sucrose solutions for warming of MII oocytes and zygotes. EG: ethylene glycol; DMSO: Dimethylsulfoxide

LN2. Liquid nitrogen is usually non-sterile and probably may contain pathogens. Additionally reactive chemical compounds are potentially present. Embryos or gametes vitrified on these “open” carrier devices are directly exposed to the LN2 during cooling and these straws are closed only in LN2 implying a constant level of LN2 in the straw (Fig. 2a).

One drawback of this strategy is the direct contact of the biological material with LN2 during cooling as well during the whole storage time. Although the question of contamination by bacteria, viruses or fungi during cooling or storage in LN2 is still under debate, the potential probability of contamination raises safety concerns [28, 29]. For this reason, great effort is taken at least to minimize the risk of contamination with pathogens during the vitrification process and when they are directly plunged into LN2 by sterilization. Various methods for sterilizing LN2, including ceramic filters [30] or UV-light simultaneously with hermetical cryo-storage [31, 32] are under development and are both technically complex and expensive. Storage in the vapor was proposed as a valuable alternative. However, even LN2 vapor is reported to be a risk factor for pathogen transmission [33]. Additionally, temperature variations within the vapor might impair the viability of cryopreserved gametes and embryos.

Even when the exposure to infectious material at the point of vitrification is avoided by using sterilized LN2 and a possible cross-contamination is circumvented in vapor tanks, the constant exposure to the LN2 inside the ‘open’ vitrification devices including reactive low molecular compounds still remains [34]. Although the probability of impairment of cellular structures by contact with LN2 is still being discussed, this risk is important and indicates that the storing system, especially in long-term means should be revised (Fig. 2b). Reactive chemical compounds in LN2 might induce biophysical injuries of the cryopreserved samples. Methods to sterilize the LN2 prior to contact with the gametes or embryos with UV light might additionally provoke the production of reactive compounds, leading in turn to an increased risk of damage due to the physical and chemical properties of irradiated LN2.

However, even the standard storage conditions and refilling of the tanks pose a hazard when oxygen from surrounding air condenses and mixes with LN2 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 LN2/oxygen mixture a synthesis of oxygen radicals resulting from ozone formation and

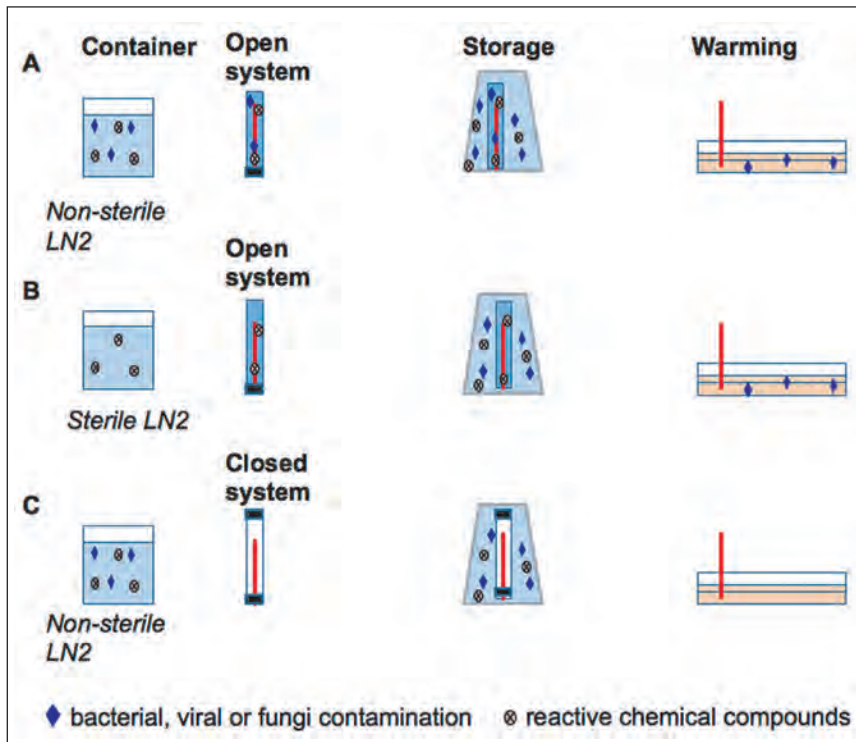


Figure 2. Vittrification in open and closed devices following contact with sterile or non-sterile liquid nitrogen (LN2).

decomposition cannot be excluded and is even enhanced by the catalytic effect of nitrogen. A recent publication reports that mouse oocytes show impaired survival, fertilization rates and embryonic development after prolonged contact with LN2 [34]. Therefore, not even the avoidance of cross-contamination in the tanks by storage of straws in nitrogen vapor can sufficiently protect the samples.

The theoretical risk of cross-contamination in LN2 containers even at -196°C has been widely debated [35]. In view of this debate, we therefore recommend a hermetically closed system, which solely guarantees optimal storage conditions. Isolation of embryos inside a closed straw assures safety against contamination from LN2 and hazardous effects that may occur during long-term storage.

The European Directive (2004/23/EC) as well as with FDA directives on tissues and cells storage dictates for cryopreservation of cells and tissues to adhere 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 valu-

able option consists to switch from an open vittrification carrier device to a protocol that entails complete isolation of the biological samples from LN2 during both the cooling process as well as storage by hermetically isolating the embryos from LN2 in the tanks (Fig. 2c).

Practical Aspect: The Vitrisafe, an Efficient Hermetically Closed Vittrification System

After mixing the biological material in the VS, oocytes or embryos are placed on the gutter of the Vitrisafe (AstroMedtec, Austria) before insertion into a protective outer straw (CBS, Cryo Bio System, France) (Fig. 3a, b). The edges of the protective 0.5 ml straw are welded ensuring a complete isolation of the biological sample from LN2 and the hermetically closed straw is directly plunged into LN2. A cooling rate of $1300^{\circ}\text{C}/\text{min}$ is achieved.

Closed Devices and Reduction of the Cooling Rate

A huge difference exists between vittrification protocols with open ($> 25.000^{\circ}\text{C}/\text{min}$) or closed ($< 2.000^{\circ}\text{C}/\text{min}$) carrier devices. This reduction in the cooling rate is responsible for a still ongoing debate – especially for the vittrification of oocytes – as the cooling rate is widely believed to be an important factor for

success of the freezing protocols. Although, several studies showed that the vittrification of blastocysts in closed carriers achieves good IVF results in clinical studies [36–39], it is still believed that open systems should be used for sensitive biological materials such as MII oocytes. Recently, Papatheodorou et al. [40] reported in a prospective randomized study comparing vittrification – warming cycles in closed vs. open devices ($n = 75$ in each group) no difference in the birth rate (BR) after vittrification of oocytes in in both groups (36% vs 24%).

Step Three: Storage in Liquid Nitrogen

The choice of long-term storage container depends on the vittrification tool: open or closed carrier devices [1]. In order to avoid possible cross contamination during long-term storage, it is recommended to store open carrier devices in the vapor phase of LN2 [41]. Special containers designed for this purpose are available. The safety of this storage still has to be proved, as there was a reported fungal transmission through LN2 vapor [33]. Moreover, this storage system needs high-technology systems to control the storage temperature implying higher costs as compared to conventional storage tanks.

One advantage of hermetically closed devices is that they may be stored in Dewar tank with non-sterilized LN2 facilitating the handling. Subsequently, the use of classical container permits in a more tangible way the level of LN2 during straw manipulation (Fig. 2 a–c).

Effect of Prolonged Storage

Little is known about risks of prolonged storage of cryopreserved cells as vittrification is the solidification of a fluid without formation of crystalline structures – a physically disorganized unstable system. This raises the question if this state changes over time, impairing survival and implantation potential of vittrified gametes and embryos. Subsequently, the impact on the health of children born is unknown.

Our results show no alterations of survival rate (SR), pregnancy rate (PR), ongoing pregnancy rate (oPR) and birth rate (BR) with vittrified blastocysts stored for 1, 2, 3, 4, 5 or 6 years. A mean PR of 43.6%; an oPR of 35.8%; and a

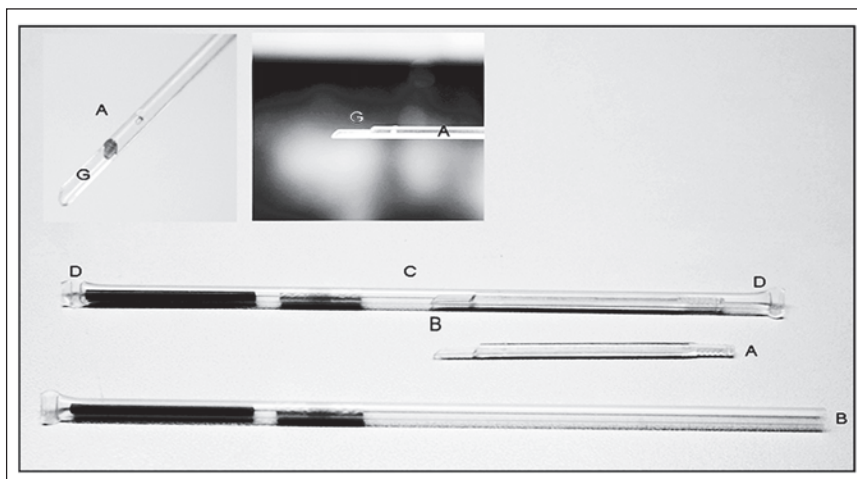


Figure 3a. Device for aseptic vitrification. The Vitrisafe carrier device consists of the carrier (Vitrisafe plug) (A) and a high security straw 0.3 m (B). G: Gutter to place embryos; B: Aseptic Vitrisafe CBS Straw; C: Aseptic sealed Vitrisafe device; D: Welding points.

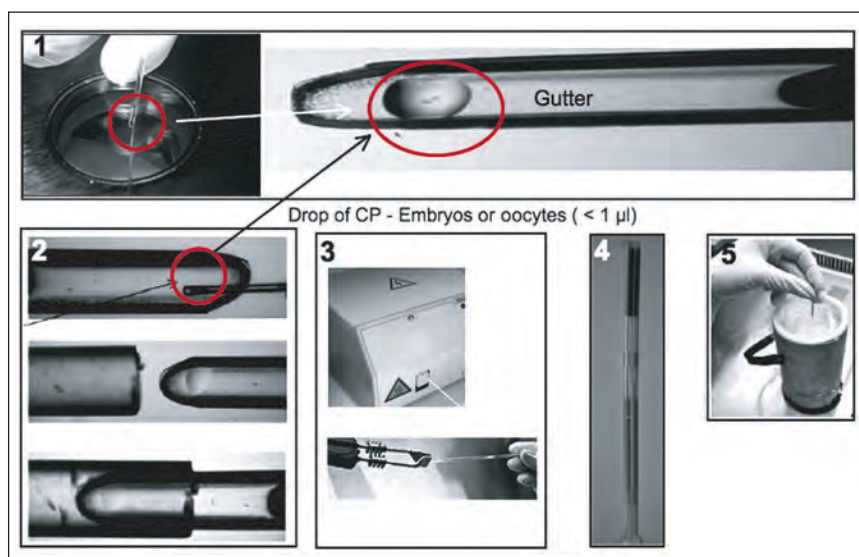


Figure 3b. Vitrification procedure with the Vitrisafe plug (1). The biological material is placed on the gutter of the Vitrisafe plug (2). The Vitrisafe plug is inserted into the high security straw (3). Thermo-sealing of the outer straw (4). Hermetically closed vitrification straw (5). Plunged into liquid nitrogen (LN2)

BR of 29.0% was observed. No malformations in any of the children born were reported.

In our setting, neither SR nor developmental potential of cryopreserved embryos seems to be impaired by long-term storage. Additionally, we see no indication for a potential risk in means of BR and health of children born after vitrification and several years of storage. These results encourage us that vitrification is a safe technique.

Step Four: The Warming – Maybe the most Critical Step?

As the rate of cooling engenders a hot debate, it is surprising that little care is

emphasized to the warming procedure. However, it has become obvious that the warming rate might play a more essential role in modulating SR after vitrification than the cooling rate.

A high warming rate prevents the vitreous water from re-crystallizing during the warming phase [42, 43]. 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 velocity into small ice crystals. Given appropriate time or too low warming rates, the small ice crystals are subjected to the phenomenon referred to as

re-crystallization that may have lethal consequences (Fig. 4).

It is well known that for any given concentration of cryoprotectants the critical warming rates are much higher than the critical cooling rates [44]. Consequently, the minimal 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 that separate 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 cells. Hence, not to increase the concentration of CP too much the biological material has to be warmed extremely rapidly.

Practical Aspect: The Vitrisafe, a Carrier that Guarantees High Warming Rates

The Vitrisafe is designed to guarantee high warming rates of $> 25,000^{\circ}\text{C}/\text{min}$. This is ensured as the gutter is extracted from the protective straw and directly emerged into 1M sucrose solution. Thereby ultra-fast warming rates are achieved.

Step Five: Dilution of the Cryoprotectants

During warming water reenters the cells and CP are 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.

Practical Aspect: Warming of Aseptically Vitrified Oocytes

A Dewar of LN2 containing the devices is placed close to the stereomicroscope. Avoiding contact with LN2, the plug is pulled out of the larger straw and the tip of the gutter holding the cells is immediately immersed in minimal 0.5 ml of 1M sucrose for 1 minute (warming rate $> 25,000^{\circ}\text{C}/\text{min}$; Fig. 1). The oocytes are then transferred to 0.75 M, 0.5M, 0.25M, and 0.125 M sucrose for 1, 1, 2 and 2 minutes respectively. All procedures are performed at room temperature. The oocytes are then washed several times in the culture medium before

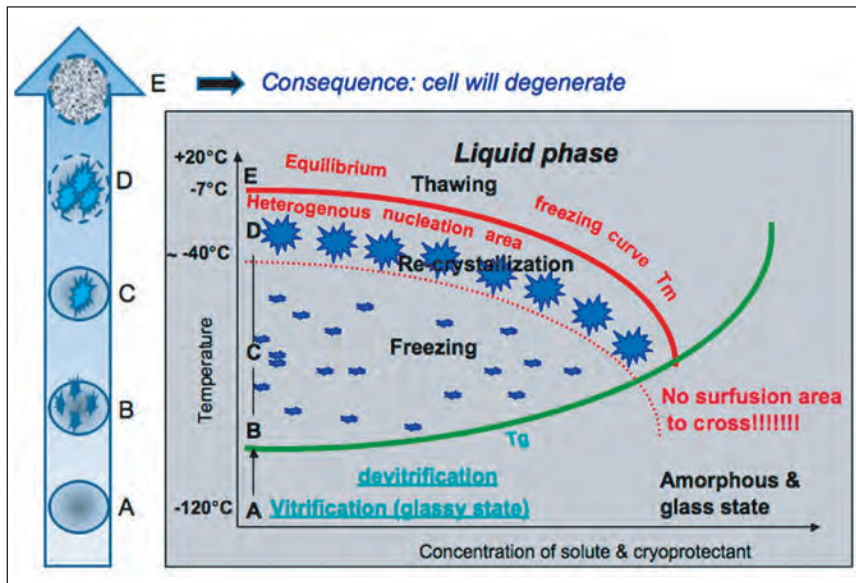


Figure 4. Low warming rates and intracellular formation of ice crystals. Warming process from (A) a vitrified state; (B) devitrification; (C) freezing; (D) re-crystallization; (E) thawing.

an additional culture period of 4 to 24 h before ET.

Clinical Applications

Vitrification of MII oocytes

Success Rates

Twenty five years since the first report of a birth after oocyte cryopreservation [45] this technique is still listed as an experimental technique. One reason

therefore might be that it took another 15 years to establish reliable protocols applicable in routine laboratory procedures. Only in the last decade with the introduction of ICSI and advances in the cryopreservation techniques, increasing success rates led to the breakthrough of human oocyte freezing [46–48]. A meta-analysis comparing vitrification of oocytes to slow freezing showed an increase in the SR as well as in the PR with

vitrification [49]. Therefore vitrification has become the most popular method.

Recent papers state that vitrification of oocytes does not decrease embryo development and implantation potential. In a study on oocyte donation cycles comprising more than 200 fresh and 200 vitrified oocytes showed equivalent fertilization rates and blastocyst development in fresh and vitrified oocytes (Tab. 1) [50]. One year later, a large randomized controlled study on 3000 fresh and frozen oocytes originating from an oocyte donation program also reported that vitrification does not impair fertilization rate and embryo development [51]. Further studies confirmed these finding in oocyte donation programs [52–54]. The SR in these studies were consistently around 90%, ranging from 88–97%, an implantation rate (IR) between 24.7% and 55.3% was reported after vitrification of oocytes in donation programs.

Comparable findings were reported in oocytes originating from standard IVF cycles. A study demonstrated outcomes from 251 fresh and 120 vitrified oocytes with a SR after warming of 99.4% showed equivalent fertilization rates, embryo development to d3 and IR in both groups [55]. Further reports con-

Table 1. Survival rate and embryo development after oocyte vitrification

	Vitrification Device	Survival Rate (SR)	Fertilization Rate (FR)	Implantation Rate (IR)	IR of Fresh Oocytes
Oocyte donation cycles					
Cobo et al. [50]	Cryotop "open"	224/231 96.9%	171/224 76.3%	20/49 40.8%	NA
Nagy et al. [52]	Cryotop "open"	134/153 87.6%	117/134 87.3%	26/47 55.3%	9/19 47.4%
Cobo et al. [41]	Cryotop "open"	3039/3286 92.5%	2256/3039 74.2%	205/513 39.9%	204/498 40.9%
Trokoudes et al. [54]	Cryotop "open"	192/210 91.4%	162/192 84.4%	20/81 24.7%	22/86 25.9%
Garcia et al. [53]	Cryolock "open"	253/283 89.4%	191/251 76.1%	NA 43.9%	NA 42.9%
Standard IVF cycles					
Antinori et al. [55]	Cryotop "open"	328/330 99.4%	305/328 92.9%	39/295 13.2%	71/685 10.3%
Cao et al. [57]	Cryoleaf "open"	268/292 91.8%	182/268 67.9%	NA –	NA –
Smith et al. [56]	Cryotip "closed"	260/349 74.5%	200/260 76.9%	NA –	NA –
Rienzi et al. [58]	Cryotop "open"	120/124 96.8%	95/120 79.2%	19/93 20.4%	69/318 21.7%
Almodin et al. [59]	Vitri-Ingá "open"	214/252 84.9%	173/214 80.8%	24/161 14.9%	61/286 21.3%
Rienzi et al. [60]	Cryotop "open"	2304/2721 84.7%	1642/2182 75.2%	NA –	NA –

Table 2. Pregnancies, obstetric and perinatal outcome after oocyte vitrification

	oPR vitrified oocytes	oPR fresh oocytes	Delivery rate per ET	Infants born	Miscarriage rate/ET
Oocyte donation cycles					
Cobo et al. [50]	11/23 47.8 %	1/1 100 %	NA NA	NA NA	3/15 20%
Nagy et al. [52]	15/20 75.0%	5/9 55.6	15/20 75.0%	26 –	0 –
Cobo et al. [41]	148/295 50.2%	144/289 49.8%	NA –	NA –	NA –
Trokoudes et al. [54]	17/36 47.2 %	18/41 43.9 %	17/41 (41.5%) –	NA –	0/17 0%
Garcia et al. [53]	21/34 61.8%	51/85 60.0%	NA –	NA –	NA 9.5%
Standard IVF cycles					
Antinori et al. [55]	39/120 32.5 %	71/248 28.6%	58/251 23.1%	NA –	8/39 20.5%
Rienzi et al. [58]	15/39 38.5 %	48/124 38.7 %	NA –	NA –	3/15 20%
Almodin et al. [59]	21/46 45.6%	41/79 51.9%	NA –	NA –	NA –
Smith et al. [56]	18/48 37.5%	NA –	18/48 37.5%	20 –	4/48 8.3%
Rienzi et al. [60]	NA –	NA –	128/436 29.4 %	147 –	NA –

firmed these findings stating that embryo quality and IR do not differ significantly between fresh and vitrified oocytes [56–59]. A recent multicenter study including three infertility clinics in Italy and Spain intended to determine the efficiency, reproducibility and consistency of outcome after oocyte cryopreservation [60]. The results from 486 warming cycles were presented. High SR, FR and BR are found comparable to fresh IVF cycles. Authors conclude that vitrification is a safe and efficient method for MII oocytes, consistent in different centers and countries.

In Table 2 the PR and BR of children born after vitrification are listed. In oocyte donation cycles an oPR of 47.2–75% was reported, showing to differences as compared to fresh cycles [51–54]. The miscarriage rate/ET ranged from 0–20% but was not listed in each publication. Similar data are published for egg freezing in standard IVF cycles with an oPR ranging from 32.5–45.6%. A BR in these patients was found between 23.1% and 37.5% [51–54].

Our experience: Vitrification of MII Oocyte using Vitrisafe as Closed System

One of the critical points after OPU is the aging of the oocytes due to inability to obtain sperm at the right time (< 3 hrs post OPU) in patients with erectile dys-

functions or in cases of azoospermia, where testicular biopsy has to be applied. Oocytes age when intracytoplasmic sperm injection (ICSI) or intracytoplasmic morphologically selected sperm injection (IMSI) has to be postponed, impacting on their capacity to fertilize and impairing embryo development. One strategy to avoid oocyte aging is to vitrify and thaw them in a cryo-cycle when sperm collection is possible.

We summarized our experience of oocyte vitrification cycles due to lack of sperm at the day of OPU. A total of 455 oocytes were vitrified from which 416 were viable and fertilized after warming. A fertilization rate of 71.4% and a cleavage rate of 95.3% on day 3 were observed. After transfer of blastocysts an oPR of 43.8% and a BR of 37.5% were reported, 28 babies were born (unpublished data).

Our results confirm that high BR can be obtained after aseptic vitrification of oocytes. This suggests that in all centers with good and standardized protocols oocyte cryopreservation using vitrification in hermetically closed devices should be applied in cases of unexpected failure of sperm production. The encouraging data suggest applying this strategy not only in cases of no sperm production but to postpone fresh embryo transfer when the sperm of IMSI candidate pa-

tients show 100% of sperm with large nuclear defects.

Is there an Adverse Effect of Vitrification on MII Oocytes?

Cryopreservation induces well known modifications in MII oocytes, such as thickening and hardening of the zona pellucida and premature cortical granule reaction [61, 62]. The negative impact of these molecular changes has been overcome by the implementation of ICSI. Concerns remain about other structural changes occurring due to the procedure. Oocyte cryopreservation could impact on subcellular structures building the spindle apparatus. The meiotic spindle is responsible for normal segregation of the sister chromosomes during meiosis II, dysfunction could result in increased incidence of aneuploidy. A recent study investigated this subject in 44 patients and 294 fresh and 294 vitrified MII oocytes. They report that although the IVF process was less efficient after oocyte vitrification in terms of fertilization rate and blastocyst development, implantation rates were equivalent and there was no increased risk of aneuploidy [63]. In a comparative study it has been proposed that vitrification as compared to the slow freezing technique might be less shocking to the meiotic spindle than slow freezing thereby having less consequences for cell functioning [64].

Obstetric and Perinatal Outcomes in Pregnancies Conceived Following Oocyte Cryopreservation

As oocyte cryopreservation is a relatively young technique in ART, the reports of pregnancy outcomes are still scarce. All reports so far are uncontrolled implying that comparison with pregnancies conceived spontaneously or in fresh ART cycles is difficult. Over 500 pregnancies have been reported in the literature so far, but the majority of the publications do not report the status of the infants in detail. A single large study presented data on congenital abnormalities following oocyte cryopreservation and reported evidence that children born following oocyte vitrification demonstrated the same obstetric and perinatal outcome as naturally conceived offspring [65]. Thereby children resulting from 165 pregnancies, 137 singletons and 28 twins were examined. A total of five congenital abnormalities were reported, including two children with ventriculoseptal defect, one club foot, on skin haemangioma and one congenital biliary atresia. This gives an incidence for congenital abnormalities of 2.5%. This is in the same order of magnitude as compared with spontaneous conceived pregnancies and pregnancies following ART [66–69].

No difference in the rate of congenital anomalies in infants born after oocyte cryopreservation was reported in a large review comprising 900 live birth [70]. In a literature search a database of 936 live born infants following oocyte cryopreservation (slow freezing as well as vitrification) between 1986 and 2008 was established. Thereof 12 children (1.3%) were noted to have birth anomalies. The reported defects were three children with ventricular septal defects, a choanal and a biliary atresia, one child with Rubinstein-Taybi syndrome, one Arnold-Chiari syndrome, one cleft palate, three infants with clubfoot and one skin haemangioma. Also this study confirms no increased risk for congenital anomalies in children born after oocyte cryopreservation as compared to naturally conceived infants.

However, more data on obstetric outcomes should be available although the results so far seem to be reassuring. No data are available at the moment on neonatal and child development. Although

we do not find any evidence to be worried, currently no data on long-term children follow-up are available.

Vitrification of Pronuclear Embryos

Vitrification has not reached the same level of popularity when it comes to the cryopreservation of human pronuclear embryos. Only a handful of cases and findings have been published [71–76] and the number of manuscripts describing the clinical application of 2PN vitrification remains extremely limited [36, 77], even though cryopreservation is justified for a wide range of different reasons as previously stated.

Our experience: Vitrification of Pronuclear Stage Embryos

In order to maximize the chances of pregnancies and to overcome impaired uterine environment one credible strategy that is more often evoked is to replace fresh embryo transfers by transfers of vitrified embryos in an optimized well prepared luteal phase.

An analysis was conducted on the BR after vitrification in aseptically closed conditions of zygotes and transfer at the blastocyst stage after 5 days of culture. Our data show that high live birth rates are obtained after ET of blastocysts that originated from vitrified zygotes. Between January 2009 and December 2010, 91 ETs of blastocysts from aseptically vitrified 2PN were performed in our institute. Only in 4 warming cycles was no ET performed due to a lack of blastocyst development (4.2%). In the 91 cycles, 165 blastocysts were transferred (mean 1.8 blastocysts/ET). An oPR per ET of 40.7% was obtained, as a result of which 32 patients (36.8%) delivered. No malformations in any of the 38 babies born were reported. An IR of 23% per embryo transferred was noticed.

Combined, good clinical results and increased safety conditions contribute to a change in transfer strategy and encourage us to increase ET rate in cryo-cycles for optimal uterine environment.

■ Conclusions

In spite of reduced cooling rates due to aseptic vitrification conditions, acceptable results are obtainable if the intracellular concentrations of CPs are well

adapted to the needs of the cells. Step-wise addition of the CPs and vitrification in an aseptic embryo carrier device enables to vitrify with satisfactory survival and pregnancy rates MII oocytes and fertilized oocytes.

Nevertheless, to date, there is no agreement on a universal vitrification procedure. The progress towards a universal vitrification- warming protocol is not yet realistic seeing all the different cryobiological and technical factors that are implicated in the process of vitrification-warming.

■ Conflict of Interest

No potential conflict of interest to this article was reported.

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