Chapter 6

The Maining of Cryopreservation for in-vitro Fertilization Patients

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Additional information is available at the end of the chapter

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1. Introduction

The necessity to cryopreserve certain tissues and cell types for their use in assisted reproduction has allowed technique developments that improve the quality of treatments and help both professionals and patients to perform these techniques. In this chapter we will talk about the importance that cryopreservation has meant to assisted reproduction techniques and about the benefit for patients of the advance and improvement of different cryopreservation techniques, for example in the recent and increasingly demanded technique Egg Vitrification, to preserve female fertility.

From the cryopreservation point of view sperm, oocytes, embryos and ovarian tissue will be analyzed, by reviewing how different cryopreservation techniques have evolved up to reaching the techniques used nowadays giving, furthermore, a vision of how they will be in the future to optimize even further, the procedure increasing survival rates and viability of gametes, embryos and ovarian tissue to 100%.

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

To understand the problem of contamination in cryopreservation we need to have an overview of the current problem in which all researchers are concerned about, seeking a cryopreservation protocol with good results but without contamination problems. Discussing the cryopre-

servation's different techniques such as slow freezing, vitrification, kinetic vitrification (extra-, hyper-, super-, ultra-fast vitrification) and the various components that help us understand the difficult balance between technique, device used and the risk of contamination.

The device used, the protocol used and the cooling solution used can change the outcome of cryopreservation and therefore we have to find a protocol for cryopreservation with a cooling solution and a secure device to provide us good results free of contamination.

2. Slow freezing and Vitrification

Two are the most utilized methods for gamete cryopreservation: slow freezing and

vitrification. Slow freezing uses low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method that decreases cold shock, without the risks of solution effects or crystallization, and uses high cooling rates in combination with a high concentration of cryoprotectant [1].

Slow freezing is a conventional cryopreservation process in which a relatively low concentratiton of cryoprotectant is used (1.5 M), it shows little toxicity to cells or tissue and requires expensive equipment. As the cryoprotectant is added to cells, it results in initial cellular dehydration followed by a return to isotonic volume with the permeation of cryoprotctant and water. Generally, cells are cooled slowly using a controlled rate freezing machine, which allows samples to be cooled at various rates; ovarian tissue is generally cryopreserved at 2° C/min prior to ice seeding and 0.3° C/min after crystallization to ensure the tissue is dehydrated before intracellular ice formation occurs. Optimal rates to minimize intracellular rates formation vary among cells and tissue types [2].

It is generally believed that cell injury at low cooling rates is principally due to the concentration of both intracellular and extracellular electrolytes and that cryoprotectants act by reducing this build-up. Experimental data support this explanation, in fact the extent of damage to human red blood cells during freezing in solutions of sodium chloride/glycerol/water can be quantitatively accounted for by the increase in solute concentration. Furthermore, a given degree of damage occurs at lower concentrations of solute in the presence of higher concentrations of glycerol; it appears that glycerol contributes as element of damage itself [3].

Although initially reported in 1985 as a successful cryopreservation approach for mouse embryos, vitrification has taken a backseat in human assisted reproduction. However, the practical advantages of this cryopreservation method have more recently caught the attention of many ART laboratories as a feasible alternative to traditional slow freezing methods. Since 1985 more than 2,100 publications can be found referring to the topic of "vitrification", which is further evidence of the burgeoning growth of interest in this cryopreservation technology. One "drawback" considered by embryologists who are not familiar with the vitrification technique, is the use of higher concentration of cryoprotectants, which does potentially mean that the vitrification solutions are more toxic than their counterpart solutions used for conventional slow freezing. However, with better understanding of the physical and biological

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principles of vitrification this has lead to numerous successful clinical applications of this technique within the field of assisted reproduction. As of today, all developmental stages of human embryos cultured in vitro have been successfully vitrified and warmed, with resulting offspring. Today, slow freezing technology still has the longest clinical track record, and greater 'comfort level' amongst embryologists. Nevertheless, vitrification with its increasing clinical application is showing a trend of greater consistency and better outcomes when compared to slow freezing technology. Therefore, when (not if) IVF programs overcome the fear of the 'unknown', and take on the challenge of the short learning curve with vitrification, then at that point vitrification will become the clinical standard for human embryo cryopreservation.

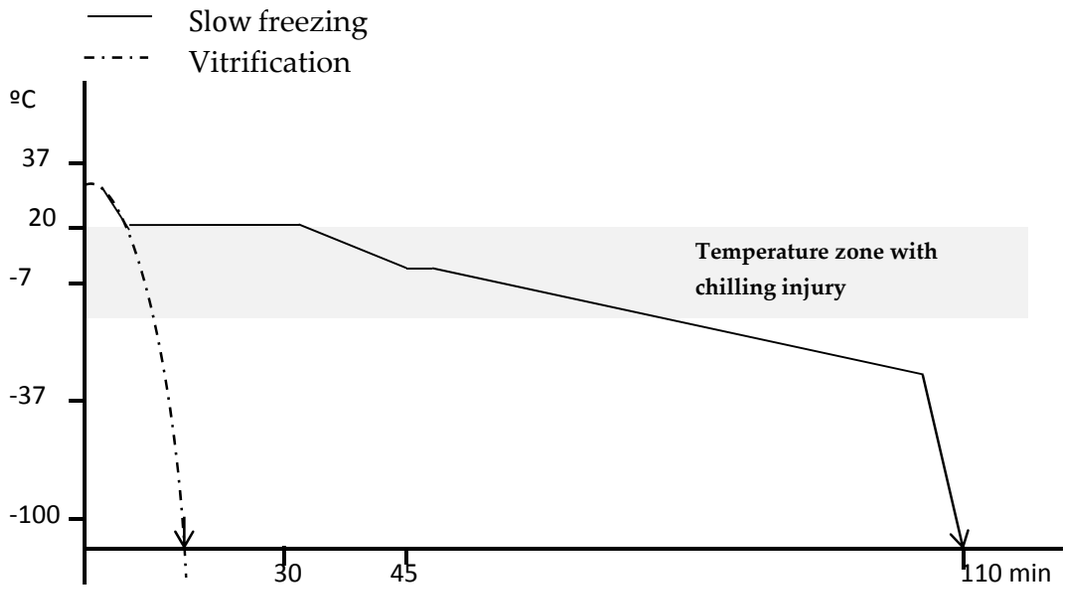

Figure 1. Cooling rates during slow freezing and vitrification. Note the relative duration of exposure of oocytes / embryos temperatures in the risk of chilling injury of each of the procedures [4].

Cryopreservation at low temperature slows or totally prevents unwanted physical and chemical change. The major disadvantage to using low temperature cryostorage is that it can lead to the crystallization of water, and thereby this approach can create new and unwanted physical and chemical events that may injure the cells that are being preserved. Although the results achieved by slow freezing in many cases seem quite successful [5, 6], ice crystal formation still renders traditional slow-freezing programs generally less consistent in their clinical outcomes. Another downside to the slow freezing approach is the time to complete such freezing procedures for human embryos, which can range from 1.5 to 5hrs. This is due to the fact that the slow rate of cooling attempts to maintain a very delicate balance between multiple factors that may result in cellular damage by ice crystallization and osmotic toxicity. Traditionally slow-freeze embryo cryopreservation has been a positive contributor to cumulative patient pregnancy rates, but ultimately the limitations of current slow-rate freezing methods in ART have become more evident in the shootout with vitrification-based cryostorage.

Vitrification is one of the more exciting developments in ART in recent years that attempts to avoid ice formation altogether during the cooling process by establishing a glassy or vitreous state rather than an ice crystalline state, wherein molecular translational motions are arrested without structural reorganisation of the liquid in which the reproductive cells are suspended. To achieve this glass-like solidification of living cells for cryostorage, high cooling rates in combination with high concentrations of cryoprotectants are used. A primary strategy for vitrifying cells and tissue is to increase the speed of thermal conductivity, while decreasing the concentration of the vitrificants to reduce their potential toxicity. There are two main ways to achieve the vitrification of water inside cells efficiently: a) to increase the cooling rate by using special carriers that allow very small volume sizes containing the cells to be very rapidly cooled; and b) to find materials with rapid heat transfer. However, one has to take into account that every cell seems to require its own optimal cooling rate, e.g., mature unfertilized oocytes are much more sensitive to chilling injury than any of the cell stages of the pre-implantation embryo. The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 [7]. In 1993 successful vitrification of mouse embryos was demonstrated [8]. Furthermore, bovine oocytes and cleavage-stages were vitrified and warmed successfully a few years later [9]. In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported [10, 11]. Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in ART, oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice crystallization using vitrification [12]. In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high cooling rates to very low temperature. Interest in vitrification has clearly risen as evinced by the almost exponential growth of scientific publications about vitrification. Vitrification is very simple, requires no expensive programmable freezing equipment, and relies especially on the placement of the embryo in a very small volume of vitrification medium (refered also as "minimal volume approach") that must be cooled at extreme rates not obtainable in traditional enclosed cryostorage devices such as straws and vials. The importance of the use of a small volume, also referred to "minimal volume approach" was described and published in 2005 [13, 14]. In general, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are in inversely related. In addition, recent publications have shown the dominance of warming rate over cooling rates in the survival of oocytes subjected to a vitrification procedure [15, 16].

During vitrification, by using a cooling rate in the range of 2,500 to 30,000°C/min or greater, water is transformed directly from the liquid phase to a glassy vitrified state. The physical definition of vitrification is the solidification of a solution at low temperature, not by icecrystallization but by extreme elevation in viscosity during cooling [17, 18]. Vitrification of the aqueous solution inside cells can be achieved by increasing the speed of temperature change, and by increasing the concentration of the cryoprotectant used. However, a major potential drawback of vitrification is the use of high concentration of cryoprotectant, and an unintentional negative impact of these cryoprotectants in turn can be their toxicity, which may affect the embryo and subsequent development in utero. It is therefore essential to achieve a fine balance between the speed of cooling and the concentration of the vitrifying cryoprotectants.

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This is necessitated by the practical limit for the rate of cooling, and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to note that recently published papers [19, 20, 21, 22] have shown that the use of relatively high concentration of cryoprotectants such as 15% (vol/vol) ethylene glycol (EG) used in an equimolar mixture with dimethyl sulphoxide (DMSO) had no negative effect on the perinatal outcomes from blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers.

Vitrification in principle is a simple technology, that is potentially faster to apply, and relatively inexpensive; furthermore, it is becoming clinically established, and is seemingly more reliable and consistent than conventional cryopreservation when carried out appropriately [23, 24].

Cryoprotectant agents are essential for the cryopreservation of cells. Basically two groups of cryoprotectants exist: 1) permeating (glycerol, ethylene glycol, dimethyl sulphoxide); and 2) nonpermeating (saccharides, protein, polymers) agents. The essential component of a vitrification solution is the permeating agent. These compounds are hydrophilic non-electrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the "freezing point" of the solution. Regarding the high concentration of cryoprotectant used for vitrification, and in view of the known biological and physiochemical effects of cryoprotectants, it is suggested that the toxicity of these agents is a key limiting factor in cryobiology. Not only does this toxicity prevent the use of fully protective levels of these additives, but it may also be manifested in the form of cryo-injury above and beyond that seen occurring due to classical causes of cell damage (osmotic toxicity and ice formation) during cryopreservation. In spite of this, the permeating CPA should be chosen firstly by their permeating property, and secondly on the basis of their potential toxicity. Because the permeating CPA is responsible for the toxicity (the key limiting factor in cryobiology), different cryoprotectants have been tested for their relative toxicity, and the results indicate that ethylene glycol (EG; MW 62.02) is the least toxic followed by glycerol. Additionally, these highly permeating cryoprotectants are also more likely to diffuse out of the cells rapidly and the cells regained their original volume more quickly upon warming, thus preventing osmotic injury. Therefore, the most common and accepted cryoprotectant for vitrification procedures is ethylene glycol (EG). Today EG is more commonly used in an equimolar mixture with DMSO. Often additives are added to the vitrification solution such as disaccharides. Disaccharides, for example sucrose, do not penetrate the cell membrane, but they help to draw out more water from cells by osmosis, and therefore lessen the exposure time of the cells to the toxic effects of the cryoprotectants. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. In addition, permeating agents are able to compound with intracellular water and therefore water is very slowly removed from the cell. Hence the critical intracellular salt concentration is reached at a lower temperature. Removal of the cryoprotectant agent during warming can present a very real problem in terms of trying to reduce toxicity to the cells. Firstly, because of the toxicity of the vitrification solutions, quick dilution of them after warming is necessary; and secondly, during dilution water permeates more rapidly in to the cell than the cryoprotective additive diffuses out. As a consequence of the excess water inflow the cells are threatened by injury

from osmotic swelling. In this situation the non-permeating sucrose acts as an osmotic buffer to reduce the osmotic shock. During warming using a high extracellular concentration of sucrose (e.g., 1.0M) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra-and extracellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling [25, 26, 27].

3. Sperm

Human spermatozoa can be successfully cryopreserved and utilized. Cryopreservation now plays an essential role in fertility preservation under the following scenarios:

- couples undergoing infertility treatment.
- cancer patients undergoing gonadotoxic chemotherapy or radiation.
- patients undergoing certain types of pelvic or testicular surgeries
- patients suffering from degenerative illnesses such as diabetes or multiple sclerosis; spinal cord disease or injury.
- men undergoing surgical sterilization such as vasectomy
- screening and quarantine of donor semen samples

Many advances in reproductive medicine in the past five decades have made cryopreservation of human spermatozoa an invaluable tool for the clinical management of infertility and sperm banking. The advent of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with microsurgical sperm handling techniques along with advances in female gamete acquisition have resulted in an increased demand for the cryopreservation of semen and tissue samples, often containing a very limited number of spermatozoa. Sperm cryopreservation also makes it possible for cancer patients to preserve their fertility prior to gonadotoxic chemotherapy or radiation. Applications of sperm banking are not limited to cancer patients but extend to patients undergoing certain types of pelvic or testicular surgeries; those who suffer from degenerative illnesses such as diabetes or multiple sclerosis; spinal cord disease or injury; and persons in occupations where a significant risk of gonadotoxicity prevails. Sperm cryopreservation is also available to men undergoing surgical sterilization such as vasectomy, in the event that children may be desired in the future. Another use for semen cryopreservation is to allow donor semen samples to be quarantined while appropriate screening is performed to prevent the transmission of infectious pathogens during therapeutic donor insemination (TDI) [28].

In cases of severe male infertility, single or lesbian women, the use of donor sperm is the only approach to address fertility issues [29, 30]. Advances in sperm cryopreservation have created opportunities for many families to achieve pregnancies through therapeutic donor insemination or IVF with donor sperm.

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At present, some 30,000 births per year worldwide are attributable to frozen donor sperm inseminations [31].

Although major improvements have been made in sperm cryopreservation, there are many unresolved technical issues. Since freezing protocols differ between types of cells, the ideal conditions for human sperm freezing and thawing need to be perfected. To add more complexity, samples with abnormal semen parameters, such as severe oligospermia or high seminal fluid viscosity, often require unique cryopreservation conditions. For example, the particular cryoprotectants can affect cooling rates. In addition, storage temperature can significantly influence cryopreservation outcome. Liquid nitrogen (LN2) can offer long-term survival of spermatozoa due to essentially absent metabolic activity, such as chemical reactions, genetic modification or aging of cells [32]. A conventional slow freezing protocol has been in use for many years and very little has changed in terms of methodology and reagents. While freezing aims to preserve cells it can also easily destroy them if certain precautionary steps are not taken into consideration. During cryopreservation cells and tissue undergo dramatic transformation in chemical and physical characteristics as the temperature drops from+37 to-196°C. The cells can lose up to 95% of their intracellular water. The concentration of solutes increases considerably, triggering the possibility of osmotic shock. Moreover, potential intracellular ice crystallization and mechanical deformation by extracellular ice may cause significant injury leading to cell death. Furthermore, if cells survive freezing, they might sustain additional damage during the thawing process due to osmotic shock, uncontrollable swelling and ice re-crystallization [33].

Remarkably, the first reference of empirical sperm freezing dates as far back as the late 16th century, but it was only with the discovery in 1937 by Bernstein and Petropavlovski that glycerol can aid spermatozoa in surviving long term freezing, that sperm cryopreservation became practical. Expansion of artificial insemination for the dairy industry led to further important research in the field of cryobiology [34]. Shortly after these practices were initiated with animals, the first pregnancies were reported in humans after insemination with frozen spermatozoa.

The next milestone was the discovery of the possibility to store human spermatozoa in LN2at-196°C, resulting in superior recovery rates compared to storage at higher temperatures between-20 and-75°C. After the era of empirical freezing; cryobiology matured to its fundamental stage, focusing on the biophysical and biochemical principals of cryopreservation, further advancing the field [35]. A comprehensive review of the historical background of sperm freezing was recently published and is recommended for readers looking for more details [36].

3.1. Cryopreservation of epididymal and testicular spermatozoa

Couples with male factor infertility represent 30 to 40% of the infertile population. Azoospermia accounts for 10% of cases of confirmed male infertility, and often requires surgical retrieval of spermatozoa. Since the introduction of ICSI, many cases of severe male infertility can now be successfully treated. Cryopreservation of surgically retrieved spermatozoa is a valuable component in the effective management of male infertility, reducing the necessity of repeat surgeries. Diagnostic sperm retrieval prior to IVF has several benefits including the possibility

of freezing spermatozoa for future use, or if none are retrieved, initiation of the IVF stimulation cycle can be postponed or avoided. Testicular spermatozoa have been utilized to achieve pregnancy in couples with severe male factor infertility, with reported pregnancy rates similar to ejaculated spermatozoa, according to a meta-analysis study [37]. In the case of obstructive azoospermia, recovery of spermatozoa by aspirations varies from 45 to 97% [38, 39]. In cases of non obstructive azoospermia recovery depends on the degree of testicular pathology and varies from 0 to 64% [40, 41]. A second or third surgery can increase the chance of complications including hematomas, inflammation, testicular devascularization, fibrosis and permanent testicular damage [42]. To avoid this, if pregnancy is not achieved during the first ICSI attempt, a repeat of the surgical procedure would not be required if a portion of the surgical specimen has been banked. Cryopreservation of surgically retrieved spermatozoa can also aid the coordination of oocyte retrieval and avoids the pressure of having the urologist available on the day of the ICSI procedure. Usually the number of spermatozoa obtained during a surgical procedure is limited, and in the case of testicular sperm they may not be fully matured. In the future, if no mature spermatozoa are recovered, spermatogonial stem cells or early germs cells could potentially be matured in vitro and used for fertility treatments [43].

There are significant technical challenges for successful cryopreservation of testicular tissue due to its complex structure and intracellular interactions. Different cells of testicular tissue will have dissimilar responses to cryopreservation and require different concentration of CPAs. Freezing larger pieces of tissue is not advisable as it would increase resistance of heat transfer and penetration of CPAs leading to variation in cooling rates within different parts of the tissue. In addition, seminiferous tubules capture liquid and increase chances of ice formation [33]. To avoid these difficulties, cryopreservation of smaller tissue fragments or mincing tissue prior to freezing has been advocated [44].

3.2. Cryopreservation in oncological patients

Quite often in clinical practice, the long term effects of cancer therapy on a patients' ability to have children in the future is not adequately addressed [45]. While the priority is to eliminate the cancer and save their life, fertility preservation especially among adolescent or young adults to ensure the potential of procreation with their own gametes after treatment, needs to be considered. Impaired spermatogenesis has been demonstrated before treatment in some patients with malignancies, depending on their location (eg. testicular cancer) or type (eg.Hodgkin's lymphoma) [46]. Current treatment options such as surgery, chemotherapy and/or radiation can impair spermatogenesis and sexual function and lead to temporary or permanent infertility [47].

The scale of negative effects of cancer treatment on spermatogenesis depend on the specific gonadotoxicity of administered chemotherapeutic agents, number of chemotherapy treatment cycles, radiotherapy field location and dosage, type and stage of the cancer, and age of the patient. Considering combination cancer therapy, uncertainty in individual esponse to treatment and the large number of confounding variables, it becomes very challenging to assess the risk of iatrogenic infertility in many patients. The ability of cancer survivors to have their own biological offspring is very important for many oncology patients, especially at

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younger ages [48]. Advances in early diagnostic investigation and treatments have led to increasing numbers of young cancer survivors.

Cryopreservation of semen has changed the reproductive prospects for young patients diagnosed with cancer. Unfortunately, banking services continue to be underutilized since cancer patients and their families are not always informed about the potential fertility risks associated with cancer treatments, or the availability of banking. According to some surveys, less than 20% of patients undergoing chemotherapy or radiation treatment are informed about the adverse effects of such treatment on spermatogenesis or are offered sperm banking for fertility preservation. Cancer patients are usually under huge physiological and time pressure to make cryopreservation decisions while dealing with a life threatening situation. To complicate matters, some young patients are unable to produce semen samples by masturbation. In such cases, PVS or electro-ejaculation under general anaesthetic might be required. Surgical retrieval of testicular tissue may be an option for prepubertal boys who are not capable of producing mature sperm. Testicular tissue cryopreservation has been reported in boys with cryptorchidism to preserve fertility [49]. Cryopreserved testicular tissues can be autografted to restore reproductive functions; however recurrence of neoplastic process is a concern in oncology patients and such procedures are still considered to be experimental [43]. A multidisciplinary team approach is important to ensure that patients have the opportunity to preserve their fertility potential if they elect to do so.

The posthumous use of semen is an entirely separate and complex ethico-legal subject. The ethical and legal aspects of posthumous assisted reproduction have been recently addressed by the European Society of Human Reproduction and Embryology Task Force on Ethics and Law [50].

4. Oocytes

The cryopreservation of human oocytes constitutes an important step forward in Assisted Reproductive Technology (ART) despite the fact that for more than 2 decades oocyte cryopreservation has long been the focus of unsuccessful efforts to perfect its clinical application. More recently, vitrification as an alternative to traditional slow freezing protocols has been shown to provide high degrees of success in vitrified metaphase-II human oocytes. Although oocyte cryopreservation historically has low efficiency mainly because of low rates of survival, fertilization, and cleavage, data on ~ 2000 "frozen oocyte" babies born worldwide since 1986 exists. The question arises as to what makes oocytes so unique compared to embryos, besides differences in cell size and membrane permeability? Oocytes have a low volume-to surface ratio; hence they are less efficient at taking up cryoprotectant and at loosing water. Other differences to be considered are a) that the maternal DNA is held suspended in the cytoplasm on the meiotic spindle & not within the protective confines of the nuclear membrane, therefore damage in the DNA and microtubules could explain the limited success of oocytes, b) the oocyte is arrested in a state primed for activation, and c) the changes in its environment can cause parthenogenetic activation. What are the applications then for oocyte cryopreservation

in the US? One application would be to preserve fertility in women with malignant/premalignant conditions who would have to undergo treatment that might negatively impact their future ability to have children (50,000 per year <40 yr old), also in women who may want to delay childbearing ('clock-tickers') because of their careers, partnership status or psychological/ emotional reasons. A very interesting approach is donor oocyte banking, which makes the donor-recipient cycle more convenient by facilitating the "egg donation" and allows quarantining of the oocytes, which provides a unique advantage in economy as well as feasibility. Other applications are if a male is unable to produce a semen sample on the day of egg retrieval and or it could also eliminate ethical/moral questions of producing extra embryos. Overall, oocyte cryostorage offers an opportunity to reduce number of embryos generated per IVF cycle, and therefore lessening the pressure on the patient to increase the number of fresh embryos transferred. In addition, while also reducing embryo cryostorage it has the benefit of helping women "retain ownership" of their ability to be genetic parents at a time of their choosing, a time of greater convenience & health. The live born babies from cryopreserved oocytes have shown no apparent increase in congenital anomalies. Although 13 years later after the first slow-freeze birth, the number of reported babies born as a result of vitrified oocytes is now approaching that of slow-frozen oocytes without any increasing risk in congenital abnormalities [51]. Vitrification of oocytes does not appear to increase risks of abnormal imprinting or disturbances in spindle formation or chromosome segregation [52]. It has the greatest potential for successful oocyte cryopreservation and with its increased clinical application is showing a trend to greater consistency and better outcomes (similar to outcomes between fresh or warmed oocytes). Vitrification of oocytes, when applied to properly screened patients, will be a useful technology in reproductive medicine practice and will constitute a major step forward in ART.

Fortunately to date, no significant increase in abnormalities has been reported from these cryostored oocyte pregnancies [53], regardless of the historical concerns that cryopreservation of mature oocytes might disrupt the meiotic spindle and thus increase the potential for aneuploidy in the embryos arising from such eggs. These concerns have mostly been allayed by publications that show no abnormal or stray chromosomes from previously frozen oocytes [54], and FISH comparison of embryos from fresh and thawed oocytes show no increase in anomalies [55]. There also appears to be adequate recovery of the meiotic spindle post-cryopreservation whether using conventional or vitrification technology [56, 57, 58]. The scientific literature on oocyte cryopreservation grows daily it seems. Most reports focus on clinical pregnancy rates [59, 60], and as such while this data is helpful to increase our confidence in the technology, it does little to research new directions for oocyte cryopreservation.

5. Embryos

In 1983, Trounson and Mohr [61] announced the first pregnancy from a previously frozen human embryo obtained from in vitro fertilization (IVF). The first live birth after embryo cryopreservation was reported in 1984 in Australia, and the first in the United States followed in 1986. Since that time, cryostorage and subsequent use of human embryos has

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become standard practice in assisted reproductive technology (ART) and is now involved in a significant proportion of all infertility treatments. In fact, the 2002 National Summary of Fertility Centers Report (NSFCR) determined that 97% of the 391 American centers reporting to the Society for Assisted Reproductive Technology (SART) offer cryopreservation [62]. Hoffman et al. [63] surveyed all SART-reporting clinics in the United States with regard to their cryopreservation practices and also found that virtually all of the 340 responding clinics freeze embryos and store them on site, accounting for over 400,000 frozen embryos as of 2002 [64].

Advances in assisted reproductive technologies have expanded procreative options for many people experiencing infertility. With the evolution of in vitro fertilization (IVF), more embryos often result from each cycle of ovarian stimulation than can safely be returned to a woman's uterus for implantation [65]. To reduce multiple gestations and their morbidity, avoid embryo destruction, improve cost effectiveness, and preserve future options for infertile couples, embryo cryopreservation (freezing) has developed as a routine practice in most IVF clinics [66]. Once embryos are frozen, they may be used for future pregnancy attempts, donated to another couple, designated for stem cell or other research, or discarded.

Cryopreservation allows the transfer of a limited number of embryos back to the uterus and the storage of the remaining embryos for future use, thus maximizing the cumulative effectiveness of an in vitro fertilization (IVF) cycle [67]. In addition, cryopreservation makes feasible the postponement of embryo transfer (ET) in a future cycle, thus decreasing the incidence of ovarian hyperstimulation syndrome in high-risk patients, while it maintains the probability of pregnancy [68].

Cryopreservation of embryos also has an enormous potential in Preimplantacion Diagnosis programmes (PGD). Therefore, not only does it allow us to conserve those normal embryos not transferred, but can also benefit hyporesponsive patients thanks to the accumulation of embryos in cycles [69]. An extension of embryo freezing is the embryo donation program, by which, following the course of Article 11 b of the current legislation (Law 14/2006, May 26), many couples that are not subsidiaries of Assisted Reproduction Techniques, may resort to these thanks to the anonymous donation of surplus embryos from IVF [69]. The embryonic stage in which we will perform cryopreservation is key in obtaining acceptable results, not only in embryonic survival rates, but in implantation and birth rates too. Besides a correct embryo selection of the best quality embryos is crucial for success in the cryopreserved embryo transfer Programme [69]

Conventional cryopreservation of pronuclear zygotes (2PN) is well established in countries such as Germany where freezing of later stage human embryos is by law or by ethical reasons not allowed. The time to complete the conventional protocol to cryopreserved zygotes is 98min. In Germany the clinical pregnancy outcomes arising from the frozen/thawed 2PN cycles is about 18%, with an implantation of around 10% per embryo transferred. The time to complete vitrification of zygotes requires approximately 12min. Recently successful vitrification of 2PN with high survival (~ 90%), cleavage rates on day-2 (>80%), and blastocyst formation of 31% and pregnancies were reported [70, 71, 72, 73]. Zygote vitrification implemented as a clinical setting can provide a clinical pregnancy rate of close to 30%, with an implantation rate of 17%

[73]. The pronuclear stage appears well-able to withstand the vitrification and warming conditions, which is probably due to the significant membrane permeability changes that occur post-fertilization; such changes to the oolemma may also make it more stable and able to cope with the vagaries of the cold-shock and striking osmotic fluctuations that occur during the vitrification process.

Activation of the embryonic genome occurs after the 8-cell stage (3 days post oocyte retrieval) is reached [74]. If the activation does not occur, the embryo will not survive further. Therefore, the improvement of human IVF outcomes requires identification of embryos that will progress beyond the 8-cell stage. Blastocyst culture (5 days post oocyte retrieval) allows for the transfer of embryos that clearly have an activated embryonic genome. This requires that the elimination of embryos in extended culture from day 3 to day 5 should depend solely on their inherited survival potential and not be a consequence of an adverse effect exerted by the sequential media used for culture beyond day 3. Additional advantages in cryopreserving at the blastocyst stage are: 1) At this stage a lower numbers of embryos can be transferred in fresh cycles, resulting in less high order multiple pregnancies, 2) The same is true for cryopreserved blastocysts showing higher pregnancy rates and implantation per thawed embryo transferred, 3) Approximately 120 hours (day five) into development the healthy human embryo should be at the blastocyst stage comprised of some 50 to 150 cells, of which about 20 to 30% make up the inner cell mass (ICM), the remainder making up the trophectoderm (TE), 4) the higher cell number allows better compensation for cryo-injuries, which results in greater viability and faster recovery, 5) the cytoplasmatic volume of the cells is lower, thus the surface-volume ratio is higher, and that in turn makes the penetration of the cryoprotectant faster, and 6) on average fewer embryos per patient were frozen-stored, but each one when thawed has a greater potential for implantation [75].

6. Cryopreservation of ovarian tissue

Cryopreservation of ovarian tissue is of interest to women who want fertility preservation beyond the natural limit, or whose reproductive potential is threatened by cancer therapy, for example in hematologic malignancies or breast cancer. It can be performed on prepubertal girls at risk for premature ovarian failure, and this procedure is as feasible and safe as comparable operative procedures in children.

At birth, the ovaries contain the lifetime complement of primary oocytes which are arrested in the prophase stage of meiosis 1 and are surrounded by a single-layered epithelium to form the primordial follicles. Ovarian cortex presents several advantages when compared with isolated oocytes:

- It contains the important pool of growing follicles.
- It does not necessitate the in vitro maturation/in vitro fertilization /embryo culture steps if it is associated with grafting.
- No previous ovarian stimulation is necessary.

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Consequently cryopreservation of ovarian cortex is an alternative to cryopreservation of isolated oocytes or embryos. It could be used as an emergency preservation and as infertility therapy method for valuable animals. Ovarian cortex cryopreservation has been developed in human in order to preserve fertility in young women submitted to gonadotoxic therapy [76, 77]. In human newborns were obtained after orthotopic autograft of frozen-thawed ovarian cortices [78].

It is obvious that, to achieve successful cryopreservation of ovarian tissue, it is essential to maintain the functional status of the whole mixture of different cell types: oocytes, granulosa cells, epithelial cells, fibroblasts… This represents a major difficulty, because the optimum kinetic of cooling is different for each cell type. Oocytes are large cells, with a low surface to volume ratio, surrounded by zona pellucida. Immediately adjacent to the oocyte are corona radiata cells that have long cytoplasmic extensions which penetrate the zona pellucida, ending in oocyte membrane. These processes and gap junctions are important in the metabolic cooperation between the oocyte and surrounding layers of granulosa cells, which form the cumulus-oocyte complex during the growth phase. Consequently, at the opposite to cryopreservation of isolated cells, a cryopreservation protocol for a tissue represents a compromise between the requirements of the different constitutive cells.

The early work on ovarian tissue cryopreservation was performed in animal studies: rabbit [79] and rat [80, 81]. The earliest positive results were obtained when glycerol (15%) plus serum were used as cryoprotective agents (CPAs) for cryopreservation of rabbit granulosa cells, via a slow cooling protocol [79]. An equilibration period was necessary to achieve CPA penetration into the tissue. For this reason small samples were recommended. A rapid rewarming by plunging the samples into a water bath at 40°C was the most effective procedure [80]. Normal offspring were obtained from mice with orthotopic ovarian grafts of tissue that had been frozen and stored at-79°C [82]. Vitrification of ovarian tissue was also investigated. Nevertheless, Isachenko et al suggested that in human, low freezing protocols were more promising than vitrification protocols [83].

This technique has also been developed in rabbit [84], mouse [85], rat [86], ewe [87, 88], cow [89]. Vanessa Neto and her group [90] have obtained newborn rabbits after autografting of cryopreserved ovarian cortex. Also, their team developed this technique in cat [90] and dog [91].

Several techniques have been applied to ovarian cortex cryopreservation: slow freezing, vitrification. Simultaneously to ovarian tissue cryopreservation, numerous researches have been conducted about ovarian tissue grafting: orthotopic, heterotopic, auto-, allo-and heterografting [92].

The most common cryopreservation method is the slow freezing procedure, consisting of an initial slow, controlled-rate cooling to subzero temperatures followed by rapid cooling as the sample is plunged into liquid nitrogen for storage (−196°C). At such a low temperature, biological activity is effectively stopped, and the cells functional status may be preserved for centuries. However, several physical stresses damage the cells at these low temperatures. Intracellular ice formation is one the largest contributors to cell death; therefore, freezing

protocols use a combination of dehydration, freezing point depression, supercooling, and intracellular vitrification in an attempt to avoid cell damage.

Currently used ovarian cortex cryopreservation protocols have been direct, or slight modifications of the methods developed for isolated oocytes and embryos. There were primarily developed by trial and error adjustments of cooling and warming rates, and choice of CPA and CPA concentrations. However, because there are a large number of protocol variables potentially affecting cell viability, an exhaustive experimental search for the optimal combination of these parameters has long been considered to be prohibitively expensive in terms of time and resources.

6.1. Chemical and physical parameters affecting equilibration and freezing processes of ovarian tissue in mammalian species

The result of a cryopreservation process is influenced by several chemophysical parameters affecting directly or not the functions and the integrity of the ovarian cells along the freezing process, from the equilibration to the thawing. Among these parameters, the method of equilibration, the freezing rate, the composition of the freezing solution and notably the nature of the permeating CPAs and the non-permeating CPAs, the concentration of each CPA, the use of serum, or the rate of thawing may be investigated to know the relative influence of each of them and the induced cell injuries.

In general, we can expect coupled flows of water and CPAs when CPAs are added, during freezing, thawing and when CPAs are removed from the cells, resulting in a series of anisosmotic conditions. During freezing, the cells dehydrate and shrink and remain shrunken during storage, but return to their isosmotic volume upon thawing. Finally, the cells are subjected to potentially lethal swelling upon CPA dilution and removal. During the controlled slow cooling extracellular ice formation is induced (seeding) at a temperature just below the solutions' freezing point, and then the cooling continues at a given rate in the presence of a growing extracellular ice phase, which raises the extracellular solute concentration in the unfrozen fraction and results in water being removed from the cell via exosmosis.

Permeating CPAs, such as glycerol, dimethyl sulfoxide, ethylene glycol or propylene glycol are typically included in the cryoprotective medium, to protect the cells against injury from the high concentrations of electrolytes that develop as water is removed from the solution as ice. During the equilibration step the inner cell water is partly replaced by the permeating CPAs. However, the CPAs can be damaging to the cells, especially when it is used at high concentrations. The toxicity can be reduced by decreasing the time or the temperature of the equilibration step [93]. But equilibration at low temperatures requires increasing the exposition time to freezing solution. Furthermore, the CPAs may have dramatic osmotic effects upon the cells during their addition and their removal.

Consequently, the use of several steps of increasing concentrations of CPAs during the equilibration allows reducing the osmotic gradient. The cells exposed to such permeating CPAs undergo initial dehydration, followed by rehydration, and potential gross swelling upon removal. This osmotic shock may generate membrane damages by mechanical means and

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predisposition of the cell to injuries during the other steps of cryopreservation, or even cell death [94]. These kinds of damages could be reduced by using cells surfactant such as serum. During the freezing step, the follicular preservation depends on the nature and the concentration of the CPAs.

Control of the cooling and warming rates is also crucial, as the freezing/thawing rates and the temperature of seeding also influence the ice properties. If cells are cooled too rapidly during the controlled slow cooling process, water does not exit the cells fast enough to maintain equilibrium and, therefore, the oocytes and other ovarian cells freeze intracellularly, resulting in death in most cases. If cooling is too slow, the long duration can cause 'solution effects' injury resulting from the high concentration of extra-and intracellular solutes, probably due to the effects of the solutes on the cellular membrane or through osmotic dehydration. During warming the small intracellular ice crystals might subsequently undergo recrystallization, forming bigger ice crystals that rupture the cell membrane, thus leading to fatal damage. Finally, the thawing and the removal of the CPA depend on the temperature and on the presence of non-permeating CPA limiting the osmotic swelling during rinsing.

7. Contamination in cryopreservation

One of the first thing we must learn is to differentiate their respective importance are the concepts of contamination and cross contamination of samples. The first relates to the contamination of the sample by freezing or by direct contact with the cooling solution and the second refers to the contamination of the sample within the common container which is in contact with all cryopreserved samples, some samples may be contaminated or the liquid nitrogen (LN2) might be contaminated producing a possible cross-contamination. The potential for disease transmission and pathogen survival through contaminated LN2 has been proposed by many authors [95-97], and the evidence of contamination in human patients has been described for different pathogens [98-104]. It has to be stated that none of the reported infections after insemination or ET in humans and domestic animals can be clearly attributed to the applied cryopreservation and storage procedure but the use of safe cryopreservation protocol is very important to avoid human cell contamination or cross contamination in common LN2 tanks.

Although cryopreservation had a boom in the mid 70's and early 80's with the opening of the first sperm banks in America and Europe, it was not until the mid-80's when we saw the need for biological samples cryopreserved in quarantine and the lack of screening leads to infection of several recipients that had been inseminated with semen samples from donors HIV+those unaware of their disease [105]. In these cases it was found that samples stored in the same containers with frozen HIV+samples were not contaminated, otherwise in 1995, six patients undergoing cytotoxic treatments hermetic problems developed an outbreak of acute hepatitis B after undergoing an autologous cryopreserved material that had been stored in the same cryogenic container as other patients infected with hepatitis B [106].

7.1. Cells and tissue contamination

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

The case in 1985 where there was infection with hepatitis B in the cryopreserved samples [106] the infection was due to an error in packaging and storage of samples. With time a deterioration of the bags containing infectious material causing the infection of the LN2 and other samples was observed. Further studies have shown that the storage of samples is decisive. There is evidence that frozen samples in hermetically sealed straws are not contaminated even if they are in contaminated containers with contaminated LN2 and LN2 does not contaminate infective biological samples that were frozen in a sealed container [107,108] During the cryopreservation, biological samples go through many processes before being cryopreserved. In the case of IVF cells are subjected to a phase of procurement, fertilization, development, transfer and finally cryopreservation. This represents an approximate 6-day process in which many factors can affect the contamination of the sample at the end of the process. We can find contamination or cross-contamination in the following cases [109]:

- Handling contaminated biological samples (semen, follicular fluid, tissue, etc.). Without precautions to avoid contamination outside the base plate to be used for conservation (cryotube, straw, etc.). It is very important to disinfect and clean the container before filling it with LN2 [110]. In this regard to ensure an adequate level of biosafety a study is needed of infectious diseases transmissible from any patient or donor who wants to freeze any samples. According to Castilla [111] the clinic policy for a donor with infectious diseases is radically different to that of a patient with any of these diseases wanting to freeze biological material for autologous use. In the first situation, the biological material at hand will not freeze. In the second, the biological material should be frozen but with measures that we discuss later. Screenings for infectious diseases that normally must be submitted are: To analyse serological studies for syphilis, hepatitis and HIV. To analyse the clinical studies infective clinical phases: toxoplasmosis, rubella, herpes virus, cytomegalovirus (CMV), Neisseria gonorrhoeae and Chlamydia trachomatis. These tests are required for donors of semen every 6 months. As the risk of disease transmission during storage in LN2 is mainly viral. Interestingly, the American Society of Fertilisation [112], ESHRE [113], British Andrology Society (BSA) [114] and the Spanish Association of Tissue Banks (AEBT) [115] also recommend serologic screening for CMV, not just clinical. The presence of CMV in semen has been associated with active disease (anti-CMV IgM+or recent seroconversion anti-CMV IgG+). Similarly, these companies recommend performing serologic tests for HTLV-I and HTLV-II. But although it is clearly demonstrated the transmission of human papilloma virus by using LN2 cryotherapy and has been shown IUI transmission of herpes simplex virus (HSV) [116], none of the scientific associations mentioned above recommend a culture for detection or serological studies of HPV donors or patients with infection who are going to freeze biological material because the analysis to detect these deceases are not very sensitive. As rubella serologic screening of donors, its low prevalence in this population

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means that serological tests have a low positive predictive value, making it unadvisable. Finally, we believe a patient who needs to freeze some reproductive biological material should have at least one serology for HIV, hepatitis B and C. This proposal is consistent with the recommendations of the AEBT for cryopreservation of semen [115].

- Use of contaminated culture media. In these cases the degree of cross-contamination would reach very high levels having an impact on many patients. Although the preparation of embryo culture media and sperm extenders from specific ingredients are avoided in human clinics, it continues to be a common practice in animal ART [117]. Nevertheless, many ingredients of embryo culture media and sperm extenders act as stabilizers for many micro-organisms at freezing temperatures (milk, serum or serum albumin, sucrose, sorbitol and other sugars). Unfortunately, the most common cryoprotectors (CPs) in applied oocyte cryopreservation and embryo (glycerol, DMSO, ethylene glycol, propylene glycol, methanol etc.) are toxic for cells. Also bacteria and viruses efficiently protect from cryoinjuries, eg Concentrations of DMSO as low as 5% enveloped viruses defend against the trauma of freezing [118]. The Fact That microorganisms survive in association with germplasm is not only important from the potential of disease transmission by embryo transfer to recipients, but also in approaches to the storage of samples for testing and health certification of embryos or international movement. On the other hand we must also bear in mind that all culture media containing antibiotics to prevent or limit survival of microorganisms.

- Conservation of contaminated material or straws cryotubes closed or sealed badly flawed causing the breakdown of the frozen straw, leaving the contaminated sample directly exposed to the LN2 tank risking contaminating the other samples. Closed systems can be sealed in many ways (thermal sealer, ultrasound sealer, radiofrequency sealer, polyvinyl alcohol powders, and solid caps). Given the sealing time and the temperature reached does not affect the cryopreserved sample, we have to ensure that the seal is airtight and that the device is built of resistant material to low temperatures of LN2 (Ionomeric resins, quartz glass capillary, Polyvinyl chloride, Polyethylene glycol tetralato, etc).

- Using contaminated LN2 during the freezing process. In this case we have proposed some solutions that we will see later.

- Poor source management of LN2 from our supplier contaminating commercial LN2 that comes to our lab in the process of manufacture or transportation and filling ourcontainers.

- For transportation of contaminated material in containers. Storage containers should be emptied and cleaned periodically due to the risk of lost straws or small particles of contaminated material that falls to the bottom of a large container [119,120]. Most of the companies of LN2 containers provide cleaning protocols. The main problem is the cleaning of transport cylinders called "dry" because the material that absorbs the LN2 in these bottles is difficult to sterilize. Bielanski [121] describes a method of disinfection of commercial dry shippers with two different types of a LN absorbent. Based on the results presented, it appears that solutions of sodium hypochlorite and ethylene oxide are equally useful for the disinfection of dry shippers constructed with a hydrophobic LN absorbent. In contrast, for dry shippers

without a hydrophobic LN absorbent it is advisable to use gas only for decontamination in order sterilization to avoid their damage by liquid disinfectants.

- The air in the room. If the air that reaches the lab comes from another area that could be contaminated and there isn't a good filter. Some laboratories do not have filtration systems or positive pressure to prevent air contamination.
- Operators. If they are infected then that can lead to contamination by contact or peeling during processing of samples or the handling of cryogenic tanks. Staff must meet certain health and hygiene conditions: negative serology for HIV, HCV, HBV and vaccination against hepatitis B and other viral diseases for which there is a vaccine available. We must also have a detailed description of their jobs, tasks and responsibilities. In addition the centre must provide the worker training in freezing techniques for updating and improving procedures.
- Use of open devices. In recent times there is much talk of closed or open system and the possibility of contamination, so many countries have banned open systems and the trend is to ban the high risk of sample contamination. In a closed or semi-closed device the nitrogen of common container is never in contact with biological material frozen on the inside so cross-contamination cannot produced. In the open system, the biological material is in contact with the common nitrogen so contamination from the sample is very easy if the LN2 is contaminated or contamination of LN2 if the sample is contaminated. The latest study done by Criado and his group [122] showed 45% of contamination in an open device (Cryotop) Vs 0% of contamination in a semi-close device (Ultravit) equal and using a contaminated laboratory LN2.

7.2. Cooling solution contamination

The cooling solution plays a significant role in avoiding contamination of biological samples. It means that we will freeze the sample and we will deposit it for a long storage until thawed and used. Normally the LN2 cooling solution is the most widely used in cryopreservation and survival of pathogens at high temperatures (-196 ° C) has already been proven by many studies [195-97,121,122] cases also involved in seeing cross-contamination of human papillomavirus [108,123].

The need for better cooling rates to avoid formation of crystals in cryopreservation has resulted in the discovery and use of new cooling solutions (slush, slurry, etc.). So there are more components to consider when contamination is to be avoided. Using these new cooling solutions gives a lower temperature than the LN2 temperature and much faster transmission. The Slush nitrogen is obtained by a vacuum pump (Telstar TOP-3; Telstar S.A., Terrassa, Spain) that solidifies part of the LN2 in a few minutes. On return to normal atmospheric pressure, the nitrogen collapses, and the subcooled LN2 has solid particles in it commonly referred to as ''slush'' [124].The advantage of Slush nitrogen lies not only in the temperature difference with respect to LN2 (-196°C Vs-210°C) but also in the reduction of the Leiden frost effect, which is the formation of a layer of vapor around the sample when immersed in the cryogenic liquid from room temperature decreasing the cooling rate [125,126]. It has not yet been demonstrated

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the survival or non survival of pathogens in this cooling solution of 15-20 ° C difference in LN2, this is obtained by vacuum pressure, which can lead to rupture of the cell wall of pathogens to balance internal and external pressure of these in the process of forming Slush. The 'Slurry' nitrogen is a mix of LN2 with different particles for example copper powder. At present investigations are being carried out as an alternative to LN2 to increase the cooling rate because with this cooling solution the thermal conduction is increased. Likewise, experiments are ongoing with various solutions to increase the thermal conduction and the cooling rate.

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

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

Table 1. Microbial contamination of embryos and semen during storage in liquid nitrogen (adapted from Bielanski *et al.,* 2003)

These cooling solutions "alternatives" are only used at the time of freezing the sample and once frozen, it passes to the general container that is filled with LN2, although these solutions where they freeze cool samples have to be sterile we have to ensure that the general LN2 container does not have contact with the frozen sample in order to not contaminate the sample and the LN2 if the sample is positive for any pathogen. Retrospective studies in which commercial LN2 cryotanks were examined after 35 continuous years of service revealed various bacterial and fungal contaminations in the LN2 detritus [117]. Many of the identified bacteria isolated in these studies were ubiquitous environmental micro-organisms and were rare opportunistic pathogens of low significance in producing disease in humans or animals (Table I). It should be acknowledged that some of the isolates may have been derived from laboratory contamination during semen and embryo processing for cryopreservation rather than genuinely being present within the sample. In agreement with Bielansky and Vajta the risk of contamination by human pathogens seems to be rather low. Components of the standard LN2 production system comprise a compressor, a cryogenerator and containers. From a practical point of view,

the complete sterilization and maintenance of sterility in such a robust system might be a very demanding task, if possible at all. Accordingly, some ubiquitous bacterial agents can be expected in any commercially produced LN2. Nevertheless, it is an 'in and out' system and only air-borne contaminants are supposed to enter it (LN2 compressor) via air used for LN2 production. As they are not air-borne, it is unlikely that viral agents of human concern such as HIV, hepatitis and herpes viruses would enter the LN2 production system.

Microbiological contamination of embryos and semen during storage in LN2 [117]

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

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

- LN2 Filtration: One of the solutions that have been developed is the filtration of LN2. Air Liquid has marketed CERALIN a liquid filtration system through LN2 ceramic filters. The CERALIN ON LINE consists of two elements of liquid filtration connected in series and inserted into a section of vacuum transfer line. The ceramic membrane is made from multiple layers formed into a multi-channel element. It is housed in a vacuum insulated pipe, itself installed close to the end-use point. The filter minimizes the pressure drop and avoids the vaporization of the LN2. Thus it avoids nitrogen losses. Several sizes are available, depending on the nitrogen flow. The efficiency of this equipment was investigated and proved in laboratory. The filter is located downstream of the nitrogen vessel. During operation, LN2 flows through the filter and over the ceramic membrane. The result is high-purity LN2 with a bacteria count of less than 1 CFU/L gas. Additionally, the large filtration area of the membrane and low level of contamination of LN2 means it is likely to be several decades before filter saturation.

- UV Sterilization: This method is based on emitting the minimum dose on UV radiation necessary to kill micro-organisms that can survive at the boiling point of nitrogen (-196°C) and which is irradiated in a temperature-controlled regimen, within a short time interval, before the LN2 completely evaporates. The extremely radiation-resistant bacterium

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Deinococcus radiodurans is inactivated (>-4log) by administe-ring 400.000 μWs/cm² per each sterilization cycle. An adequate amount of UV radiation deactivates the growth of all kinds of micro-organisms, from viruses like Hepatitis (which require an 8.000 UV dose) to fungi like Aspergillus Niger (330.000 UV dose) [127]. At CRYO 2011 Dr. Parmegiani spoke about a new dispositive of UV sterilization of the common containers with cells or tissues inside but the scientific community thinks that is too dangerous biological samples exposed to UV rays without any protection. Although his group is proposing special canisters "not transparent" I think they have to do many more tests to rule out damage to the samples because the common view is confirmed that UV light is harmful, even if used just overnight decreased embryo developmental rates.

- LN2 Steam: As an alternative to hermetical storage in LN2, cryostorage contamination might be avoided by storing the carrier containing the vitrified oocytes in LN2 vapour [128, 129]. However, Grout and Morris [130] maintain that storage in the vapour phase of LN2 still carries a risk of sample contamination. Storage of semen in LN2 vapours was discarded early in the development of sperm cryopreservation techniques and it was found that long-term viability of sperm was reduced compared with LN2 storage [131,132]. However, recent experiments with new materials have succeeded in developing the technique with acceptable results for both semen and embryos [133,134] and in our last experiment we demonstrated 0% of contamination in vapor nitrogen in an experimental contaminated laboratory LN2 (non published). The drawback of the generalization of this form of storage is the need for careful monitoring of temperature in different parts of the container, which makes the marketing of these containers type [134] more difficult.
- Before entering discussions regarding the sterility in LN2 used for vitrification, we should debate the use of communal containers, which is where cross-contamination can be found, as there is a possibility that the “contaminated cells” could come into contact with each other, and where a number of viruses and bacteria are found, which would never be found in the commercial LN2.

7.3. Contamination in transport

To carry out a safe transportation of biological material we should clearly distinguish a number of concepts [111].

1. Infectious substances: those that contain viable microorganisms (bacteria, virus, prions, parasite, fungus) or bacterial toxins that are known or believed to cause disease in animals or humans.
2. Diagnostic specimens: human or animal materials (body fluids, blood, tissue, tissue fluids, etc.). Obtained for diagnostic or investigational [135].

Most often transported biological reproductive materials are cryopreserved semen donor and follicular fluid when the laboratory is separated from the follicular puncture site. In both cases, we consider the recommendations to follow are those of diagnostic specimens. There are several documents related to the transport of biological material, such as the Universal Postal

Union (UPU), the International Aviation Organization (ICAO) and International Air Transport Association (IATA) [136-138].

At European level, all documents related to transport are based on the recommendations of the Committee of Experts of the United Nations Dangerous Goods (UN) [139]. There is also a european agreement on international transport of dangerous goods by road (ADR), approved by RD 2115/9838 [140]. We will describe some aspects of the mentioned regulations on the transport of diagnostic specimens. The basic system consists of packaging:

1. Primary container, watertight, leak proof, labeled and contains the sample. This container should be wrapped in absorbent material. In terms of labeling, according to AEBT, if it is a semen sample from a donor, must contain an alphanumeric code that identifies the donor and the sample number of the donor. On the other hand, if the sample is for autologous use may be noted also the surname of the patient [115].
2. Secondary container, sealed, leak-proof and protects the primary container. You can place multiple primary containers wrapped in a secondary container. This should be sufficient absorbent material used to protect all primary containers and avoid collisions between them.
3. Outer shipping container: the secondary container is placed in a shipping package that protects the secondary container and its contents from outside elements, such as physical damage and water. The data forms, letters and other identifying information of the sample should be placed taped outside the secondary container. The label for submitted materials consists of:
    - **a.** Basic triple packaging.
    - **b.** Does not require signs from United Nations (UN).
    - **c.** No substances require pictogram or declaration from the sender.
    - **d.** Biological material for clinical use" must be indicated.
    - **e.** Tag address:
        - Name, address of destination, as detailed as possible, and phone number.
        - Name, address, telephone number and contact person at the semen bank.
    - **f.** The documents included with the storage conditions and special instructions for shipping. One of the special considerations that we must have in mind when transporting a sample of semen is not breaking the cold chain, so you must use a container or LN2 as well as avoiding the possible use of dry ice.
    - **g.** Permission for import / export and declaration.
    - **h.** Label orientation.
    - **i.** Date and time of departure of Semen Bank [115].

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

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

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

According to AEBT [115], the possibility of returning a material that hasn't been used should be avoided, as a rule, the return of the semen that has been provided by the Bank, as it will only accept the return of the displayed when you meet the following 3 conditions:

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

8. New techniques for in-vitro fertilization patients: Ultravitrification

Today the differences between Slow freezing and Vitrification are known worldwide. We all know that slow freezing is characterized by a prolonged cooling curve and the use of low concentrations of cryo-protectors generally ''non-toxic'' for the cells (1–2 M) with cell injury due to ice formation [141] and that Vitrification is characterized by the rapid procedure and the use of a high concentration of cryo-protectors (4-6M) to prevent cell damage that is toxic to most mammalian cells [7,142-149].Thus, vitrification with a semi-close devices have a better cooling rate without cross-contamination or novel cyopreservation techniques are needed that allow rapid cooling to achieve vitrification in the absence of high concentration of CPA or if is possible without CPA.

The requirements and relationships for conditions to achieve satisfactory vitrification in the area of mammalian ART are well displayed in the equation of Yavin and Arav [151]

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

Probability of vitrification by Yavin and Arav [151]

The main points to be gathered from this relationship are that the smaller volume of the vitrification solution in which the cellular material is placed for the vitrification process, the faster cooling and warming rate that can be achieved and the lower concentration of CPAs needed reducing the detrimental effect of the inherent toxicity of CPAs and increasing the overall success of the procedure.

What would happen if We could vitrify without CPA's or with a low concentration of CPA's? What would happen if We could combine the advantages of Slow freezing and Vitrification and vitrify with low concentrations of CPA's with a secure and free contamination device? That is Kinetic vitrification (Ultra-vitrification). Perfecting the techniques of Vitrification has been achieved a morphological survival rate comparable to normal Vitrification protocol [123] or a 59.1% of blastulation rate in mouse embryo [151] with Kinetic vitrification and concentrations of CPA's typical of Slow freezing.

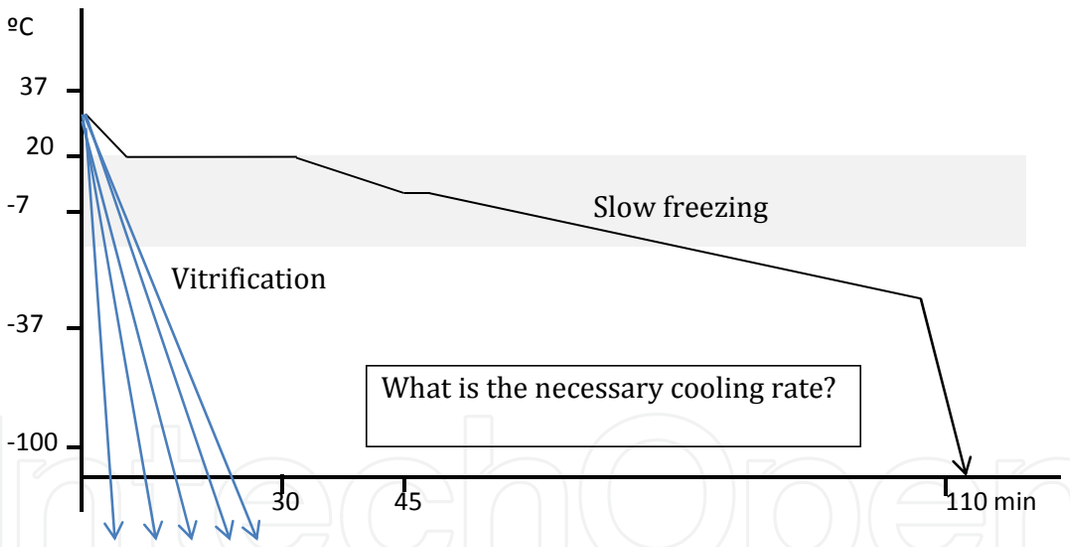

Figure 2. Necessary cooling rate to have a good probability of vitrification

Previous studies have tried to achieve high cooling rates for cell vitrification. However, none of them utilized low CPA concentrations (1.5-2 M). In 1985, Rall and Fahy successfully vitrified mouse embryos in 6.5 M cryoprotectant cocktail solution [7]. In that case the method consisted in a 0.25 ml straw container plunged into LN2; the cooling rate was 2.500 °C/min. When this container was plunged into Slush nitrogen, the cooling rate increased up to 4000 °C/min [152]. The use of OPS (instead of the 0.25 ml straw) in LN2 increases this cooling rate up to 5.300 °C/min [152] and to 10.000–20.000 °C/min if plunged in Slush nitrogen [152,153]. Similar cooling rates were achieved in the case of a Cryoloop quenched in Slush nitrogen [154]. The use of

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electron microscope copper grids has also been investigated, but the cooling rates were in the same order of magnitude that the afore mentioned works: 11.000–14.000 °C/min in the case of plunging the grid in LN2 [155] and 24.000–30.000 °C/min if plunged in Slush nitrogen [126, 155]. From Boutron's theory, none of these approaches reaches the critical cooling rate to achieve vitrification with low concentration of CPA (1.5-2M). It´s impossible to use open devices with Slush nitrogen as the cell is on the outside and there is a possibility of detaching from the device.

Adjusting to Yavin and Arav formula the Ultra-vitrification technique arose achieving a cooling rate above 250.000 °C/min and of 90.000 °C/min in thawing. This rate is one order of magnitude higher than the highest cooling rate achieved in different strategies (electron microscope copper grids in Slush nitrogen [126,155], whilst keeping all the advantages of a straw-like form for the container and being in the range of the necessary cooling rate to achieve vitrification. To have this increase in the cooling rate a few changes were made to the normal vitrification process:

Slush Nitrogen

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

It was shown for oocytes and embryos that increasing the cooling rate would improve survival rates by up to 37% [156]

Figure 3. Slush Nitrogen

Quartz Micro-capillary

Another determining factor to achieve a high cooling rate is the device used. To increase the thermal conductivity and minimize the volume, this technique has used a quartz microcapillary. This has a 0.2-0.3 diameter allowing to ultra-vitrify 0.1-0.2 μl with a 0.01mm wall, a lot thinner than any other device (0.075 mm in OPS). Another important characteristic is the material it is made from: Quartz. The thermal conductivity of quartz glass is a lot higher than that of plastic of which other devices are made of. This converts it in one of the materials that best conducts the temperature. [157]

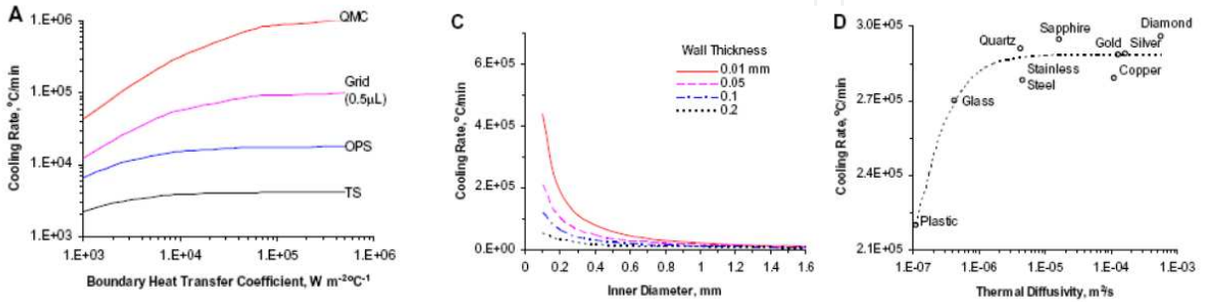

Figure 4. Xiaoming He et al [157]

In a thermal performance of quartz capillaries for vitrification done by Risco and his group [158] a commercially available version of the OPS (MTG Medical Technological Vertriebs, GmbH) was used. The thermal conductivity of these PVC straws was 0.19 W m_1 K_1. The inner diameter is 0.800 mm and the thickness of its wall is 0.075 mm (Fig. 1a). The QC used (The Charles Supper Company, Inc.) have an inner diameter of 0.180 mm and a wall thickness of 0.010 mm. These geometrical improvements (4.44 times smaller in diameter and 7.50 times thinner) translate not only into a faster heat transfer, but also into a 20 times reduction in volume of the contained solution (for a given height). This is beneficial because the thermal conductivity of the quartz glass is 1.3 W m_1 K_1, that is almost one order of magnitude higher than that of PVC.

A clear heat release peak is present during cooling as well as melting during rewarming. (b) Thermal history for QC when filled with a 1.5M propane-1,2-diol and 0.3M sucrose cryoprotectant solution quenched in LN2 and then thawed in a water bath at 37°C. Crystallization of water is not obvious during cooling, but melting is shown during rewarming. (c) Thermal history for OPS when filled with a 1.5M propane-1,2-diol and 0.3 M sucrose cryoprotectant solution quenched in Slush nitrogen and then thawed in a water bath at 37°C. In this case, crystallization during cooling and melting during rewarming was not recorded. However, visual inspection reveals the presence of ice. (d) Thermal history for QC when filled with a 1.5Mpropane-1,2-diol and 0.3 Msucrose cryoprotectant solution quenched in Slush nitrogen and then thawed in a water bath at 37°C. The sample keeps its transparency over all the cooling–rewarming cycle, an indication of the capability of this approach to vitrify the studied solution. All these changes have allowed us to maintain a concentration of cryoprotectors typical of slow freezing, 2 M PrOH+0.5 M sucrose, obtaining a morphological survival rate of

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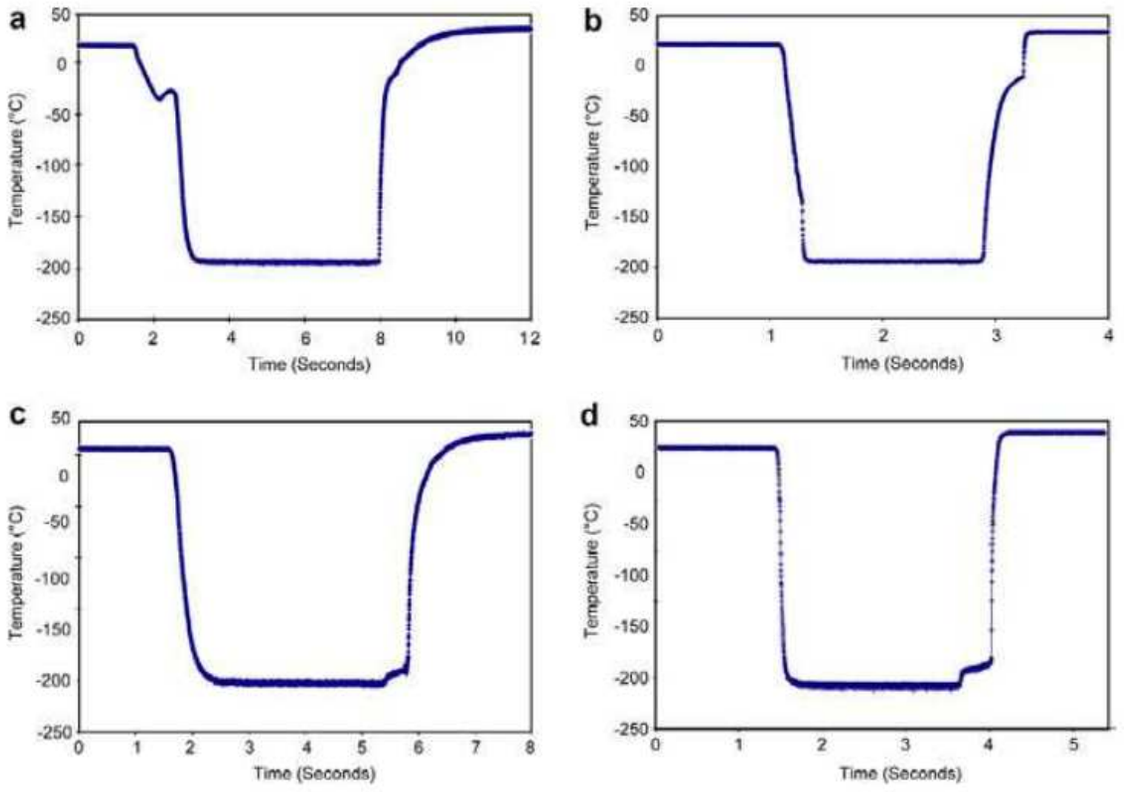

Figure 5. a) Thermal history for the OPS when filled with a 1.5M propane-1,2-diol and 0.3M sucrose cryoprotectant solution quenched in LN2 and then thawed in a water bath at 37°C.

92 % in human oocytes [124]. Dr. Ho-Joon Lee et al [151] tested this new technique on mouse ooctyes and they saw that using Ultra-vitrification with low concentrations of cryoprotectors improved the fertilization rate and above the blastulation rate. Only the use of Ultravit device in this technique ensures the non contamination of the sample or cross-contamination in communal containers.

%	Slow freezing [159]	Vitrification [159]	Ultravitrification mourine oocytes [151]	Ultravitrification human oocytes [124]
Surv. rate	61	91.8	92.5	92
Fert. Rate	61.3	67.9	75	?
Blast. rate	12	33.1	59.1	?

Table 2. Comparison between slow Freezing, Vitrification and Ultravitrification [162,152,124]

This comparison demonstrates the use of low concentration of cryoprotectant in the Ultravitrification protocol favours the morphological survival (92%) and increases the blastulation rate (59.1%). Thus confirming the hypothesis that cryoprotectants are toxic to the biological

sample and if we could find a vitrification protocol that would allow us to vitrify without cryoprotectant, we would achieve a better embryo development and a greater chance of pregnancy in the case of freezing eggs or embryos. A lot more studying is needed regarding this new technique but a priori the results indicate that we can hopefully lower the concentration of the cryoprotectants decreasing the toxicity in cells.

9. Conclusion

Cryopreservation has always been a fundamental tool in assisted reproduction but increasingly assumed a more important role because it serves not only to optimize ART treatments but thanks to the possibility of both cells and tissues cryopreserved successfully, we can offer the possibility reproductive future of cancer patients.

Cryopreservation takes many utilities; the most traditional approach is optimization of IVF treatments. In this sense it has been used in the cryopreservation of sperm for sperm banks or uses it to treatment when there is a problem to get it on the day of egg retrieval. The sperm freeze has been doing for decades due to the relative ease of the technique and the good results are achieved, therefore it is more widespread use of sperm cryopreservation in cancer patients or patients who are going to be vasectomized getting preserve their fertility for the future.

From the revolutionary emergence in the late 70's in vitro fertilization (IVF), the possibility of storing surplus embryos from IVF programs was a need and offered an excellent alternative Cryobiology. Other situations where cryopreservation is very useful are reported when an embryo transfer is dangerous for the health of the patient for risk of ovarian hyperstimulation (OHSS).

Another key point in recent years is gaining more importance is the freezing of oocytes. Because of its large size and the amount of water that contains inside, the oocyte has always presented difficulties for cryopreservation. The need for better cooling rates to avoid formation of crystals in cryopreservation has resulted in the discovery and use of new cooling solutions (slush, slurry, etc.). These new cooling solutions and the new vitrification media with little or no cryoprotectant has become possible that cell cryopreservation excellent results after thawing.

Up to date, the studies that have evaluated the health of children born through the procedure of egg thawing have reflected that there is no increase in the incidence of congenital abnormalities. And it remits us to prestigious investigations such as that published by Noyes in 2009 [160] with nearly 900 babies born through freezing and thawing of oocytes, in which it reflected that these children were completely normal. More recently Levi Setti in 2013 [161] published another study in which it stated that, after studying 954 pregnancies, there is clear evidence that the children born by this technique have the same probability of malformation and the same complications during a pregnancy and delivery as children born by other assisted reproduction techniques in which fresh eggs have been used.

Therefore, up-to-date no published investigation makes us believe that oocyte vitrification is dangerous for the children that will be born as a benefit of the technique. Although 13 years

later after the first slow-freeze birth, the number of reported babies born as a result of vitrified oocytes is now approaching that of slow-frozen oocytes without any increasing risk in congenital abnormalities [160]. Vitrification of oocytes does not appear to increase risks of abnormal imprinting or disturbances in spindle formation or chromosome segregation [162]. It has the greatest potential for successful oocyte cryopreservation and with its increased clinical application is showing a trend to greater consistency and better outcomes (similar to outcomes between fresh or warmed oocytes). Vitrification of oocytes, when applied to properly screened patients, will be a useful technology in reproductive medicine practice and will constitute a major step forward in ART. Today these results and the constant research to continue advancing to achieve even further improvements in the cryopreservation protocols have allowed the creation of egg banks around the world like OVOBANK that provide patients do not have to travel to find donors in cities where there isn´t a high variability of egg donors with similars results to those treatments with fresh eggs.

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