ΠΑΝΕΠΙΣΤΗΜΙΟ ΙΩΑΝΝΙΝΩΝ
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ
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ΤΟΜΕΑΣ ΧΕΙΡΟΥΡΓΙΚΟΣ
ΜΑΙΕΥΤΙΚΗ ΚΑΙ ΓΥΝΑΙΚΟΛΟΓΙΚΗ ΚΛΙΝΙΚΗ

Κρυοσυντήρηση ανθρώπινων ωαρίων με τη μέθοδο της υαλοποίησης (vitrification). Η επίδραση δυο συστημάτων υαλοποίησης (ανοικτού και κλειστού) στην αναπτυξιακή ικανότητα ωαρίων και εμβρύων και μελέτη της εφαρμογής τους σε κλινικές περιπτώσεις

ΑΧΙΛΛΕΥΣ ΠΑΠΑΘΕΟΔΩΡΟΥ
ΒΙΟΛΟΓΟΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΙΩΑΝΝΙΝΑ 2017

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7. Βρεκούσης Θωμάς Επίκουρος Καθηγητής Μαιευτικής-Γυναικολογίας με έμφαση στη Γυναικολογική Ογκολογία του Τμήματος Ιατρικής του Παν/μίου Ιωαννίνων

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Πασχόπουλος Μηνάς
Καθηγητής Μαιευτικής-Γυναικολογίας

Η Γραμματέας του Τμήματος
ΚΑΠΙΤΟΠΟΥΛΟΥ ΜΑΡΙΑ

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Στους γονείς μου
τη γυναίκα μου
και τον μικρό Νικόλα

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PART 1 – INTRODUCTION

1.1 Cryobiology

Cryobiology deals with life at low temperature (1,2). The word cryobiology is relatively new. Literature search indicates that cryobiology was first used in the early 1950s to describe the newly developing field of low temperature biology (3–6). Living creatures must be able to adapt to the changing surface environment of the earth in order to preserve the existence of life itself. The two main effects of cold on living tissue are either destruction of life or preservation of life at a reduced level of activity. Both of these effects are demonstrated in nature. Death by freezing is a relatively common occurrence in severe winter storms. Among cold-blooded animals, winter weather usually results in a coma-like sleep that may last for a considerable length of time. Therefore, the definition of cryobiology is to study living organisms at low temperature. In other words, cryobiology is the branch of biology involving the study of the effects of low temperatures on organisms (most often for the purpose of achieving cryopreservation).

In cryobiological applications, much lower temperatures are used than those presented in natural environments. Liquid nitrogen (at -196°C) can either destroy living tissue in a matter of seconds or preserve it for years, and possibly for centuries, with essentially no detectable biochemical activity. The final result, when heat is withdrawn from living tissue, depends on processes occurring in the individual cells. Basic knowledge of the causes of cell death, especially during the process of freezing, and the discovery of methods which prevented these causes, have led to practical applications for long-term storage of both living cells and living tissues. In the industrial food area, the microorganisms used in cheese production can be frozen, stored, and transported without loss of lactic acid-producing activity. In the medical field, it is commonly known

that whole blood or separated blood cells can be cryopeserved and stored for their valuable applications.

1.2 Principles of cryopreservation

1.2.1 Water: Major factor in the process of cryopreservation

Water is the fundamental molecule of life. The biochemical components of a cell are either dissolved or suspended in water. Water is essential for the survival of all known forms of life; without an aqueous environment, life would not exist. Water has many distinct properties that are important for the proliferation of life and these separate it from other substances. It enables the proliferation of life by allowing organic compounds to react in ways that ultimately allow replication. Water is essential both as a solvent for many of the body's solutes and as an essential part of many metabolic processes within the body. Water is vital and central to these metabolic processes. Temperature affects metabolic processes. When the temperature falls, cells may slow down or stop all metabolic processes, and extremely low temperature may cause cell death.

During the freezing process, water tends to crystallize in pure form, while the dissolved or suspended materials concentrate in the remaining liquid. In the living cell, this process is quite destructive. During a slow-freezing process, ice first begins to form in the fluid surrounding the cells, and the concentration of dissolved materials in the remaining liquid increases. A concentration gradient is established across the cell wall, and water moves out of the cell in response to the osmotic force. As freezing continues, the cell becomes relatively dehydrated. Salts may concentrate to extremely high levels. In a similar manner, the acid-base ratio of the solution may be altered during the concentration process.

Dehydration can affect the basic organization of the cell as well as the molecular relationships, some of which depend on the presence of water at particular sites. The collapse of the cell that results from water loss may bring into contact intracellular components that are normally separated to prevent any destructive interaction. Finally, as the ice crystals grow in size, the cell walls may be ruptured by the crystals themselves or by the high concentration gradients that are imposed upon the walls. To prevent dehydration, steps must be taken to stop the separation of water in the form of pure ice so that all of the cell fluids can solidify together.

Cryobiology is the core of fertility cryopreservation. The earliest application of fertility cryopreservation was in the storage of animal sperm cells for use in artificial insemination. The principal application for human fertility cryopreservation was also begun with sperm freezing, and then with embryo and oocyte as well as gonadal cryopreservation. Knowledge and medical achievement have steadily advanced in the field of fertility cryopreservation, especially with recent oocyte and ovarian tissue cryopreservation. These historic accomplishments in the application of the scientific method can provide overwhelming support for continuing on this path. This chapter will try to set out briefly the scientific background and our current basic knowledge of cryobiology.

1.2.2 Water: Nature and physical properties

Water appears in nature in all three common states of matter: vapor, liquid, and solid. Water is a tasteless, odorless liquid at standard temperature and pressure. The maximum density of water occurs at 3.98°C. Water becomes even less dense upon freezing, expanding 9%. This causes an unusual phenomenon: ice floats upon water, and

so organisms can live inside a partly frozen pond because the water on the bottom has a temperature of around 4°C. The boiling point of water is 100°C at sea level and one atmosphere pressure. The freezing point of water is very close to 0°C. This temperature may be lower if super cooling occurs or if there are substances present in the water which could cause freezing point depression to occur.

The transition between liquid water and solid ice is one of the most commonly observed events in nature. As mentioned above, when water is cooled, it often is taken substantially below the freezing point before ice begins to form. This is because of the need for nucleation to occur before an ice crystal can begin to grow. Nucleation refers to the process by which a minimum crystal is formed, which can then expand. The continued expansion of the crystal is a process known as growth. When an ice nucleus begins to grow, any solutes that are present in the liquid will be excluded from this growing ice front. If the rate of crystal growth is faster than the rate at which diffusion of the particular solutes can carry them away from the ice front, then a concentration gradient will very quickly form in the liquid that surrounds the ice crystal. The concentrated solute will then lower the freezing point of the solution. When a certain amount of ice has formed, the solution at the interface will have a freezing point equal to the temperature of the interface. At this point, ice growth will be limited by diffusion of the solute away from the crystal. If the temperature is reduced to far below the melting point with supercooling speed, the solution may be prevented from reaching this situation of ice crystal nucleation and growth. If water is cooled sufficiently fast enough so that nucleation cannot occur, it is possible to avoid ice crystal formation (7); this process is known as vitrification.

1.2.3 Glass transition temperature

The glass transition temperature *(Tg)* is the temperature at which an amorphous solid becomes fragile on cooling or soft on heating. Glass transition is a pseudo- phase transition in which a liquid that is being cooled produces a glassy structure with properties similar to those of crystalline materials. Below Tg, amorphous solids are in a glassy state, and most of their joining bonds are intact (figure 1).

At a certain temperature, the average kinetic energy of molecules no longer exceeds the binding energy between neighboring molecules, and growth of an organized solid crystal begins. Formation of an ordered system takes a certain amount of time since the molecules must move from their current location to energetically preferred points at crystal nodes. As the temperature falls, molecular motion slows down further and if the cooling rate is fast enough, molecules never reach their destination: the substance enters into dynamic arrest and a disordered glassy solid form.

Water can be transformed to glass. Glass formation of water below the melting point can occur, usually through very rapid cooling or the introduction of agents (e.g. cryoprotectants) that suppress the formation of ice crystals (figure 1).

1.2.4 Vitrification

Vitrification is defined as the process of glass solidification of a liquid. The liquid is in a metastable state (isomer nuclei) until it gets below a characteristic temperature, Tg, which is indicated by a sharp exothermic event. This heat loss occurs because of the loss of metastable complex. This complex that has more energy than can be held by the bonds which is able to form will oscillate for a short time and then disintegrate. Upon reaching

Tg, the excess energy will be lost, thereby stabilizing the complex. Once below Tg, the system is not merely a viscous liquid but is also a solid that is in a stable thermodynamic state. Achieving vitrification with pure water requires very small amounts and incredibly fast cooling. However, it is important to mention that vitrification can also occur in aqueous solution during slow freezing.

1.2.5 Osmosis

Water can be transported across semi-permeable membranes separating compartments containing different concentrations of solutes. The membrane must be impermeable to the solute but permeable to water. This process is called osmosis and has enormous significance for living organisms. The most important and most widely occurring process for water transfer in and out of living cells is osmosis.

1.2.6 Cell permeability

Changes in the extracellular osmotic pressure will create a situation in which a cell will attempt to attain equilibration by either gaining or losing water until there is no osmotic gradient across the plasma membrane. If the cell volume is measured as a function of time, then it can be seen that equilibrium is only achieved after a certain amount of time has elapsed. The kinetics of water movement out of the cell is determined by the physical structure of the membrane.

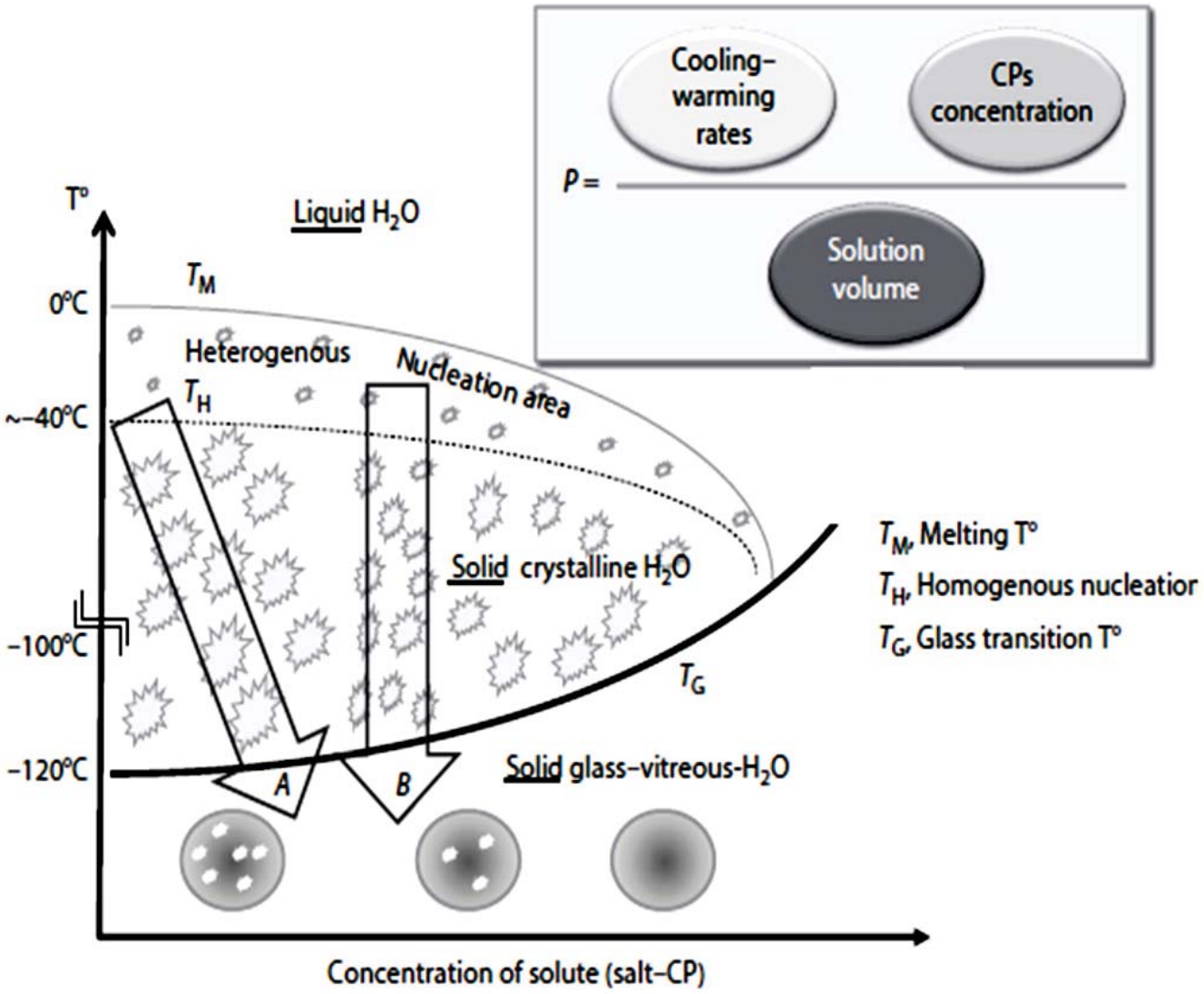


Figure 1: Phase diagram presenting the different phases of water according to the temperature. The equation represents the factors that influence the probability of achieving the vitrified state. The arrows represent the cooling speed and the size of ice crystal formation that are associated. Arrow A represents a less fast cooling than arrow B. (CP = cryoprotectant.)

1.2.7 Cryoinjury

Cryoinjury is the damage of the cell during freezing or thawing(7). Many theories and mechanisms have been proposed for cryoinjury, but none may exactly explain the nature of the phenomenon. For example, it is not surprising that survival rates can vary from cell type to cell type for the same cooling rate and freezing solution.

1.2.8 Cryoprotectants

Cryoprotectants are substances that can reduce the injury of cells during freezing and thawing. They are divided into two basic classes based on their ability to diffuse across cell membranes. Penetrating cryoprotectants are able to move across cell membranes whereas non-penetrating agents cannot.

Although a good survival rate of deep-frozen cells has occasionally been observed without a protective agent, suitable cryoprotectants usually increase the survival rate. The discovery that glycerol, first, and later dimethyl sulfoxide protect eukaryotic cells against freezing damage marked the beginning of modern cryobiology (5). Today, the most commonly used cryoprotectants in the field are glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol. The cryoprotective action of each type of cryoprotective agent must be similar, but although many hypotheses have been proposed to explain their mechanism of action, it is still unclear what role they do actually play in the freezing or vitrification solutions.

1.2.9 Toxicity of cryoprotectants

The toxicity of cryoprotectants refers to at least two effects. The first is the chemical reacting with cells before cryopreservation, and the second is the chemical

causing the change of osmosis of freezing solutions. Relatively low concentrations of cryoprotectants are usually used in cryobiology and, therefore, the chemicals themselves may not be a major concern for toxicity, although the concentration of cryoprotectants used in rapid cooling is relatively high.

1.2.10 Equilibration

It is common practice to suspend cells in aqueous (freezing) solution containing permeable cryoprotectants for the time that is required to equilibrate intracellular solutes before freezing. Many cells, especially eukaryotic cells, are sensitive to osmotic stress. Therefore, the permeating cryoprotectants are added gradually to the freezing solution in order to minimize osmotic stress as well as to allow removal of the cryoprotectants from the suspensions gradually. It means that the cells need time to balance and to adapt to osmotic shock. Based on the permeating speed of the cryoprotectants and the cell types, different equilibration times will be required. Normally, lower temperature requires a longer period of equilibration, and higher temperature needs a shorter period. The success of cryopreservation is determined by whether or not the cell undergoes intracellular ice formation during freezing.

1.2.11 Cryoprotectant toxicity and damage

The purpose of cryoprotectants is to prevent damage during cooling/freezing. However, these compounds can themselves induce damage to cells. The conditions under which cryoprotectants can cause damage to the cells, are relate to three factors: the concentration of the cryoprotectant, the exposure time and the temperature of the exposure. Cryoprotectants are generally non-toxic when used for short periods of time. On the contrary, extended exposure or exposure at elevated concentrations can result in

metabolism of the cryoprotecting agents, which can subsequently disrupt cellular function and viability (8) . Concerning oocytes, compounds such as DMSO and PROH can cause depolymerization of tubulin and meiotic spindle malformations (9–11). Besides concentration and time of exposure, the temperature at which cryoprotectant are used can also impact cellular damage. For example at 37°C cryoprotectants could cause perturbed microtubule actin microfilament arrangement in mouse oocytes, which was less apparent when cooled (9). Generally, lowering the temperature of cryoprotectant exposure has as a result a decreased uptake and metabolism of the toxic agents from the cell.

1.2.12 Avoiding toxicity

The selection of a suitable cryoprotectant and optimizing its concentration, as well as monitoring the time and temperature of exposure, is a fundamental principle that will determine the possibility of cell damage by a cryoprotectant. Therefore, a common approach to reduce toxicity is to combine various cryoprotectants, both permeating and non-permeating, thereby reducing individual concentrations and minimizing damage while maintaining the overall protective effects (12). Combining multiple cryoprotectants could induce a counteract on the negative impact of one agent to the negative impact of another agent (13). Methods of introducing and removing cryoprotectants offer another means to reduce a potential damage. Stepwise addition of cryoprotective agents, or gradually increasing concentrations, has been useful. Additionally, stepwise removal of these compounds upon warming/thawing helps to minimize osmotic stress.

1.3 Cryopreservation Theory

The aim of cryobiology is to avoid the death and maintain the immortality of the cell at low temperatures. The first theoretical basis for cryopreservation of cells was proposed by Mazur (7,14). The discovery of cryoprotectants made possible these theories. The main principal of cryopreservation is to eliminate the main causes that are associated with cell death i.e. intracellular ice crystal (7) and lethal concentrations of solutes (15)while maintaining the functional capacity of intracellular organelles.Removal of intracellular water and, consequently, reduction in intracellular ice formation is achieved by exposure to cryoprotectants. Cryoprotectants either permeate the cell membrane displacing water via an osmotic gradient (e.g. propanediol -PROH, dimethyl sulphoxide -DMSO or ethylene glycol –EG) or provide a continuous gradient but do not permeate the cell membrane, (e.g. sucrose). Permeating cryoprotectants also aid in balancing other intracellular solutes which are lethal at high concentrations. These hyper osmotic lethal conditions also occur in domains between ice crystals in the extracellular environment as the temperature is reduced and, as a consequence, further dehydration of the cell occurs. The extent of dehydration is dependent on the rate at which the temperature is decreased (16) and the permeability of the cells to water (17).The above principles are fundamental for cryobiology but they are differently applied in slow freezing and vitrification, the two methods of cryopreservation in assisted reproduction technology.

1.3.1 Slow cooling method

In slow cooling procedure, initially the cells are exposed to a simple solution with a permeable cryoprotectant (< 1.5 MPrOH) and usually, a low concentration of non-

permeable cryoprotectant (<0.3 Msucrose). After a brief exposure time period(up to 10 min) to allow uptake of cryoprotectant, the cells are loaded into plastic straws (in a volume of ~200 ml)which are sealed at both ends. Straws are then placed in the chamber of a programmable freezing machine which slowly reduces the temperature (~0.3°C/min). When the temperature is slightly below the melting point of the solution (usually around -7°C), the straw is 'seeded' so that ice nucleation to be induced at the extracellular solution. Then the slow cooling continues and the osmolarity of the extracellular solution increases as water freezes out as ice. With the increasing tonicity, the cells dehydrate. Dehydration continues during slow cooling until the cells are plunged into liquid nitrogen, usually at a temperature below -30°C. At this point, the intracellular cryoprotectant concentration is high enough that the remaining intracellular water will vitrify, preventing intracellular ice formation (IIF). During thawing, ice can form once again if the vitrified solution warms at a rate permitting the process of devitrification (figure 2). This happens when the temperature is high enough that the molecular mobility of water has increased to a point where the water molecules can move and rearrange themselves from a disorderly amorphous vitrified position to an orderly crystalline position. This occurs well below the melting point, and is therefore a potentially lethal problem(18,19). During thawing, the dehydrated cells are exposed to hypotonic conditions and rehydrate along with cryoprotectants removal usually in the initial presence of increased concentrations of non-permeating cryoprotectants (20).

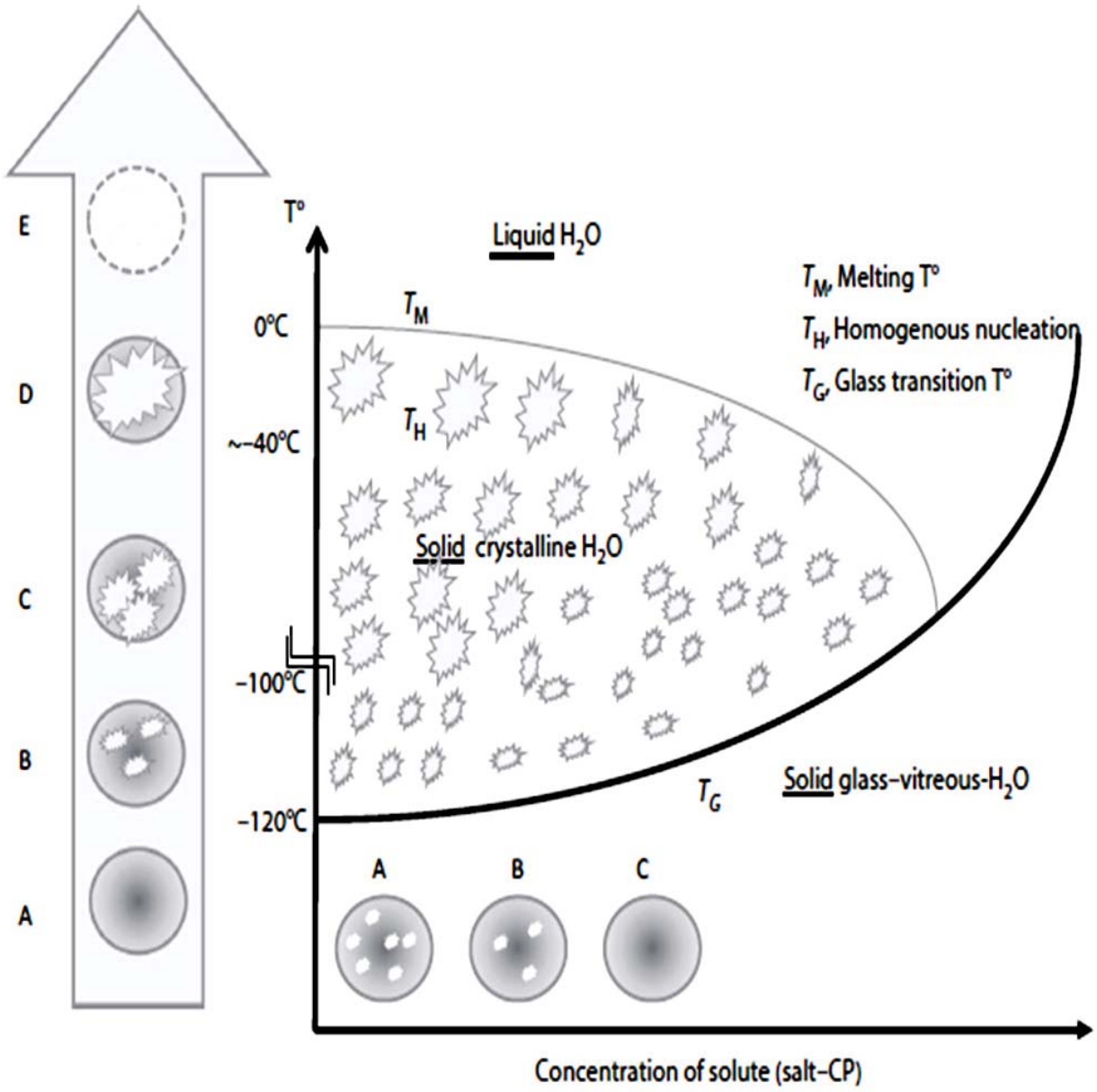


Image 2: Devitrification is a phenomenon where the glass state during warming procedure

1.3.2 Vitrification method

To achieve vitrification (i.e. a glass-like state) within a cell, both a reduction in water content and a highly viscous cytoplasm are necessary (21). This is generated by exposure to high concentrations of permeating (>4 M, DMSO, EG, Glycerol) and non-permeating (>0.5 M, sucrose) cryoprotectants which result in extreme shrinkage. However, in order to minimize the impact of the hyper-osmotic conditions the exposure time is reduced to < 1 min (22). Since a high concentration of a single permeating cryoprotectant, however, may in itself be lethal (23) or may result in impaired development (24), multiple cryoprotectants (25) have been used in combination to reduce the individual cryoprotectant toxicity while achieving a highly viscous solution (23). The high osmolarity of the vitrification solution rapidly dehydrates the cell and submersion into liquid nitrogen quickly solidifies the cell, so that the remaining intracellular water does not have time to form damaging ice crystals.

1.3.3 Why vitrification is better that slow cooling

Vitrification has been proven to be a more successful method than slow cooling because it's a procedure that eliminates ice crystal formation inside the cells, leaving intact the domains of the cells. In order to explain why vitrification is a better cryopreservation technique we need to understand the diagram in figure 1,which is based on the work of Fahy and colleagues (26) who introduced in 1984 vitrification as a potentially efficient cryopreservation method in embryology.

Y-axis represents the temperature of a liquid (e.g. water or isotonic solutions that we use to dilute cryoprotectants). X-axis represents the concentration of the solutes (e.g. cryoprotectants) that a solution contains. T_M is the melting temperature of a solution.

Above the curve of T_M, the solution is in a liquid state. T_G is the glass transition temperature. Under the T_G the liquid is transformed to a solid – glass like state. One of the physical properties of a liquid is that it can be transformed into glass if the temperature drops suddenly too much. For example when an amount of water, that is in 25^{o}C (room temperature), is instantly found below -120^{o}C, it will become solid glass. This is happening because the cooling rate is very high and the water molecules do not have time to be rearranged in the space that they take over. If the temperature of the liquid is dropping slowly, then the water molecules have time to be rearranged and they take over more space and therefore produce nucleus of ice crystals. So if the water is found in temperatures below T_M and above T_G it will yield ice crystals. T_M and T_G curves create a zone, the crystalline zone. The quicker the transition from T_M to T_G through the crystalline zone, the lesser changes there are so as ice crystals to be formed. Vitrification has been found to be more successful technique because reassures the main principal in order to avoid ice crystal formation inside the cells: extremely high cooling rates. While slow cooling procedure has a cooling rate of ~0.3°C/min, vitrification yields cooling rates around 20.000 °C/min.

According to figure 1, the use of cryoprotectants could minimize the range of the crystalline zone and could help the generation of the vitrified state with smaller cooling rates. When the concentration of cryoprotectants is higher, the generation of the vitrification state is easier. But, cryoprotectants in high concentrations are toxic for the cells; therefore there is a limit in their use.

Finally, the volume of the solution that is being cooled plays a significant role in

the formation of solid glass from water. The smaller the solution, the easier it gets to vitrify a solution.

Arav has proposed the following equation (27) that describes how the probability of yielding vitrification is affected by the above parameters:

$$P=\frac{(Coolingrates)x(Concentrationofcryoprotectants)}{Volumeofsolution}$$

According to this equation, vitrification is easier achieved when a small volume of a solution with the appropriate concentration of cryoprotectants is being cooled rapidly. The vitrification method is ensuring all these parameters and this is why finally has been proven to be more successful than the slow cooling technique.

1.4 History of oocyte cryopreservation

The first research concerning the effect of low temperatures on the survival of oocytes was performed in mammals. Chang in 1952(28) reported that there was a two-fold reduction in the developmental competence of mature rabbit oocytes after being held at 0°C for 24 hours. A few years later, similar studies were conducted in the mouse by Sherman and Lin (29,30) who demonstrated that mouse oocytes suspended in a medium containing glycerol and cooled to -10°C and held for one or two hours could be used to produce pregnancies in mated recipients. However, a few oocytes survived, which were then fertilized with the oocytes held at 0°C or -10°C for six hours or longer. After these milestone studies there was little progress until the late 1970s when the first live mice were produced from frozen, and thawed oocytes (31). These investigators used the same approach as with mouse embryo cryopreservation (12,32), specifically exposing

oocytes to 1.5 M DMSO followed by slow cooling to -80°C before freezing in liquid nitrogen. There have been subsequent efforts to improve survival rates. In 1978, Leibo and colleagues (16) performed direct microscopic observation of intracellular ice formation in matured mouse oocytes. They concluded that cell survival after freezing to -150°C in 1 M DMSO depended on the cooling rate and was negatively correlated with intracellular ice formation. Most importantly, survival was poor when oocytes were cooled faster than -1.5°C/min. This observation together with Mazur's mathematical model of the physiochemical process during cooling described earlier, led several other investigators to adopt slow cooling as the best method for cryopreserving oocytes from the human (33,34).

Regarding immature oocytes, the first offspring (in cattle) from a cryopeserved germinal vesicle (GV) oocyte (that was subsequently matured and fertilized in vitro) was produced in 1992 (35). Likewise, the first human baby born after a similar procedure was reported six years later (36).

While slow cooling had been used somewhat (30-70%) successfully to freeze human oocytes (37,38), poor results were reported for other species including pig, cattle, sheep, and horses(39). As a consequence, causes of damage and poor development of cryopreserved oocytes had become the focus of several studies conducted in the following decade (40).

These studies pointed out that mammalian oocytes have several unique features that contribute to extreme susceptibility to damage during cryopreservation procedure. As a result of these studies, a vitrification method,applied in 1985 in mouse embryos (21),

was adopted in late 1980s for the cryopreservation of mouse, rat, hamster, rabbit, human, cow, and pig oocytes (40). Although some progress was made in the cryopreservation of mouse oocytes (24), very little improvement was achieved in humans (41). Until that point, it was well recognized that mammalian oocytes were sensitive to cooling and the degree of chilling sensitivity was directly correlated with the survival after freezing and thawing. In the early 1990s, Steponkus (42) and Mazur (43) reported that Drosophila embryos, a cell type known to be highly susceptible to chilling injury, could be successfully cryopeservedby the use of an extremely rapid cooling rate. The results of these investigations led Martino et al. (44) to pioneer an ultra-rapid cooling method for bovine oocytes. In that study, oocytes contained in minimal volume of a concentrated cryoprotectans solution were placed on electron microscope grids and plunged directly into liquid nitrogen. Using this approach, the cooling rate was estimated to be more than -100,000°C/min, which was 40 times faster than conventional vitrification (i.e., cooling in plastic straws). This extreme cooling rate appeared to be critical for the success of bovine oocyte cryopreservation, because exposure of bovine oocytes to 0°C for only five seconds significantly reduced developmental competence (42% vs. 20% blastocysts development). Ultra-rapid cooling permits the oocytes to bypass the critical temperature zone (between 0°C and 15°C) so rapidly that there is no time for chilling injury. This improved survival and function of cryopeserved oocytes has led other investigators to derive alternative cooling devices, including open pulled straws (45), solid- surface vitrification (46), micro drops (47), closed pulled straws (48), cryoloop (49), nylon mesh (50), multi-thermal gradient (51), and cryotop (52), all of which permit cooling rates exceeding -100,000°C/min. Since then, this techniques which is generally referred to as “minimum

volume vitrification" (MVV) has been widely adopted to preserve oocytes in several species, including the human (53,54), and it has been recently suggested that MVV is the method of choice for oocyte and embryo cryopreservation (55,56). Quantification of the metabolome and proteome of the oocyte has revealed that slow freezing has a dramatic effect on cell physiology, whereas vitrification appears to have limited detrimental effect (55). This is possibly achieved by the limited exposure to cryoprotectans. Analyses of meiotic spindle dynamics and embryo development following IVF indicate that vitrification is less traumatic than slow freezing, and therefore, has the greatest potential for successful oocyte cryopreservation (55). Despite these quantitative differences in in vitro assessments, there remain no differences in overall implantation or live birth success in humans after transferring embryos produced by slow cooling versus vitrification. Novel approaches that were evaluated to improve oocytecryopreservation success include intracellular injection of trehalose (57), utilizing choline-substituted medium to reduce the potentially harmful buildup of sodium (58), and treatment with cholesterol-loaded methyl-beta cyclodextrin to stabilize the plasma membrane (59). Basic investigations into the cryosensitivity of mature human oocytes (60) conducted in the 1990s have paid dividends in the birth of several healthy children (53,54). The advent of intracytoplasmic sperm injection (ICSI) to fertilize thawed human oocytes—a process that circumvents zona hardening (preventing sperm penetration) caused by premature release of cortical granules— has significantly contributed to the improved outcomes of human oocyte cryopreservation (61). In the mid-2000s there were 200 healthy children born worldwide with the use of cryopeserved immature or mature oocytes (62–64). As a consequence to these reports, oocyte cryopreservation has emerged as one of the

feasible options for fertility preservation in women in the 21st century. However, the room for improving the technique was huge and that is why oocyte cryopreservation was considered, until recently, as an experimental technique.

Over five decades of investigation, it was learned that it is far more difficult to cryopreserve oocytes at the metaphase II (MII) stage than the embryos. The main reasons were first identified and described in the late 1990s. First, the oocyte is actually the largest mammalian cell type because of which it has a relatively lower surface area to volume ratio compared with an embryo (17,40,65). As a consequence, oocytes have low permeability to water and cryoprotectans and are highly susceptible to osmotic damage (17,40). To make it even more complicated, it has been demonstrated that water permeability of mouse oocytes differs among mouse strains (66), indicating the significant role of genotype in dictating oocyte cryosurvival. Second, oocytes are extremely susceptible to chilling injury (67). Cooling MII oocytes to room temperature or below induces irreversible disruption of the meiotic spindle and dispersal of chromosomes (67). Exposure of oocytes to low temperature subtly alters membrane structure and function, caused by thermotropic phase transition and lateral phase separation of membrane lipid components (40). Furthermore, cooling also induces premature exocytosis of the cortical granule, which in turn causes zona hardening (40). Third, the exposure of oocytes to conventional permeating cryoprotectans, such as propanediol, ethylene glycol, and DMSO, disrupts cytoskeletal structures and increases intracellular calcium, which in turn initiates oocyte activation and zona hardening (40,68). Thus, it is perceived that cryopreservation of oocytes faces several challenges in order to be successful.

1.5 Cryopreservation in Assisted Reproduction Technology

Cryopreservation is of crucial, and increasing, importance in reproductive medicine. Since the mid-1980's, cryopreservation and storage of cleavage stage human embryos, deriving from assisted reproduction techniques, has been used in order to avoid simultaneous transfer of multiple embryos without discarding this valuable human biological material . In the past decade, this approach has helped to drive practice towards single embryo transfer and consequently to reduce dramatically the multiple pregnancy rates in many countries. At that period of time, the relatively clinical success of cryopreserved embryos raised a challenge for embryologists and cryobiologists. The challenge was, not only to yield the most sufficient method for cryopreserving embryos at different stages (2PN, cleavage stage, blastocyst stage), but also to evolve a method for successfully cryopreserving human oocytes. This need was driven by a desire to preserve fertility in young women with cancer that would undergo gonadotoxic treatments and also by restrictive legislation which, in some countries, forbidden embryo cryopreservation. Finally, the last few years, reproductive scientist has realized that oocyte cryopreservation could be a procedure - key for success in oocyte donation treatments. The formation of banks with cryopreserved oocytes that would give physicians, embryologists and patients freedom and material to work with, was a scenario - utopia for several years. The reason was that cryopreservation of metaphase II (MII) oocytes was widely viewed as inefficient methodology. Soon, it was perceived that a methodology that would improve the sufficiency of frozen MII oocytes was a necessary prerequisite for progress in this ART and fertility preservation area. The introduction of vitrification as the principal method for cryopreserving oocytes has totally changed the game by improving dramatically the clinical results.

1.5.1 Vitrification in ART

Vitrification is the physical phenomenon that takes place when the solidification of a solution occurs not by ice crystallization but by extreme elevation in viscosity, by which a vitreous consistency is achieved, very similar to that of glass (69). Vitrification is a method of cell and tissue cryopreservation.

Vitrification was firstly described in 1938 (70) when they reported the successful cryopreservation of frog's spermatozoa. In mammals, the first successful cryopreservation procedure was achieved at 1949 with the slow freezing of spermatozoa using glycerol as cryoprotectant (71). Vitrification was applied to embryology forty years later in mouse embryos (21). Oocytes were successfully vitrified in 1991 (72). Interestingly, its application in assisted reproductive technologies has been limited until recently. For many years, slow cooling was the primary option for cryopreserving oocytes but with limited success (73,74). Due to these results, oocyte cryopreservation was considered as an experimental technique and it was used in emergency cases where fertility of women had to be preserved, like cancer patients who suffer the chronic adverse effects of their treatment, including gonadal failure and infertility. Recently, vitrification has been proven to be a very useful tool, giving outstanding results in terms of survival and clinical outcome, on the contrary to slow-cooling protocols (75,56,76).

1.5.2 Application of vitrification in ART

1.5.2.1 Fertility preservation

Fertility preservation in women with cancer in their reproductive age is an aspect with an increase interest 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. Leukemia, lymphomas, cancer of the central nervous system, breast and cervical cancer are the most common forms of cancer. Nowadays, young patients with cancer have very good prognosis to survive the disease due to early diagnosis and improvement of the available treatments. However, due to chemotherapy and/or radiotherapy the patients often face the probability of irreversible 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 premature ovarian insufficiency (POI) in 97% of all reported cases (77). Administration of chemotherapy might cause loss of follicles within the ovary, lead to hormone deficiency and infertility (78).

Besides the possibility to cryopreserve embryos in order to preserve fertility in these patients, which is only applicable for couples, three main options exist. The first option is ovarian cortex ablation and cryopreservation with subsequent autografting which is the only strategy for pre-pubertal girls and is still considered as an experimental approach with limited results. Another option is oocyte vitrification after minimal invasive ovarian stimulation, which 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. The third option is to combine these two techniques.

Besides cancer, there are more diseases that include treatment with chemo- or radiotherapy. In autoimmune diseases, such as systemic autoimmune rheumatic diseases and in haematological diseases, cyclophosphamide therapy is required (79). Additionally, there are non-oncological medical indications for egg freezing like 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(80). Genetic predispositions leading to risk of fertility loss at young age is another indication as reported for Turner syndrome mosaicism (81).

In the daily IVF routine, oocyte cryopreservation helps couples where no semen can be retrieved at the day of pickup due to medical or psychological reasons. In the past, the oocytes harvested after pick-up should be discarded after about 8 hours due to the aging process. Now they can be vitrified, warmed 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(82).

1.5.2.2 Oocyte Vitrification for Ethical and Social Reasons

There are couples with moral or religious objections about cryopreservation of embryos. In addition, some countries have law restrictions regarding the cryopreservation of embryos. At the same time the same countries allow the cryopreservation of surplus oocytes that result from ovarian stimulation. (83). A very young application of oocyte cryopreservation is the so called “social freezing” (84). For many women it is not cancer but the passage of time (aging) that denies them a chance

of motherhood. There are social, educational, and financial pressures that force them to delay forming a family until their late 30's. In this age their chance for a pregnancy is limited 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. Cryopreserving their eggs at young ages, women get the chance to postpone childbirth without taking risks of a negative impact on oocyte quality due to aging process.

1.5.2.3 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 premenopausal 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's and recipient's 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. With oocyte cryopreservation the need for donor/recipient synchronization is eliminated. Egg banks

can be established facilitating the logistics of coordinating egg donations (85). Temporary quarantine of donor eggs to test the donors for transmissible diseases becomes possible with application of freezing.

1.6 Vitrification – The technique

As mentioned above, the intracellular crystallization 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(86).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 guarantee an amorphous state throughout the cooling as well as during the warming process.

1.6.1 Vitrification - Methodology

Vitrification/warming procedure of oocytes consists of 5 steps:

a. Exposure of gametes to the cryoprotectant solutions (CPs)

b. Loading on the carrier and plunging in LN2

c. Storage in LN2 containers

d. Extraction of the carrier from the protective straw and warming of the cells

e. Dilution of the intracellular cryoprotectant (CP) concentration

a. Exposure to Cryoprotectant Solutions

The high concentrations of CPs and extremely fast cooling/ warming rates are two conditions that support the formation of a glass-like state. Independently 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 (75). This means that the success of vitrification 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, changes 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.

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, almost all vitrification methods consist of exposing the biological material in a minimal of two steps to gradual increasing concentrations of (NVS) non-vitrifcation and vitrification solutions (VS). The NVS is exclusively composed of permeable CP. In contrast, in the VS additionally non-permeable CPs with low and high molecular weight exists.

The oocytes are firstly exposed to one or more NVS. During this step, a certain amount of CPs 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 (17,87,88).

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.

b. Loading on a Carrier device, plunging in LN2.

After their exposure to cryoprotectants, oocytes must be placed to a device that will hold them during cooling and during storage in the LIN2 tanks. There are two types of devices for vitrification of oocytes: open and closed devices.

Open devices are consisted of a carrier in which the material is being loaded. As it, the carrier is being plunged in LN2 and the oocytes are in direct contact with it. This direct contact with the LN2 yields cooling rates of a high range (> 20.000 ^{o}C) which is a fundamental precondition in order to achieve vitrification.

Closed devices, like the open ones, are consisted of a carrier that will hold the oocytes and in addition they have an external part which will enclose the carrier and will be hermetically closed. The external part is usually a high security straw. Then the closed device (carrier and straw) that contains the oocytes will be plunged to LN2. In this way oocytes are not in contact with LN2 during the cooling procedure. For many scientists this kind of isolation that the closed system provides to the biological material is of great importance. On the other hand, the cooling rates that are being generated in closed system devices are significantly lower (around 2.000 ^{o}C) and this fact brings a great skepticism to scientists in order to use this type of devices.

The loading of the oocytes to the carriers is being performed by the means of a small volume of VS solution that contains the material. As seen above, the volume of the vitrification plays an important role to the possibility of generating vitrification. The smaller the volume the easier the formation of a vitreous state.

c. **Storage of oocytes in LN2**

As mentioned above, the oocytes, during the cooling procedure are being held in carrier devices. After they are plunged in to LIN2, these carriers are being transferred in large containers that are full with LIN2. Oocytes can be stored in these containers from a few months to several years, as long as those containers are being filled with LIN2 periodically. Oocytes that were vitrified using an open system vitrification will be constantly in touch with LIN2 during their storage period. On the other hand, oocytes that were vitrified using a closed system, will be constantly isolated from the LIN2 of the container that includes them.

d. Warming procedure

Cooling rate has always been considered as the most critical condition for a generating a successful vitrification procedure. It is surprising how little attention has been given to the warming procedure. However, it has become obvious that the warming rate might play a more essential role in modulating survival rate after vitrification than the cooling rate.

A high warming rate prevents the vitreous water from recrystallizing during the warming phase (89,90). 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 a recrystallization that may have lethal consequences.

It is well known that for any given concentration of cryoprotectants the critical warming rates are much higher than the critical cooling rates (91). 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, in order not to increase the concentration of CP too much, the biological material has to be warmed very rapidly.

Practically, in order to achieve these warming rates, a dewar of LN2 containing the devices is placed close to a stereomicroscope. Then the carrier with the oocytes is being transferred as soon as possible from the LIN2 to a dish with an appropriate volume (at least 0,5ml) of warming solution. In order to achieve even higher warming rates, the warming solution is being pre-warmed in 37^{o}C.

e. 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.

1.7 Open and Closed Vitrification Devices

There are the two categories of vitrification devices and they are easily distinguishable. Open systems allow while closed systems eliminate the direct contact between the sample-containing medium andliquid nitrogen.

As stated before, the principle of vitrification in cryobiology is to eliminate the chance of ice formation in the medium that contains the sample during cooling, storage and warming (21). This can be achieved either by increased cooling and warming rates or by increased concentration of cryoprotectants. Practically, both approaches are applied. The higher the cryoprotectant concentration the lower the cooling rate required and vice versa. However, since highly concentrated cryoprotectants may cause toxic and osmotic injury, the preferred strategy is to use the highest possible cooling and warming rates and

subsequently to apply the lowest concentration of cryoprotectants that ensures safe ice-free solidification under these circumstances (65,75,83).

Therefore the easiest way to achieve high cooling and warming rates is to use the smallest solution volume and to expose this solution directly to liquid nitrogen (Arav, 1992). This direct exposure with LIN2 is what distinguishes open from closed system vitrification and yields a huge difference in the cooling rate that is achieved in open vitrification protocols (> 25.000°C/ min) comparing to closed vitrification protocols (< 2.000°C/min).

This reduction in the cooling rate is responsible for a still ongoing debate - especially for the vitrification of oocytes - as the cooling rate is widely believed to be an important factor for success of the freezing protocols and that it why is still believed that open systems should be used for sensitive biological materials such as MII oocytes (92). 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

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 (93,94). 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 (95) or UV-light simultaneously with hermetical cryo-storage (96) 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 (97). 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 (98). 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. 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 in the standard storage conditions or in therefilling of the tanks and whenever straws are added or withdrawn poses a hazard when oxygen from surrounding air condenses and mixes with LN2. 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 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 (98). 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 (99). In view of this debate, a hermetically closed system, which solely guarantees optimal storage conditions, was recommended (100). 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) (101) as well as with FDA directives on tissues and cells storage (102) 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 valuable option consists to switch from an open vitrification 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

1.8 Perinatal, neonatal and epigenetic outcome

Oocytes not only contribute genetic information for the developing embryos, but also provide them with energy, nutrients and a mitochondrial genome. Altered expression of the genetic information could be caused by disturbances in DNA, protein-histones,

cytoskeleton system, DNA repair system and systems that regulate gene expression (imprinting, transcription apparatus, etc.). The eggs of most species are giant cells, containing sets of all the materials needed for initial development of the embryo until they reach the stage of implantation in mammals or self-feeding in others. Eggs are typically spherical or ovoid, with a diameter of 100 mm in humans and sea urchins. A typical somatic cell, in contrast, has a diameter of only 10 or 20 mm. The egg typically contains nutritional reserves in the form of yolk, which is rich in lipids, proteins, polysaccharides, cortical granules, mitochondria, spindles and microtubules and microfilaments. The higher content of lipids is relevant for cryopreservation, since it could affect the efficiency of this process (103). Another distinctive characteristic of the egg is its coat or shell, which is a specialized extracellular matrix consisting largely of glycoprotein molecules, some secreted by the egg and others by surrounding cells. The volume of the highly hydrated nucleus is also much bigger in oocytes than in somatic cells. These peculiarities of oocytes make them different from other cells and have created problems in applied cryobiology. A few years ago, the efficiency of oocyte cryopreservation remained low across most species; the reported pregnancy rate per thawed oocyte after slow freezing in humans up to 2005 was 2.3% (104). The introduction of vitrification has given more promising results with a 96.9% survival rate and 65.2% pregnancy rate per embryo transfer reported (76) and more reassuring benchmarks and key performance indicators quoted generally (105). The advances in cryobiology became so pronounced that in some centres, authors claim that vitrified oocytes are as good as fresh. The controlled randomized clinical trial in a single centre failed to demonstrate the superiority of using fresh oocytes compared with vitrified

oocytes in terms of ongoing pregnancy rate in donor cycles (106). The number of births derived from vitrified human oocytes was quoted as 800 in 2009 (104) since the first report. This number is certainly likely to be much higher in 2014, with a single centre reporting the birth of at least 1027 babies (107); however, no papers could be found reporting the worldwide estimate of children born to date.

PART 2 - Study conception and design

2.1 Study conception and design

Cryopreservation of oocytes has been considered as an experimental procedure until recently (108). When the scientific question of this thesis was conceived, cryopreservation of oocytes was far away for being characterized as a routine technique in IVF labs. On the contrary, the performance of oocyte cryopreservation was used only in emergency cases, when no other option was available. For instance, in women with cancer that had to follow emergency therapeutic treatments (e.g. chemotherapy, radiation), the only available strategy for preserving their fertility was to freeze their oocytes. A second example of an emergency case that leads to oocyte cryopreservation is the unpredicted lack of sperm sample the day of the oocyte collection in IVF cases. When the sperm sample is not available, the only way to preserve oocytes is to cryopreserve them. Finally, there are countries that their legal or moral background does not allow the formation of many embryos during an oocyte stimulation IVF cycle. Therefore, after the egg collection, the IVF laboratory proceeds to fertilization of maximum of three oocytes and the rest can be cryopreserved. As mentioned above, the clinical results after oocyte cryopreservation were very low. At that period, an emergency oocyte cryopreservation was almost considered as a catastrophic end up of a clinical case.

The main reason for these poor embryological and clinical results was the fact that slow freezing was the preferred technique for cryopreserving oocytes. Cryopreservation of unfertilized oocytes was firstly reported in the late1980s 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 25years (109) despite the fact that the clinical results remained very low. The introduction of vitrification as a very successful technique for

cryopreserving human embryos (56) enhanced our knowledge on this technique even in a clinical level. The successful outcome after embryo vitrification and warming of embryos challenged scientists to test the efficiency of the technique in human oocytes. In 2005 and 2007 there were some reports with acceptable biological and clinical outcome using e vitrified human oocytes (52,56). However, cryopreserving oocytes was not considered a safe technique because of the limitation that has the oocyte due to their low surface to volume ratio, fact that makes the survival of this cell very difficult task to achieve.

The vitrification procedure that was used in the first clinical trials for cryopreserving human oocytes was always the open system technique. As mentioned above, in this method the biological material is in direct contact with LIN2. This direct contact with liquid nitrogen ensures the fundamental principal for achieving successful vitrification: extremely high cooling rates. Therefore, it is easily understood that the high cooling rates is the most important feature for cryopreserving human cells and embryos without harming them. The high cooling rate is the reason why vitrification is more successful technique than slow freezing in cryopreserving human biological material.

However, the past decade, there has been skepticism in using open system vitrification for human biological material. It has been reported that the transmission of cross contamination from one sample to another under the liquid nitrogen is a possibility (93). Although this possibility is debatable, the European council released a guideline which dictates the use of the safest method for the cryopreservation and storage of human biological material (101). This directive led scientists to introduce closed systems for human oocyte and embryo vitrification. Nonetheless, the use of closed systems for

vitrification is not the preferred option in the IVF laboratories. Due to thermo insulation, the cooling rate in closed system (2000^{o}C) is significantly lower than the one in open system (>20.000^{o}C). Based on this fact, the closed system compared to open system is considered as a less efficient technique for cryopreserving human embryos and even less appropriate for human oocytes which generally are more difficult to be vitrified.

Although a lot of attention has been paid to the freezing procedure the warming procedure was overlooked. It was in 2008 and 2009 that the importance of the warming rates as more significant of the cooling rates has been reported by two important cryobiologists (89,90). Their work has established vitrification as successful even by using lower cooling rates, as long as the warming rates are high. This means that the cooling rates that are achieved by closed system are high enough in order to induce the desirable vitrification state and at the same time to avoid the formation of harmful ice crystals inside the cells.

The primary aim of our work was to compare: a) open vs closed (aseptic) vitrification system as well as b) fresh vs vitrified oocytes using closed system (aseptic) regarding their biological and clinical results.. The wider implication of the study was to give an answer in a still ongoing debate: Can we use safely, successfully and in routine basis closed system in order to vitrify human oocytes in an aseptic way?

For the purposes of this thesis:

A. We set up a set of experiments in order to analyze:

- The concentration of the cryoprotectants that are needed in order to protect the oocytes during vitrification and to avoid toxicity

- The time and the way of exposure in each cryoprotectant in order to establish the right protocol for closed system vitrification.

B. Then we set up a clinical trial in order to compare open vs closed vitrification system for oocytes vitrification.

C. We compared the results of closed vitrified oocytes with the ones of fresh oocytes and after critical evaluation we decided to change the warming protocol

D. We set up a second set of experiments in order to determine the best protocol for warming and rehydration of the oocytes

E. We set up a clinical trial in which we compared in a direct way closed vitrified and fresh oocytes

F. We set up a clinical trial in order to evaluate if the use of laser hatching before transfer of embryos deriving from closed vitrified oocytes can improve the clinical result

PART 3 – Material, methods and results

3.1 Oocyte pick up

All the experiments and the clinical trials took place between May 2011 and May 2015 in IAKENTRO fertility center in Thessaloniki. The oocytes that were used for our scientific purposes derived from the oocyte donation program of the clinic. The advantage of using donated oocytes from a well established oocyte donation program was that this biological material was of top quality since they were retrieved from young, healthy donors of proven fertility. Thus, every result we had was of high importance since we reassured the good oocyte quality and eliminated any bias factor that would compromise our findings.

In order to determine the appropriate protocol for oocyte cryopreservation, we applied different concentrations and exposure times of specific cryoprotectants in donated oocytes which were immature 2h after the oocyte pick up and therefore were not applicable for use in the oocyte donation program. These oocytes were left 12 to 24 hours in order to be matured in vitro and then were used as an experimental material. Once the vitrification/warming protocol was formulated, it was used in oocyte donation cases in which oocyte cryopreservation was applicable. Prospective clinical trials were set up in order to answer our scientific question. For the purposes of these trials we used the oocytes that were matured 2h after their retrieval. The oocytes were vitrified and then warmed and donated to a matching recipient.

3.2 Donors' characteristics

A detailed medical history of the donors was taken. The oocyte donors were ≤32 years old, had body mass index <30 kg/m2, regular menstrual cycles of 25–35 days, two

normal ovaries based on transvaginal scan findings, no polycystic ovary syndrome, not known endometriosis, no gynecological or medical disorders and agreed to donate their oocytes for treatment anonymously and altruistically. Oocyte donors were of known fertility and good ovarian response. Blood sample was collected for karyotyping and screening for previous viral infections (hepatitis B and C, human immunodeficiency virus, syphilis) thalassemia and cystic fibrosis.

3.3 Recipients' characteristics

The recipients and their partners underwent blood screening similar to the donors, while a hysterosalpingogram and a diagnostic hysteroscopy eliminated cases presenting hydrosalpinx or intrauterine related pathology. The recipients went through a mock transfer in a cycle previous to their donation cycle and if difficulty was encountered a cervical dilatation was performed (110) In both groups, the recipient couples did not suffer from any form of severe male infertility indication. All the enrolled cases had normal values for concentration, motility and morphology. The WHO criteria (111) were used to evaluate the sperm quality.

3.4 Donors' Ovarian stimulation

The ovarian stimulation of the donors was performed with a fixed 6^{th}day GnRH antagonist protocol. The stimulation was starting with a daily administration of 225 IU of recombinant Follicle Stimulation Hormone on the second day of the cycle. Cycles were monitored using vaginal ultrasound scanning and serum estradiol (E2) levels, beginning the second day of menstruation and repeated after 3 days of stimulation. The daily dose

of the rFSH was adjusted according to the donor's ovarian response based on serum estradiol levels in combination with the number and size of ovarian follicles as measured by transvaginal ultrasonography. To prevent an early LH surge, the protocol we used included the daily administration of GnRH antagonist (Orgalutran 0.25 mg; NV Organon) starting on the afternoon of the 6th day of stimulation. When there was a monitoring of 3 or more leading follicles with diameters >17mm and the same time the serum estradiol levels were >1500pg/ml, then 10.000 IU of human Chorinonic Gonadotrophin (hCG – Pregnyl; NV Organon) was performed. The donors who were at risk of hyperstimulation were administered twice (morning and evening) with antagonist the day before the administration of hCG (112). There was no administration of GnRH antagonist the day of the hCG administration.

3.5 Recipients' endometrial preparation

Women with ovarian function were first down-regulated in the luteal phase with a single-dose of GnRH-agonist depot (Arvekap 3,75mg) beginning on the 21st day of the previous cycle. One day after the announcement of the donor's period onset, the recipients were informed to start estradiol valerate (Cyclacur or Progynova,) 2 mg per day for the first 4 days, 4 mg per day for days 5–8 and 6 mg per day until the pregnancy test. The afternoon of the oocyte donation pickup, recipients were administered with 200mg progesterone (Utrogestan) intravaginally and continued with 200 mg, three times a day, until fetal heart beat was observed by ultrasound. The recipients without menstruation followed the same protocol without GnRH agonist. Endometrial development was evaluated by ultrasound scan and it was considered mature when the endometrial thickness was >8 mm. Recipients that were allocated with vitrified/warmed oocytes followed the same

treatment as fresh group recipients. Their preparation started with GnRH agonist on the 21st day of the previous cycle. After confirming down-regulation, by measuring serum oestradiol and progesterone concentrations, estrogens were administered as mentioned above. Endometrial development was evaluated by ultrasound scan and it was considered mature when the endometrial thickness was >9 mm. When the endometrium was < 9mm, the transfer cycle was canceled. In vitrified oocyte group, when endometrium was lower than 9mm, the warming of the oocytes was not performed and the cycle was postponed for the future (new endometrial preparation).

3.6 Oocyte pick up, fertilization and embryo assessment

The oocytes retrieved from each donor were equilibrated in fertilization media (Sage Inc., A Cooper Surgical CompanyTM; Bedminster, NJ, USA) for 2 h and then enzymatically denuded (Sage Inc., A Cooper Surgical CompanyTM). Metaphase II (MII) oocytes were evaluated and selected under a stereomicroscope. Those with dark cytoplasm, a centrally located granular area, vacuoles or a large polar body were excluded from the study.

On the day of the fertilization, a fresh sample was obtained from the male partners and specimens were processed. Intracytoplasmic sperm injection (ICSI) was performed on all cases. Fertilization was assessed 16-20h post ICSI by visualization of the two pronuclei. Embryos were cultured in 30 µl droplets of culture medium, overlaid with mineral oil (Fertipro, Bernem, Belgium) in a MINC™ benchtop incubator (Cook Medical) with provided triple gas of 6% CO2, 5% O2 and 89% N2. Embryo quality was assessed on Day-3 and on Day 5. Embryo quality was assessed on Day-3. For day 3 embryos, number

of cells, the appearance of blastomeres and the presence of cytoplasm defects or fragmentation were evaluated (113). According to this system, top quality cleaved embryos were considered those with 8 -12 symmetric blastomeres with absence of cytoplasm defects and without or with negligible fragmentation. For the evaluation of day 5 embryos we used the Gardner and Schoolcraft criteria (114) in which the thin zona pellucid (ZP), smooth trophoectoderm, equality and close adhesion of blastomeres, clearly visible blastocyst cavity and the well developed inner cell mass with many closely aggregated cells are the most important parameters correlating to the top blastocyst quality. Embryo transfers were performed on day 5, under ultrasound guidance as previously described (115) The remaining embryos were vitrified on day 5 using closed vitrification system.

3.7 Outcome measures definition

The definitions used for the outcomes of our clinical studies were adopted form the revised glossary that was proposed by International Committee for Monitoring the Assisted Reproduction Technology (ICMART) and the World Health Organization (WHO) in 2009 (116).

Pregnancy rate: The rise of serum b-HCG concentrations, 14 days after embryo transfer expressed per 100 embryo transfers.

Clinical pregnancy rate: the number of clinical pregnancies (the appearance of a gestational sac and visualization of fetal heart beat 7 weeks after the transfer) expressed per 100 embryo transfer cycles.

Ongoing pregnancy rate: the number of pregnancies with fetuses displaying heart activity beyond 12 weeks of gestation, expressed per 100 embryo transfers.

Biochemical pregnancy rate (preclinical spontaneous abortion/ miscarriage): a pregnancy diagnosed only by the detection of HCG in serum or urine and that does not develop into a clinical pregnancy expresses per 100 embryo transfer cycles.

Delivery rate: the number of deliveries expressed per 100 embryo transfer cycles. It includes deliveries that resulted in the birth of one or more live babies and/or stillborn babies. *Note: The delivery of a singleton, twin or other multiple pregnancy is registered as one delivery.*

Live birth delivery rate: the number of deliveries that resulted in at least one live born baby expressed per 100 initiated embryo transfer cycles.

Implantation rate: the number of gestational sacs observed, divided by the number of embryos transferred.

Miscarriage rate: was considered the loss of clinical pregnancies before 20 completed weeks of gestational age, expressed per 100 embryo transfers.

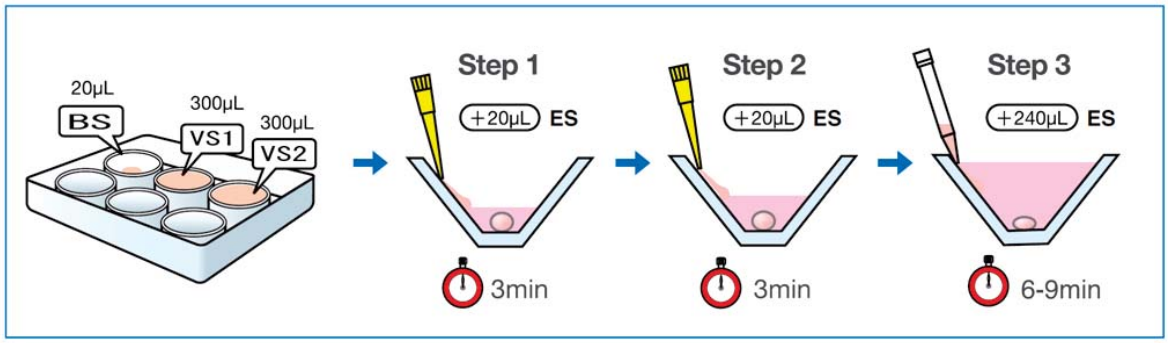
3.8 Vitrification Protocols

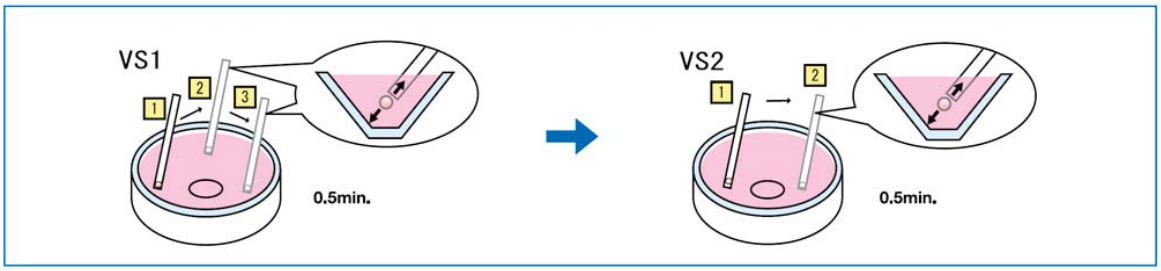
3.8.1 Open system vitrification

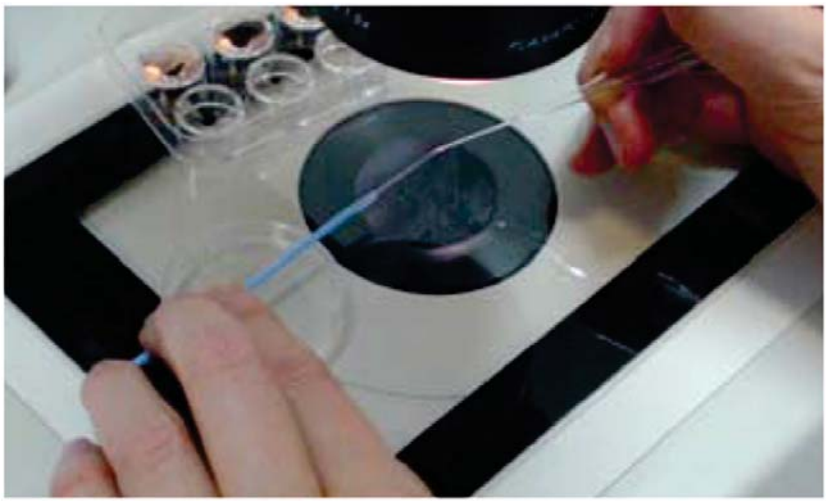
The open vitrification/warming protocol that was used for our studies was firstly proposed by Kuwayama (52) and since 2007 (56) it has been the protocol of the Kitazato kit for open system vitrification. This protocol has gained enormous popularity and many clinics in the world are using it (92). The cryoprotectants that are being used in this protocol are DMSO and EG (52).

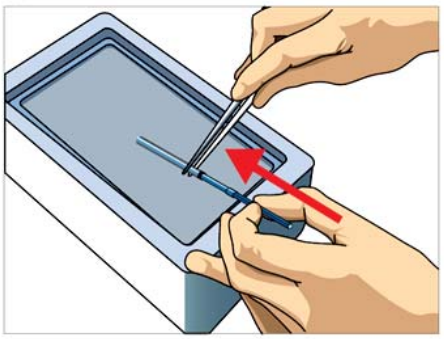
The open vitrification procedure has two phases. Equilibration phase and Vitrification phase. In the first phase Equilibration Solution (ES) is being used. The ES solution of the open system protocol contains 7.5% of DMSO and 7.5% of EG diluted in a basic Hepes Medium (BS). In the second phase, Vitrification solution (VS) is being used. The VS solution of the open system protocol contains 15% of DMSO and 15% of EG diluted in (BS).

During equilibration phase (figure 3), oocytes were initially placed in 20μl of basic hepes medium that does not contain cryoprotectants. Then, 20μl of equilibration solution (ES) were added and we waited for 3 min. Another 20μl of ES were added and we waited for another 3 min. Finally 240μl of ES were mixed with the previous solution and we waited for 9 min. Thus, the total duration of the equilibration phase was 15 min (table 1). When the ES step was over, the oocytes were placed in a drop with 300μl of VS for around 40 seconds. Then, within 20 seconds the oocytes were placed in the tip of a carrier called Cryotop (figure 3), and then the carrier was plunged in liquid nitrogen. Thus, the total duration of vitrification phase was 1 min (table 1). Under the liquid nitrogen, the carrier was inserted in a protective cap and then the whole device was stored in tanks with LIN2.

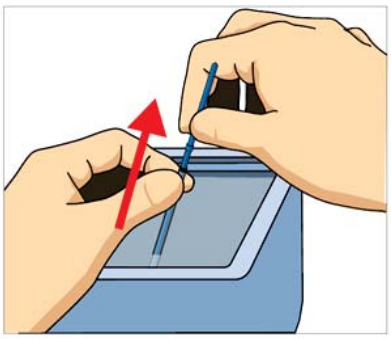




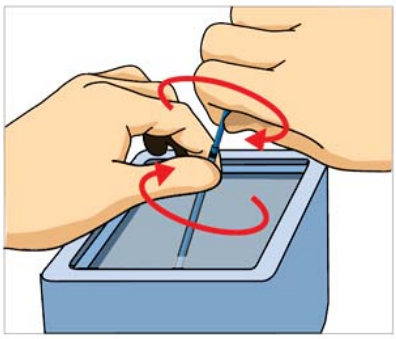




Hold the straw cap with tweezers and insert the Cryotop into it.



Hold the straw cap with fingers and fit it.



Twist it and make sure if the straw cap fits tightly to the Cryotop.

Figure 3: Open vitrification protocol, adapted from Kitazato kit (Dibimed)

VITRIFICATION					
Solution type			ES1	ES2	ES3	VS
Time of Exposure			3min	3 min	9 min	1min
WARMING						
Solution Type			TS	DS	WS1	WS2
Time of Exposure	1 min	3min	5min	1min		

Table 1: Protocol for vitrification and warming using open vitrification devices. All procedures are performed in room temperature except the TS stage which is performed in 37^{o}

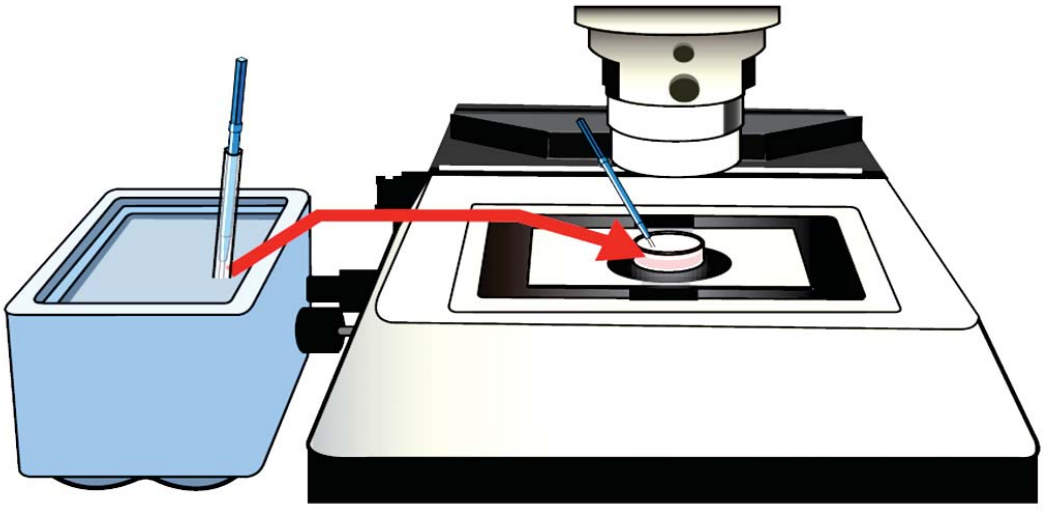


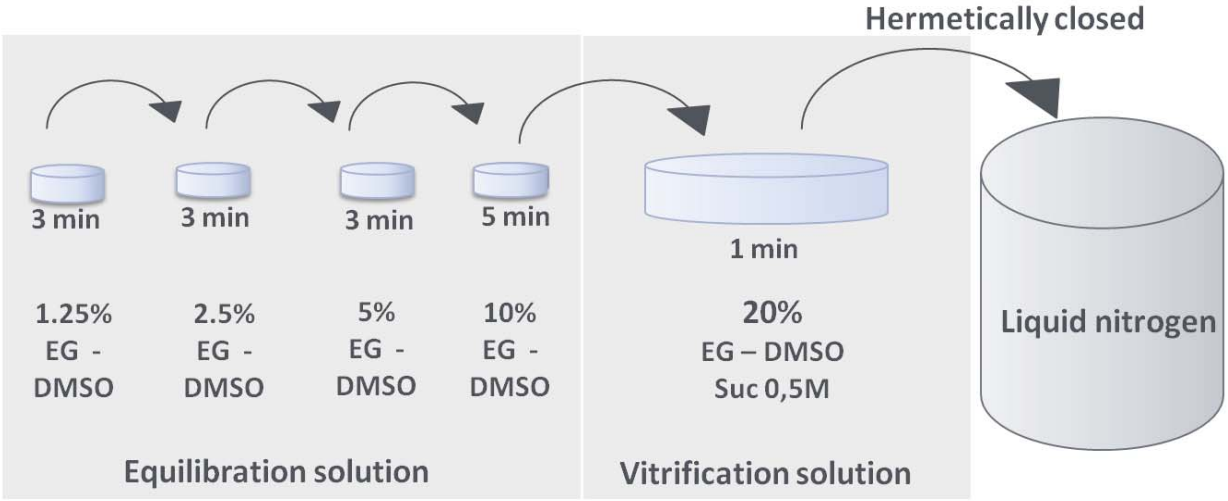
Figure 4: Warming protocol

The warming procedure started with the positioning of the Cryotop in a dewar full with LN2 (Figure 4). The cap of the device was held with a forceps and the carrier was pulled out and then directly plunged in a dish containing 1 ml TS which was previously incubated in 37^{o}C (Figure 4). The oocytes, which were in the tip of the carrier, were expected to be floating in the TS solution. After 1 min in the TS the oocytes were moved to a 300μl drop of DS solution and were kept there for 3 min. Then they were placed for 5 min in WS1 and finally in WS2 medium for 1 min (table 1). When the warming protocol was over, the oocytes were placed in fertilization media until they were inseminated.

3.8.2 Closed system Vitrification

The closed system vitrification protocol that we used was a modification of the protocol that was described by Vanderzwalmen in 2009 who used it for aseptic vitrification of blastocysts (100). Below, in experimental part 1, it is described the methodology used for the configuration of the oocyte closed vitrification protocol.

Likewise to open vitrification protocol, this protocol contains DMSO and EG and has 2 phases: Equilibration and Vitrification phase (Figure 5). The difference between them is that closed vitrification protocol has an equilibration phase with more steps (4 instead of 2). Therefore in the first phase there are four solutions, Cooling 1, 2, 3 and 4, containing respectively 1.25%, 2.5%, 5% and 10% of each cryoprotectant. In the second phase, the solution used – Cooling 5- was consisted of 20% of each cryoprotectant.



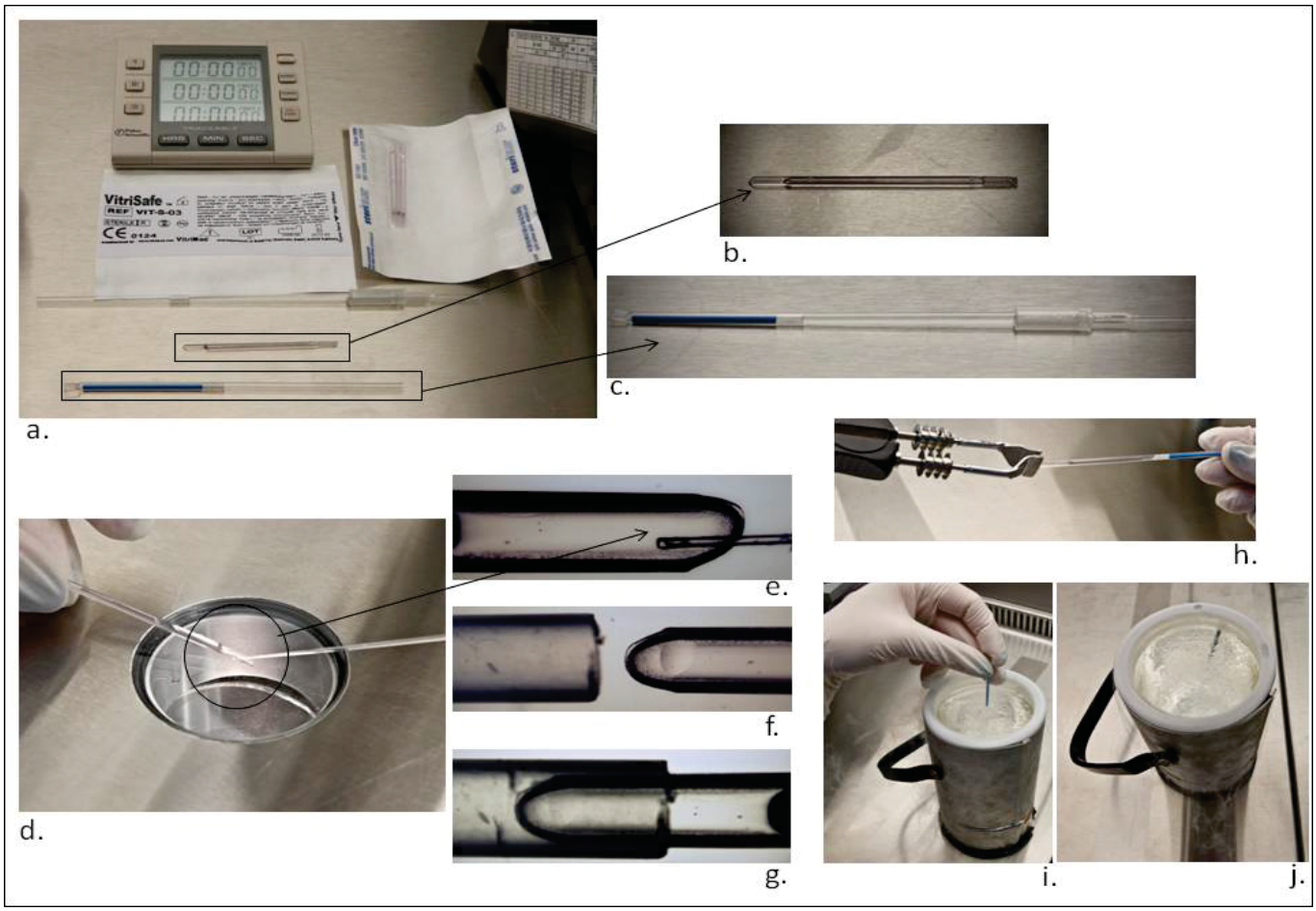


Figure 5: CP exposure protocol and Vitrisafe device. The vitrisafe is consisted of two parts: a carrier called Vitrisafe (a, b) and a high security straw 0.3m (a,c). The carrier has to be inserted in the high security which has a protective action (d, e, f, g) and then to be thermo-sealed (h) and plunged (I, j) into a dewar with LIN2

The MII oocytes were placed for 2-3 min in Cooling 1, 2 and 3 solutions and for 5-6 min in Cooling 4 solution (table 2). All steps were performed using 100µl drops of the equilibration solutions and they were covered by oil. The last step included the exposure of the oocytes in vitrification solution for 1 min in a free-oil drop of 100µl volume (table2). When this step was over, the oocytes were loaded in the tip of carrier called Vitrisafe (Vitrimed^{TM}). This carrier was inserted in a high security straw and sealed hermetically. The whole device was then plunged in LN2 and stored in cryogenic tanks (figure 5).

For the warming procedure a six step protocol was used. This protocol is almost the same protocol as the Vanderzwalmen protocol (100) with a minor modification (see experimental part 1). The warming solutions that were applied to vitrified oocytes were Warming 1, 2, 3, 4, 5 and 6 and contained 1M, 0.75M, 0.5M, 0.25M, 1.25M and 0M concentration of Sucrose respectively. For the procedure, the closed device was put in a dewar full with LN2. The upper end of the high security straw was taken out of the LN2 and was cut with a sterile scissor. With the help of an extractor tool, the Vitrisafe was taken out of the protective straw and directly placed in 1ml of W1 solution for 1 min (Figure 6). Then the oocytes were consequently exposed to W2 for 1 min, and to W3, 4, 5 and 6 for 2-4 minutes (table 2). The exposure of the oocytes in W2 to W6 solutions was done by means of 100µl drops under oil. When the warming protocol was over, the oocytes were placed in fertilization media until they were inseminated.

VITRIFICATION					
Solution type	Cooling 1	Cooling 2	Cooling 3	Cooling 4	Cooling 5
Time of Exposure	2-3min	2-3min	2-3min	5-6min	1min
WARMING					
Solution Type	Warming 1	Warming 2	Warming 3	Warming 4	Warming 5	Warming 6
Time of Exposure	1min	1min	2-4min	2-4min	2-4min	2-4min

Table 2: Vitrification and warming protocol using closed devices. All procedures were performed in room temperature except the W1 stage which is performed in 37°C

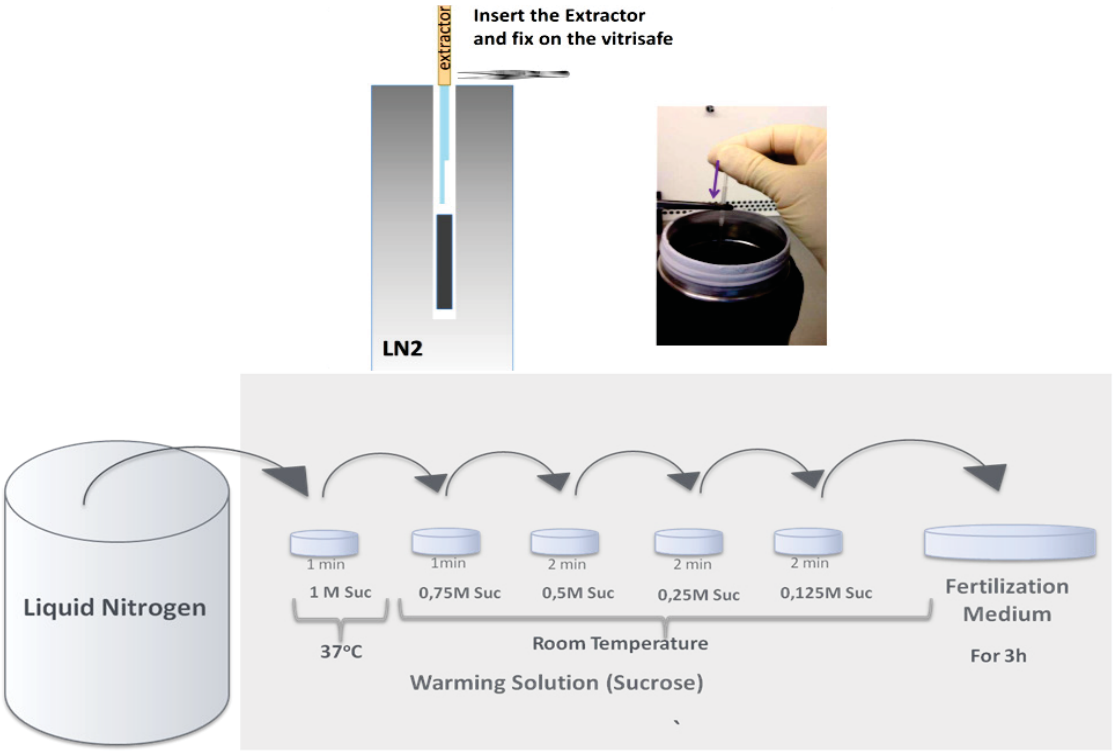


Figure 6: Warming procedure and rehydration process

3.9 Experimental part 1

Before applying clinically the vitrification of MII oocytes in closed carrier device, a set of experiments was designed, aiming to settle a suitable procedure for vitrification of MII oocytes in hermetically closed conditions. Therefore, we wanted to determine, by means of observational study, a suitable and non traumatic protocol of exposure and addition of cryoprotectant solutions.

Experiment 1: Cinematographic analysis

The first experiment was based on behavioural observation of oocytes in presence of cryoprotectant solutions (CPs) and aimed to the formation of a less traumatic protocol in terms of volume variation consequent to hypertonic cryoprotectant solutions exposure. This experiment was based on a previous report (100) in which the same approach was used in order to determine the appropriate protocol for closed system vitrification. When an oocyte is exposed to hypertonic solutions of cryoprotectant, it reacts first in a way to adjust the osmolarity by loosing water (shrinkage of the oocyte) and then the concentration by CP permeation (swelling of the oocyte) (figure 8).The degree of shrinkage – swelling will have an impact on the viability and further development and is related with the way of addition of the CP and their concentrations.

The aim of this experiment was to define an appropriate protocol with reduced shrinkage –swelling stress during exposure to the cryoprotectant solutions which was still providing results compatible for clinical application. To achieve this objective, the behaviour of the oocytes in contact with cryoprotectant solutions was assessed using cinematographic analysis (figure7). Using this analysis, we achieved to observe the

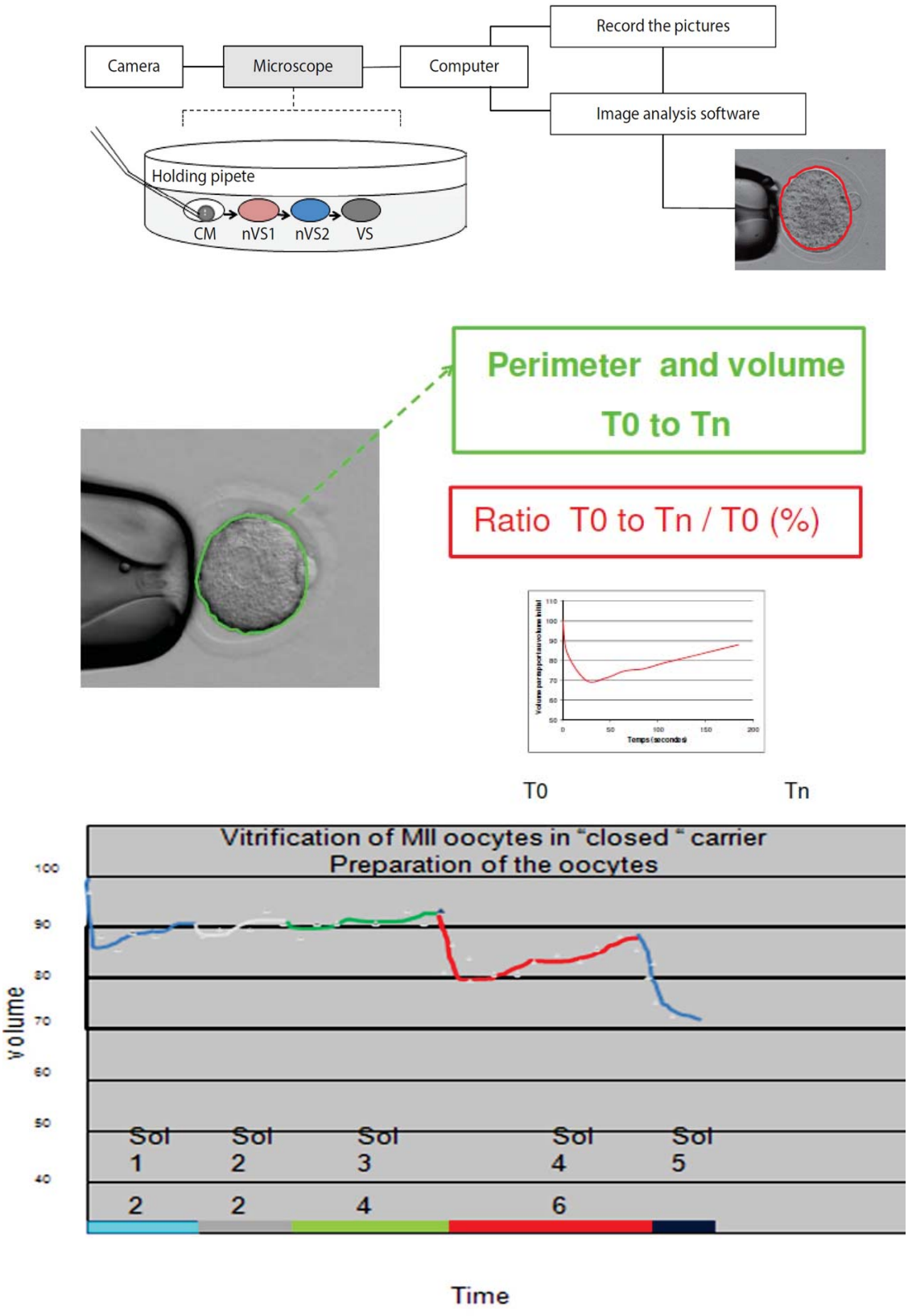


Figure 7: Cinematographic analysis

shrinkage/swelling process of oocytes during the exposure to different combiations of non-vitrification and vitrification cryoprotectant solutions (CPs). The reaction of the cells at different concentrations of CPs and the variations of the volume of the oocytes during time gave us the necessary information in order to form the closed system vitrification protocol. MI and unfertilised MII oocytes were exposed to different solutions of vitrified and non vitrified CPs. The oocytes were fixed on a holding pipette through the passage in the different baths in order that they stay on the same focus plane (figure 7). A photo was taken every 10 seconds during the exposure in the different solutions (figure 8).

After recording the different phases of exposure with the CPs, a morphometric analysis of the perimeter and volume of the oocytes was calculated (figure 7). The evolution of the volume in relation with the concentration and time of exposure in the different solution is plotted on a draft (figure7). Our main aim was to select a protocol of addition of cryoprotectant inducing a decreasing in the volume of maximum 30% after exposure to the non-vitrification (NVs) and vitrification (VS) solutions.

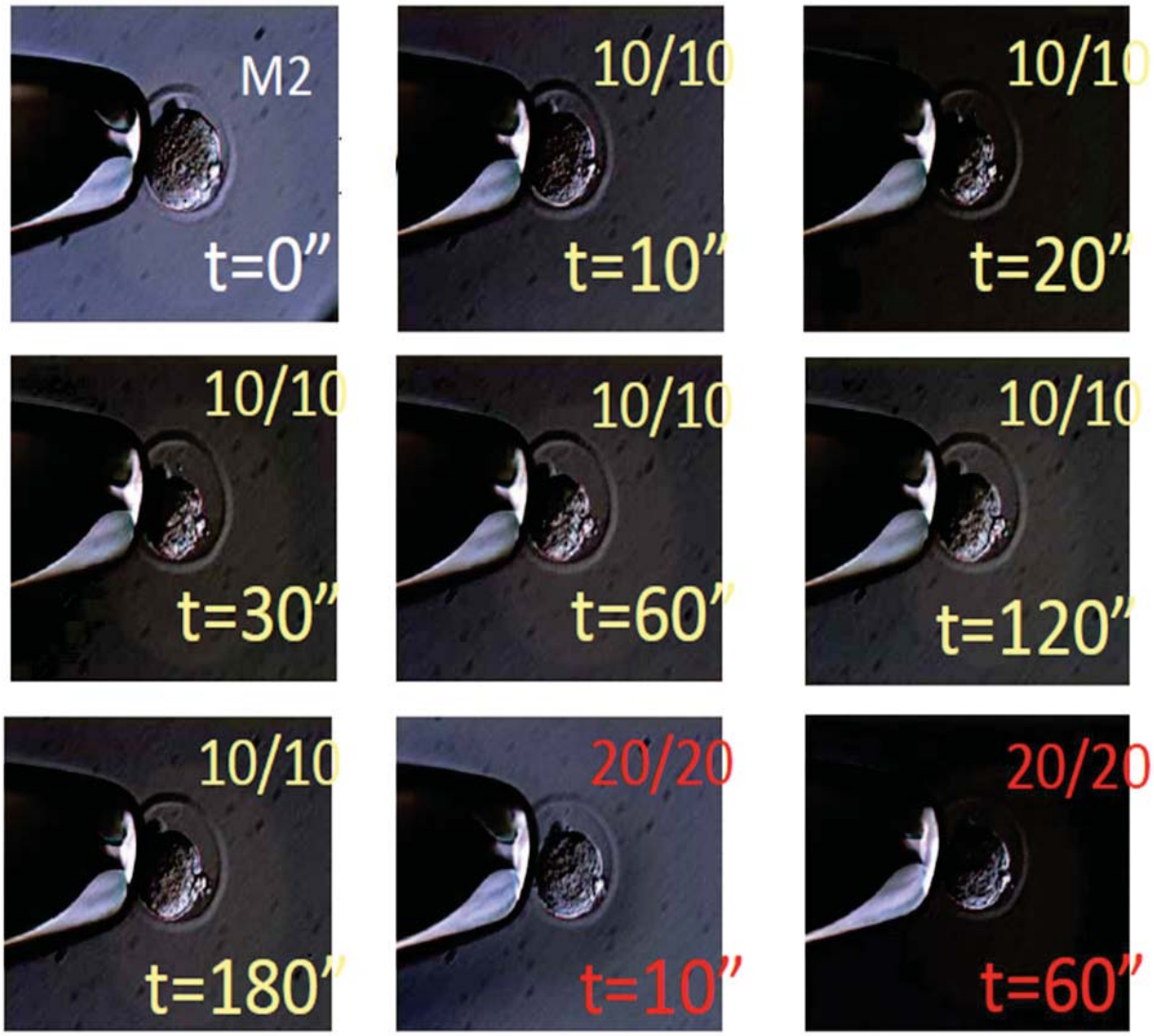


Figure 8: Shrinkage and swelling of the oocytes during exposure to different concentrations of cryoprotectants

Results of experiment 1

The results of this experiment showed that oocytes, due to their large volume, need more cryoprotectants in order to maintain their integrity after vitrification and warming. From the cinematographic analysis (figure 9) we see that the exposure of the oocytes directly to a concentration of 10% of each CP (one step approach) results to a shrinkage of the ooplasm which is more than 50% of the initial volume of the cell. This reaction is considered as an intense dehydration step that raises osmotic stress. At the same time that water goes out quickly, the CPs are entering the cell very fast. Due to these movements we could observe a higher frequency of apparition of blebs (figure10). These blebs are the visualization of high osmotic stress. On the other hand, when we performed a gradual exposure of the oocytes to CPs then the osmotic stress that is being induced in Equilibration and Vitrification phase was lower. Furthermore, we noticed that blebs are not present when the volume of the ooplasm looses maximum 30% of its initial volume.

The most important information that we had from the cinematographic analysis is the time that is necessary in order an oocyte to gain equilibrium of its volume after the exposure in a solution with CPs. What we observed was that in low CP concentration solutions (like the 1.25%, 2.5% and 5%) the oocytes were instantly shrinked (within 5 seconds) by losing a small amount of their volume (around 15%) and then re-expanded to 90% of the initial volume and reached a plateu.(figure 11). This procedure lasted around 2 to 3 min and in this way we calculated the appropriate time exposure to CPs.

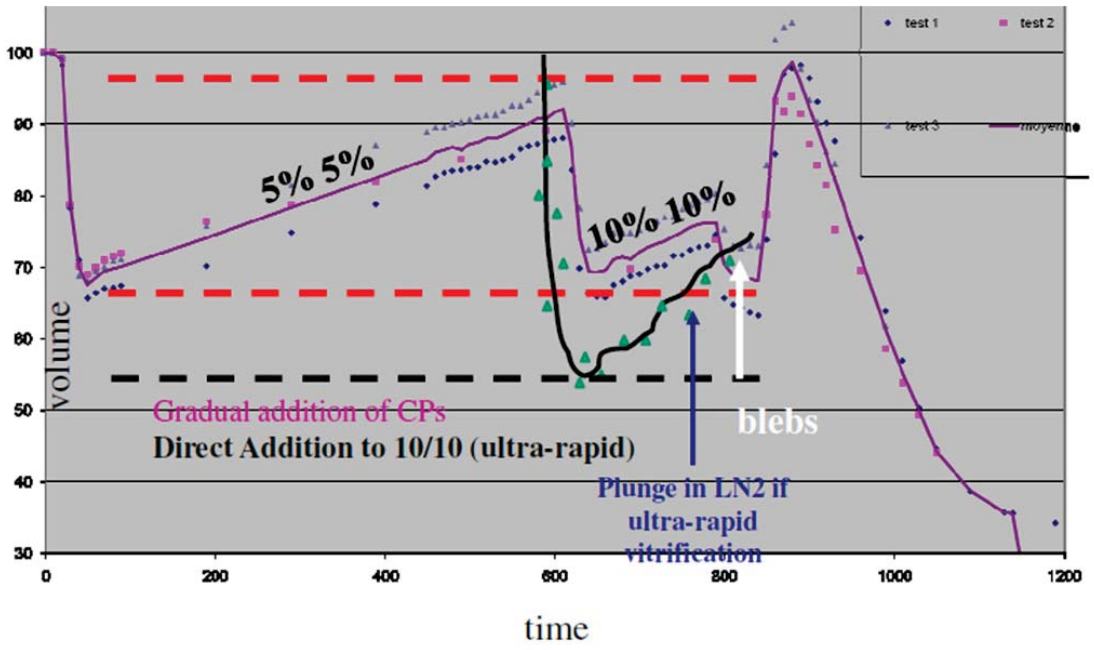


Figure 9: One – step addition of the CPs results to more than 50% change of volume and thus to an increased osmotic stress

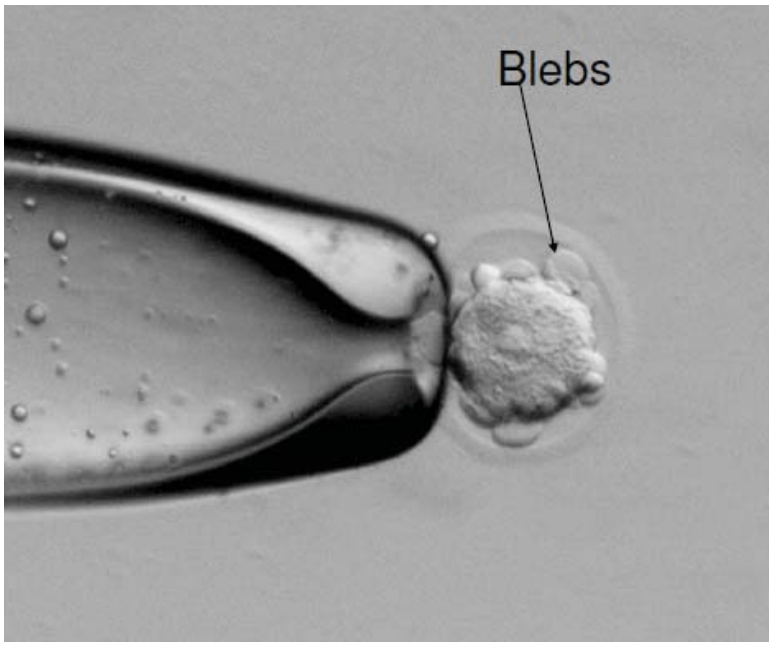


Figure 10: Blebs formation. A result of osmotic stress during fast exit of water and fast entrance of cryoprotectant

In the same way we calculated that the time exposure in a solution of 10% DMSO and 10% EG was 5-6 min. In this period the oocytes lost initially around 30% of their volume and then regained the 80% of the volume and reached a plateu. Finally, when the oocytes were exposed to 20% solution (which also contains non permeable CPs – so more hypertonic) lost the 30% of their volume in about 60sec, and then their ooplasm started to re-expand for some seconds but then again started losing volume. Thus, from this analysis, we calculated that the best moment to plunge the oocytes in LN2 is after 60 sec exposure to vitrification solution.

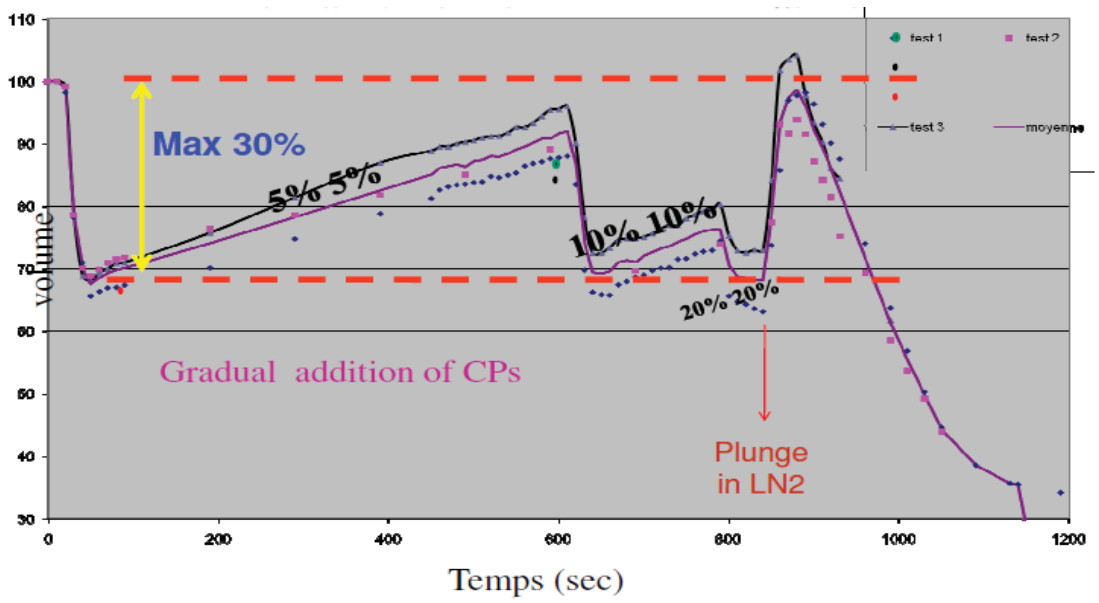


Figure 11: Gradual addition of the CPs results to a max 30% change of volume and thus to a minimum osmotic stress

Experiment 2: Selected protocol of CP exposure before vitrification

The protocol that we selected according to the cinematographic analysis involved a gradual addition of the cryoprotectant in 5 steps (table 1). Four non-vitrifying solutions

(NVs), 1.25/1.25, 2.5/2.5, 5/5, were prepared by diluting the 10% ethylene glycol (EG - Sigma) (v/v) – 10% dimethylsulphoxide (DMSO - Sigma) (v/v) solutions. The base solution for the dilutions was the Quinns Advantage Medium with Hepes (Sage Inc.) plus 20% Serum Protein Supplement (SPSS) (Sage Inc.) or HTF – HEPES 20% HSA (Sage Inc.). The vitrification solution (VS) was composed of 20% EG (v/v) – 20% DMSO (v/v), 25 μM (10 mg/ml) Ficoll (70,000 MW), and 0.75 M sucrose (Vs 20/20). The oocytes were exposed to the different non-vitrification and vitrification solutions for various times.

The aim of this experiment was to apply the findings of the experiment 1 and finally to determine the ideal protocol for CP exposure before closed system vitrification of oocytes. In table 3 it can be seen the different protocols that were applied to oocytes. 15 groups of oocytes (group A to group 0) were formatted. Every group contained 20 oocytes. In group A and B we applied a protocol similar to the one for open system vitrification of embryos (106). In group C and D we applied a protocol that has been used for closed system vitrification of embryos (117). In groups F and G we add an extra equilibration step. In group G, H, I and J we added two extra equilibration steps. These protocols (G to H) were the ones closed to the findings of the cinematographic analysis. In groups K and L we applied protocols with elevated exposure times in the first three solutions. In the groups M, N, O we increased exposure time in the 10/10 equilibration solution.

In all groups, after the exposure to the CPs, the loading of the oocytes to the carrier and then to the outer protective straw is described in the previou section. The warming procedure that was followed was adopted by Vanderzwalmen (100) protocol for

blastocyst warming. Briefly, oocytes were rehydrated in solutions 1M/ 0.75M/ 0.5M/ 0.25M/ for 1/1/4/4/ min respectively. After warming the survival rate was assessed. Two hours after warming, oocytes were reassessed for survival (table 3).

Group / Protocol	N oocytes	Time in solution					Survival at	
		1.25	2.5	5	10	20	0h	2h
A	20				5min	30s	1 (5%)	0 (0%)
B	20				5min	60s	2 (10%)	1 (5%)
C	20			4min	5min	30s	4 (20%)	1 (5%)
D	20			4min	5min	60s	5 (25%)	3 (15%)
E	20		2min	4min	5min	60s	12 (60%)	10 (50%)
F	20		2min	4min	6min	60s	13 (65%)	12 (60%)
G	20	2min	2min	4min	5min	60s	15 (75%)	14 (70%)
H	20	2min	2min	4min	6min	60s	16 (80%)	16 (80%)
I	20	3min	3min	3min	5min	60s	19 (95%)	18 (90%)
J	20	3min	3min	3min	6min	60s	20 (100%)	20 (100%)
K	20	5min	5min	3min	5min	60s	14 (70%)	12 (60%)
L	20	5min	5min	3min	6min	60s	14 (70%)	13 (65%)
M	20	3min	3min	3min	8 min	60s	16 (80%)	4 (20%)
N	20	3min	3min	3min	10 min	60s	15 (75%)	2 (10%)
O	20	3min	3min	3min	14 min	60s	15 (75%)	0 (0%)

Table 3: Different approaches used to determine the best protocol for closed system vitrification

Results of experiment 2

The best protocol for closed system vitrification was protocol J and protocol I. Both of them had significantly higher survival rate than all the other groups. Group J had

100% survival rate just after the warming (0h) and after 2h incubation in culture medium. The lower survival rate was observed in protocols A, B, C and D with survival rates ranging from 5% to 25% when assessed in 0h and even lower (0% to 15%) after 2h of incubation. In protocols E and F, where we added an extra equilibration step, we observed a significantly higher survival rate (60-65% in 0h and 50-60% in 2h) which became even higher when a second extra equilibration step was added in protocols G and H (75-80% in 0h and 70-80% in 2h). When the exposure times in equilibration solution was increased (protocols K,L,M,N,O) we observed again a decrease in the survival rate at 0h which was dramatically lower after2h of incubation and reached 0%.

3.10 Clinical trial #1

In order to validate the effectiveness of a closed and aseptic vitrification approach for oocyte cryopreservation a prospective randomized study was set up comparing the open and the closed vitrification techniques in a population of oocyte recipients sharing sibling oocytes donated from the same donor. Oocyte fertilization rates after ICSI, implantation, pregnancy and live birth rates per transfer cycle were evaluated as primary outcomes.

Sibling oocytes donated from the same donor were randomly and equally assigned into the closed group (oocytes vitrified in a closed system) or the open group (oocytes vitrified in an open system). Later on, these oocytes were warmed and donated to recipients who were randomly allocated to receive vitrified oocytes either from the closed or open group. The study was approved by the Institutional Review Board (Ref. N. 2/2007, 19/1/2007) and informed consent was obtained from all women.

3.10.1 Power analysis and sample size calculation

Our previous data (unpublished) have shown that 63% of good quality embryos on Day 3 (6-8 even sized cells with appearance of maximum 10% fragmentation of the total volume of the embryo – Veeck, 1999) were obtained from fresh inseminated oocytes in our oocyte donation program and 38% of good quality embryos resulted from donated oocytes vitrified with the open vitrification procedure. Consequently a decrease of more than 25% in good quality embryos after vitrification with either the open or the closed vitrification procedure would be inacceptable. Thus, power analysis calculations showed that 78 cycles would be needed for each group in the study in order to achieve statistical significance at a 5% level with power 80%. Data are expressed as the mean ±SD. Chi-squared test and independent sample t-test were used to compare the biological and clinical outcomes of fertilized oocytes which were initially vitrified with two different systems. P values below 0.05 were considered as statistically significant. Analysis was performed using the Statistical Package for Social Sciences version 17.0 (IBM/SPSS Inc., Chicago, IL, USA).

Seventy-eight oocyte donors were evaluated at the Iakentro IVF centre from February 2007 to December 2010. A single attempt was included for each donor. When lower than expected ovarian response observed and less than ten oocytes were retrieved, donors (n=3) were not included in the statistical analysis.

A total of 150 recipients matched with their donors were included in the study. The recipients had a mock transfer in a cycle previous to IVF and if difficulty was encountered a cervical dilatation was performed (110). Recipients were randomized into

two groups that received either oocytes vitrified in a closed system (n=75) or oocytes vitrified in an open system (n=75), according to a computer generated allocation sequence prepared from our statistician (figure 12). All patients entered the study once.

The oocytes retrieved from each donor were equilibrated for 2 h and then enzymatically denuded (Sage Inc., A Cooper Surgical CompanyTM). Metaphase II (MII) oocytes were evaluated and selected under a stereomicroscope. Those with dark cytoplasm, a centrally located granular area, vacuoles or a large polar body were excluded from vitrification. Selected oocytes were randomly and equally allocated to for the closed or open vitrification group. One hour later, metaphase II (MII) oocytes were vitrified either in a closed or open system.

The vitrified oocytes were warmed, fertilized and transferred in their recipients in the course of an Hormonal Replacement Treatment (HRT) cycle as it was previously described (118). When the recipient's endometrium was adequately prepared (>9mm) for embryo transfer, progesterone administration was started and the same day the vitrified oocytes of the randomly selected closed or open group were warmed and fertilized (figure 12).

3.10.2 Vitrification and warming Procedures

Oocytes were vitrified by using the Vitrisafe device (figure 5) in both protocols (open and closed). This device is consisted of two parts: a carrier called Vitrisafe (VitriMed, Austria) and a high security straw 0.3ml (Cryo Bio System, France). For the vitrification procedure the carrier has to be inserted in the high security straw which has a protective action. The base for the solutions used in the vitrification (Equilibration and

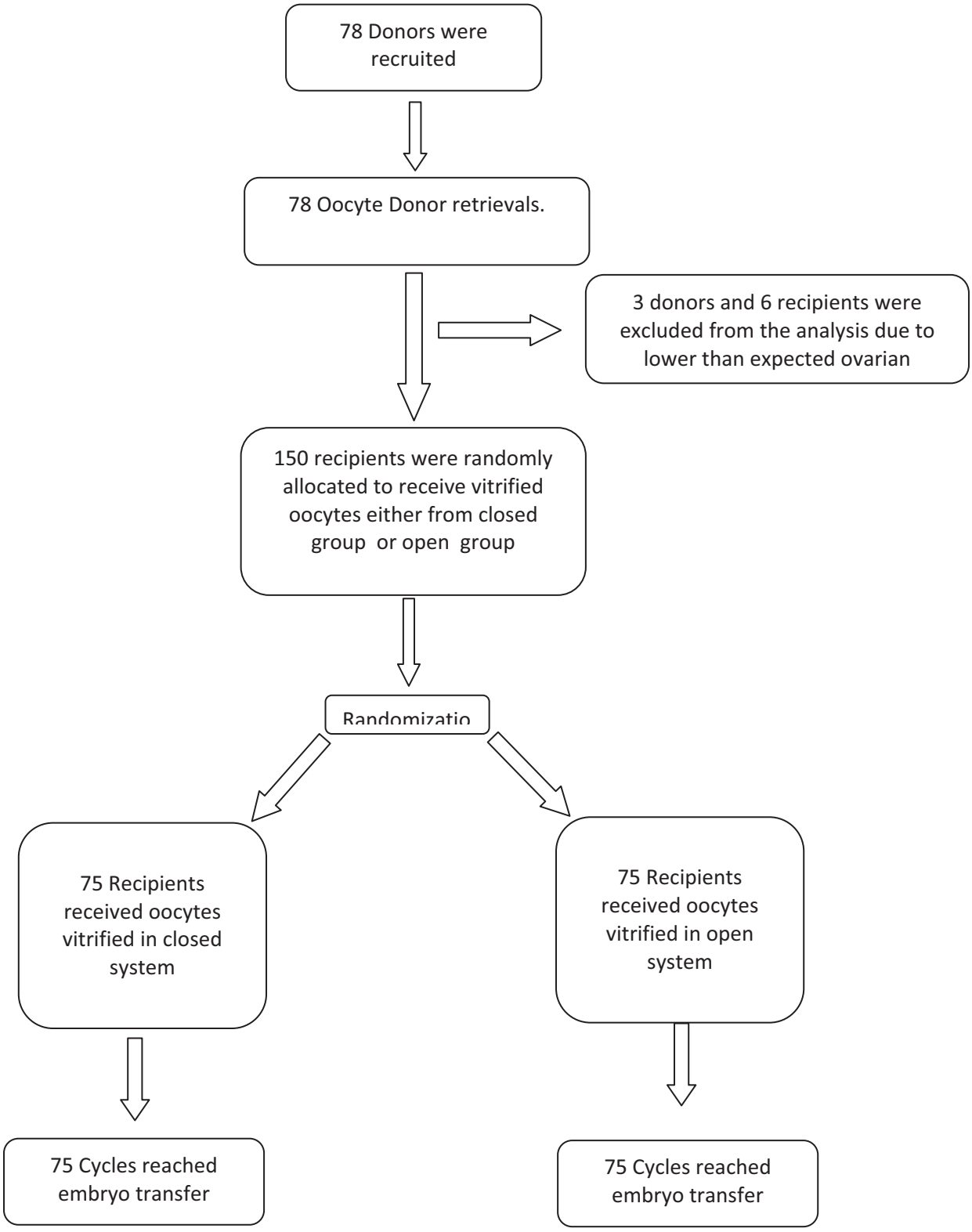


Figure12: Flow chart of inclusion and randomization of patients treated in trial 1.

Vitrification) and warming procedure of the oocytes were the Quinns Advantage Medium with Hepes (Sage Inc., A Cooper Surgical Company TM) plus 20% Serum Protein Supplement (SPSS) (Sage Inc., A Cooper Surgical Company TM). All solutions were produced in our laboratory.

3.10.2.1 Open system

In the open protocol, vitrification of oocytes was performed according to Kitazato protocol (56) but using the vitrisafe device. Equilibration was performed gradually in 7.5% ethylene glycol (EG) and 7.5% dimethylsulphoxide (DMSO) at room temperature for 15min. Subsequently, oocytes were transferred in 15% EG, 15% DMSO and 0.5 M sucrose for 1 min, and then placed on the vitrisafe in a single small drop. The excess solution was removed to leave just a thin layer around each oocyte and the carrier was submerged directly into liquid nitrogen. The vitriplug was inserted in the protective straw that had already been in the LN2. This maneuver was performed inside the LN2 by means of a forceps. Once the carrier was into the protective straw, the open edge of the protective straw was merely pulled out of the liquid nitrogen; heat sealed (Thermo Scientific) and finally plunged in the nitrogen. For the warming procedure, the extremity of the protective straw was cut; the carrier was immediately removed from the LN2 and quickly submerged in 1 ml of 37o C warming solution containing 1.0 M sucrose for 1 min. Then, the oocytes were transferred to a room temperature solution containing 0.5M sucrose, and incubated for 3 min. After two subsequent washing procedures in basic medium at room temperature for 6 min in total, oocytes were transferred into fertilization medium (Sage Inc.).

3.10.2.2 Exposure to the CP solution using the closed system

Vitrisafe is a closed vitrification device enabling a cooling rate of < 2.000oC /min (100). In order to avoid the risk of intracellular ice crystal formation (51) we had to use higher intracellular concentrations of cryoprotectants.

The protocol used for closed system was a modification of a previously reported protocol used in blastocyst vitrification (100,119). In the previous section we demonstrated in details how we formed the new protocol, adopted for closed system vitrification. As it concerns the procedure, the equilibration solution (ES) consisted of 10% DMSO (v/v) (Sigma-Aldrich) and 10% ethylene glycol (EG) (v/v) (Fluka) while the vitrification solution (VS) consisted of 20% DMSO (v/v) (Sigma-Aldrich), 20% ethylene glycol (EG) (v/v) (Sigma) and 0.75mol/L sucrose (Sigma-Aldrich). Additionally, three dilutions (1/8, 1/4, 1/2) of the ES were prepared. Similarly, four warming solutions (WS) were prepared containing 1, 0.75, 0.5 and 0.25 mol/L sucrose (Sigma-Aldrich) respectively. One hour after their denudation, 3 oocytes at a time, were transferred successively through the four equilibration solutions by means of 50μl drops under oil. This step lasted for 14 min, that is 2min in 1/8 dilution of ES, 2min in 1/4 ES, 4min in 1/2 ES and 6 min in ES. Subsequently, the oocytes were transferred for 1 min into an oil-free 100μl drop of Vitrification solution. The oocytes, by means of a single drop, were initially transferred on the Vitrisafe. The excess solution was removed to leave just a thin layer around each oocyte and right away, the carrier was inserted in the protective straw which was thermo-sealed and plunged into a dewar with LN2. For the warming procedure, the protective straw of the Vitrisafe was cut and the open pull straw was immediately

removed from the LN2 and placed in the warming solution containing 1.0 M sucrose at 37o C for 1 min. Then, the oocytes were placed in a 0.75 M sucrose warming solution at room temperature for 1 min, 4 min in 0.5M and for another 4 min in 0.25M sucrose warming solution. Finally, in order to recover, the oocytes were placed into a fertilization medium (Sage Inc.) and incubated for 2 hours at 37oC. Degenerated oocytes were removed from the cohort. Survived oocytes were cultured at 37o C (6% CO2 and 5% O2) for 2h. Intracytoplasmic sperm injection (ICSI) was performed on all surviving oocytes within 2h post warming; regardless of semen parameters.Embryo culture was performed using the SAGE sequential media. On day 3, embryos were evaluated for their quality. The embryo transfer was performed on day 3.

3.10.2.3 Statistical analysis

All cases were under clinical follow up in order to gather the data. Chi-squared test and independent sample t-test were used to compare the biological and clinical outcomes of fertilized oocytes which were initially vitrified with two different systems. P values below 0.05 were considered as statistically significant. Analysis was performed using the Statistical Package for Social Sciences version 17.0 (IBM/SPSS Inc., Chicago, IL, USA).

3.10.2.4 Results

Although 78 donors (matched with 156 recipients) were included in the study, due to lower than expected ovarian response (≤10 oocytes retrieved), 3 oocyte donor cycles with their 6 matched receivers were excluded from the statistical analysis (figure 12).

All recipients were nulliparous. The mean age (44.97±1.42 vs 44.82±1.53 years) and the mean endometrial thickness (10.15±1.15 vs 10.48±1.34 mm) of the recipients were not different between the two groups (table 1).

A total of 1206 MII oocytes were retrieved. The closed (aseptic) vitrification system was used for 598 of them while 608 were vitrified with the open system (Table 4). There was no difference in the mean number of oocytes vitrified with the closed or the open system (7.97 ± 2.42 vs 8.29±2.61). Although the survival rate was significantly lower in the closed compared to the open group (82.9%% vs 90.9% $p<0.05$), the fertilization rate (82.5% vs 73.4%), the cleavage rate (94.1% vs 93.1%) and the percentage of Day 3 top quality embryos (38.1% vs 39.4%) show no difference between the compared groups (table 4). Seventy-five transfers were performed in each group. The mean number of embryos transferred was similar in both groups (2.81±0.46 vs 2.79±0.47). Moreover, there was no statistically significant difference in the b-HCG positive rate (42.7% vs 33.3%), clinical pregnancy rate (36.0% vs 28.0%), implantation rate (13.8% vs 10.1%), ongoing pregnancy rate (36.0% vs 24.0%) and live birth rate (36.0% vs 24.0%) between closed and open group (table 5).

Twenty seven healthy babies from closed group and eighteen from open group were born. Two twin pregnancies and two miscarriages were reported in the closed group. No twin pregnancies and three miscarriages were observed in the open group. No differences were observed between the groups.

	Closed System ***n=75***	**Open System** ***n=75***
Age of recipient (years)	44.97 ± 1.42	44.82±1.53
Endometrial Thickness (mm)	10.15±1.15	10.48±1.34
Vitrified Oocytes	598 (7.97 ± 2.42)	608 (8.29±2.61)
Survived Oocytes[a] (Survival rate)	496 (6.61 ± 1.73) (82.9%)	553 (7.48±2.47) (90.9%)
Fertilized Oocytes (Fertilization rate)	409 (5.45 ± 1.81) (82.5%)	406 (5.52 ± 1.94) (73.4%)
Cleaved Embryos (Day 3) (Cleavage rate)	385 (5.13 ± 1.66) (94.1%)	378 (5.04 ± 1.77) (93.1%)
Top quality (grade 1, Veeck 1999) embryos on Day 3	147 (1.96 ± 1.33) (38.1%)	149 (1.99 ± 1.22) (39.4%)
Embryonic Score (according to Veeck, 1999)	2.62 ± 1.25	2.59 ± 1.25

Table 4. Recipient age, endometrial thickness and embryological parameters. Values presented as mean ± SD. [a] Survival rate was significantly lower in the closed vitrification group, $P<0.05$.

	Closed System ***n=75***	**Open System** ***n=75***
N embryos transferred	210 (2.81±0.46)	209 (2.79±0.47)
Positive b-HCG /cycle	32/75 (42.7%)	25/75(33.3%)
Clinical Pregnancy rate/cycle	27/75 (36.0%)	(21/75) (28.0%)
Implantation rate	29/210 (13.8%)	21/208 (10.1%)
Ongoing pregnancy rate/cycle	27/75 (36.0%)	18 /75 (24.0%)
Live birth rate/cycle	27/75 (36.0%)	18/75 (24.0%)

Table 5. Clinical parameters. There were no statistically significant differences between the two groups.

3.11 Comparison of clinical results of fresh vs closed vitrified oocytes

The biological and clinical results of the clinical trial #1 (cases of donated and vitrified/warmed oocytes) were compared with those of fresh donated oocytes. The data used were from the same clinic (same lab and embryologist) in order to have comparable data and to avoid any confounding factors that could induce any bias. Therefore we chose to compare our finding with the ones that are reported in randomized control study using fresh donated oocytes (118).

Fertilization rate, cleavage rate, top quality cleavage rate, pregnancy rate, clinical pregnancy rate and implantation rate were analyzed (Tables 6).

3.11.1 Results

The fertilization rate was similar between the two trials (77.7% vs 78.7%, p=0.6). Accordingly, the cleavage rate was not different between the two trials (72.7% vs 74.6%, p= 0.65). On the contrary, we observed a significant difference in the top quality cleavage rate in favor of the trial which used fresh donated oocytes (28.2% vs 42.1%, p=0.0001). Moreover, the pregnancy rate was significantly lower in the clinical trial #1 when compared with the pregnancy rate that was reported in Prapas et al, 2009 trial (38% vs 54.1%, p=0.003) . The same stands for the clinical pregnancy rate (32% vs 55.1, p=0.003).

The above results after oocytes vitrification, although promising and acceptable by the scientific community, were not satisfactory. The fact that oocytes vitrification provided lower quality embryos and lower pregnancy and clinical pregnancy rates, made us reconsider our protocol. Another fact that motivated us to change our protocol was a

clinical trial that was published that period (106) in which 55.9% pregnancy rate was achieved using vitrified donated oocytes while the top cleavage rate on day 3 was 36.1%.

After critical evaluation (see discussion part) of the data obtained in clinical trial #1 we decided to modify the warming protocol and we set up a new set of experiments in order to do so.

	Clinical Trial #1	Prapas et al, 2009	p value
Fertilization rate	815/1049 (77.7%)	555/705 (78.7%)	.60
Cleavage rate	763/1049 (72.7%)	506/705 (74.6%)	.65
Top Quality Cleavage Rate	296/1049 (28.2%)	297/705 (42.1%)	.0001
Pregnancy rate	57/150 (38%)	56/98 (54.1%)	.003
Clinical Pregnancy rate	48/150 (32%)	54/98 (55.1%)	.003

Table 6: Comparison of biological and clinical data between fresh and vitrified oocytes

3.12 Experimental part #2

In order to achieve even better biological and clinical results we decided to change the timing of the warming steps and thus to change the pattern of rehydration of the cells. In clinical part #1 we applied a protocol where oocytes were placed for 1min in 1M Suc, then 1 min in 0,75M Suc, 4 min in 0,5M Suc, 4 min in 0,25M. This was a protocol that was used in blastocyst warming and we assimilated for our research. After critical evaluation we believed that this protocol might induce an osmotic stress to the oocytes

and this could be the reason why vitrified oocytes ini clinical trial #1 gave us lower results than fresh oocytes used to give us.

Therefore we set up a set of experiments, similar to experimental part 1, where we used standard vitrification protocol (3/ 3/ 3/ 5.5/ 1 min in 1.25 / 2.5/ 5/ 10/ 20 % of each CP accordingly) but we reduced the timing of the rehydration of the cells. The set of experiments are seen in table7. We formed 10 study groups and applied 10 protocols. Every group had 20 oocytes. The oocytes were fresh donated oocytes. After warming the oocytes were fertilized using the same sperm donor. Fertilization rate, cleavage rate/ used oocytes and top cleavage rate/ used oocytes were assessed.

3.12.1 Results

The most efficient protocol was protocol G. The fertilization rate was 90%, cleavage rate/oocytes used was 85% and top quality cleavage rate/used oocytes was 45%. Another efficient protocol was protocol F with rate beign lower than protocol G but not significantly lower. Protocol A was the protocol used in clinical trial #1. When the exposure time was diminished (protocols B and C) all rates increased. Even when exposure time in 0.5M was only for 1 min (protocols D, E, H) all rates were higher. When the exposure time in solutions 0.5M and 0.25M were high, the results were detrimental for the cells and the embryos (table 7).The addition of an extra step during the rehydration (step 0.125M in protocols F and G), favors not only the survival rate but also the biological parameters of the resulting embryos.

Group	N oocytes	Time in solution					Survived	Fertilization/ oocytes used	Cleavage D3/ per oocytes used	Top Cleavage Day 3/ per oocytes used
		1M	0.75 M	0.5 M	0.25 M	0.125 M				
A	20	1	1	4	4		16	11/20 (55%)	8/20 (40%)	4/20 (20%)
B	20	1	1	3	3		18	14/20 (70%)	10/20 (50%)	5/20 (25%)
C	20	1	1	2	2		20	14/20 (70%0)	12/20 (60%)	6/20 (30%)
D	20	1	1	1	1		18	13/20 (65%)	11/20 (55%)	5/20 (25%)
E	20	1	1	1	2		18	13/20 (65%)	10/20 (50%)	4/20 (20%)
F	20	1	1	2	1	1	19	16/20 (80%)	14/20 (70%)	7/20 (35%)
G	20	1	1	2	2	2	20	18/20 (90%)	17/20 (85%)	9/20 (45%)
H	20	1	1	1			16	12/20 (60%)	9/20 (45%)	5/20 (25%)
I	20	1	1	5	5		10	6/20 (30%)	4/20 (20%)	2/20 (10%)
J	20	1	1	6	6		8	5/20 (25%)	0/20 (0%)	0/20 (0%)

Table 7: Different approaches in order to determine the best warming /rehydration protocol for oocytes that were vitrified in a closed system

3.13 Clinical Trial #2

In order to evaluate the efficiency of hermetically closed devices in oocyte vitrification we compared the clinical outcome after transfer of embryos derived from sibling oocytes, to recipients being synchronized (fresh oocytes) or not (aseptically vitrified oocytes) with their donor in our oocyte donation program.

A prospective, observational, cohort study was performed at IAKENTRO Fertility Center from January 2014 until December 2014. The recruitment start date was on 19/1/2014 and the completion date was on 15/12/2014. All procedures were performed

at the same laboratory. The study was approved by the Institutional Review Board of the Ioannina Medical School (Ref: 808a/8-3-2011). This trial was registered in ISRCTN registry with identification number ISRCTN56275481. Additionally, this study was approved by IAKENTRO Review Board (Ref. N. 1/2014, 19/1/2014) and informed consent was obtained from all women.

3.13.1 Non-inferiority test, sample size and study design

Based on a positive hCG/transfer baseline rate of 61 % among controls and 59 % for study subjects, a sample size of 92 transfers per arm would be required to be able to reject the null hypothesis that one-sided 95% confidence interval (or equivalently a 90% two-sided confidence interval) will exclude a difference in favor of the standard group of more than 20% with a significance level of 5% and a power (1-β) of 80%. Power calculation was performed with Sealed Envelope Ltd. 2012.(39)

Ninety two (92) oocyte donors participated in our study. A single stimulation cycle was included for each donor. Pairs of recipients, sharing sibling oocytes from the same donor, were included in the study. Each pair consisted of a recipient to whom fresh oocytes were used for their donation cycle and another recipient to whom oocytes were vitrified and used after a short period of time. A single donation cycle was included for each recipient. One hundred and eighty four couples, who received sibling oocytes donated from the same donor, were allocated to receive fresh (92 couples) or vitrified sibling oocytes (92 cases). Biological and clinical parameters were evaluated. Pregnancy rates were a secondary tracked outcome, and that the intervention did not depend on it nor did it affect the execution of the study in any way.

The current study contains a randomization procedure. During the donor's oocyte pick up, two separate dishes for oocyte collection were used. The retrieved cumulus oocyte complexes were randomly and equally assigned into the two dishes during oocytes retrieval. Odd number oocytes were allocated to the fresh group (dish 1-Group 1) and even number oocytes were allocated to the closed vitrified group (dish 2 – Group 2). This allocation method could potentially have the drawback of recruiting higher number of oocytes in group 1 as in all odd-number cases the group 1 would have more oocytes enrolled. However, according to our experience, as bigger-size follicles which are firstly retrieved in each procedure are more likely to contain higher quality (mature) oocytes, it is likely that the lastly retrieved odd oocytes are of poor quality (immature) and would not finally result in a significantly different number of MII oocytes in each group. Furthermore, it would demand an additional randomization process to decide which cases' oocyte allocation would begin from group 1 and from group 2. Therefore, in order to avoid complexity of procedure and because of the profound lack of clinical impact, it was initially decided to recruit oocytes in the same manner for all cases. Fresh group oocytes (Group 1) were fertilized the same day while vitrified group oocytes (Group 2) were stored in liquid nitrogen tanks. The warming and the fertilization of group 2 oocytes were performed when the recipient, in which those oocytes were allocated, had their endometrium properly prepared.

In this study, were included patients with a minimal of 8 mature oocytes, which is a basic requirement offered to recipient couples in our donation program. Consequently, after the denudation of retrieved oocytes, if a recipient was ended up with 7 or less mature oocytes, these cycles were excluded from our analysis since one of the two recipient

cycles had to be canceled. A 0total of 184 recipients, matched with their donors (n=92), were included in the study (Figure 13).

In both groups, the recipient couples did not suffer from any form of severe male infertility indication. All the enrolled cases had normal values for concentration, motility and morphology. The WHO criteria (111) were used to evaluate the sperm quality. For each oocyte donation cycle, there was a recipient allocated to receive the fresh and another to receive the vitrified oocytes, with an identical procedure: our center has a list of patients available for fresh oocytes and another list of patients available only for vitrified oocytes. Among all patients that had the same blood type and similar morphological characteristics with the oocyte donor, there was one recipient from each list allocated by computerized random select software for each oocyte donation cycle.

3.13.2 Laboratory procedures

The oocytes retrieved from each donor were equilibrated in Single Step Medium™ (SSM™, Irvine Scientific) for 2 hours and then enzymatically denuded (Hyaluronidase Solution, Irvine Sientific). Only MII oocytes were included in the study groups. Fresh group (group 1) oocytes were injected one hour post denudation. Closed vitrification group (group 2) oocytes were vitrified half to one hour post denudation. After their warming, oocytes were cultured in Single Step Medium™ (SSM™, Irvine Scientific) for 3 hours before they were injected. Intracytoplasmic sperm injection (ICSI) was performed in all cases. Fertilization was assess7ed 16-20h post ICSI by visualization of the two pronuclei. Embryos were cultured in 30 µl mineral oil (Fertipro, Bernem, Belgium) in a MINC™ benchtop incubator (Cook Medical) droplets of Single Step Medium™ (SSM™, Irvine Scientific), overlaid with

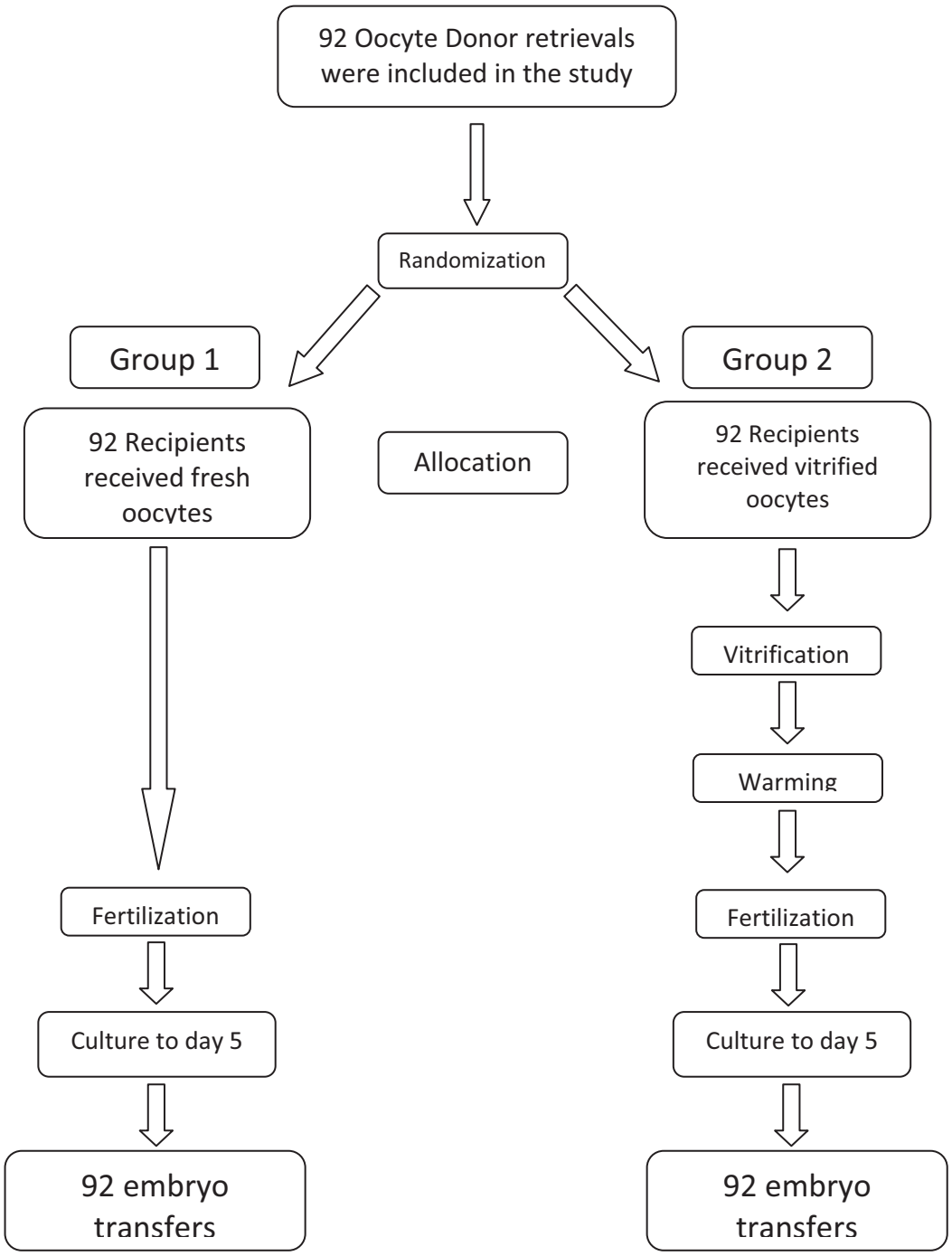


Figure 13: Flow chart of clinical trial 2

provided triple gas of 6% CO2, 5% O2 and 89% N2. Embryo quality was assessed on Day-3 and on Day 5. For day 3 embryos, number of cells, the appearance of blastomeres and the presence of cytoplasm defects or fragmentation were evaluated(113). According to this system, top quality cleaved embryos were considered those with 8 -12 symmetric blastomeres with absence of cytoplasm defects and without or with negligible fragmentation. For the evaluation of day 5 embryos we used the Gardner and Schoolcraft criteria (114) in which the thin zona pellucida, smooth trophoectoderm, equality and close adhesion of blastomeres, clearly visible blastocyst cavity and the well developed inner cell mass with many closely aggregated cells are the most important parameters correlating to the top blastocyst quality. Embryo transfers were performed on day 5, under ultrasound guidance as previously described (115). The remaining embryos were vitrified on day 5 using closed vitrification system.

3.13.2.1 Oocyte vitrification in a closed system

Oocytes were vitrified by using the Vitrisafe carrier (100) (VitriMed, Austria) after a maximum of 3 hours post OPU. Denuded oocytes were exposed to 4 equilibration solutions with 1.25% , 2.5% , 5% and 10% concentration of DMSO and Ethylene Glycol (Fertipro^{TM}) for 3, 3, 3 and 5:30 min respectively and 1 vitrification solution with 20% DMSO and 20% Ethylene Glycol plus 0,75 mol/l Sucrose and 10 mg/l Ficoll (Fertipro^{TM}) for 1 min. After the exposure to the cryoprotectants the oocytes, by means of a single drop, were initially transferred on the Vitrisafe and then the carrier was inserted in the protective straw which was thermo-sealed and plunged into a dewar with LN2. All procedures were performed at room temperature (~ 25°C).

Warming was performed in 5 steps as described before (120). Briefly, oocytes were exposed to gradually decreased concentration (1M, 0.75M, 0.5M, 0.25M, 0,125M) of sucrose for 1, 1, 2 ,2 ,2 min, respectively (Fertipro^{TM}). The rehydration of the oocytes was performed at room temperature (~ 25°C) with an exception of the first step, in which the vitrisafe was immersed in a warm solution (37^{o}C) of 1M Sucrose for 1 min. In order to recover, the oocytes were placed into culture medium (SSM™, Irvine Scientific) and incubated for 3 hours at 37^{o}C (6% CO2 and 5% O2) before they were injected. Degenerated oocytes were removed from the cohort.

3.13.2.2 Outcome measures

The number of survived oocytes, fertilized oocytes, cleavage embryos, top quality cleavage embryos, blastocysts, top quality blastocysts and embryos transferred were analyzed. Oocyte survival rate was defined as the number of oocytes survived out of the total number of oocytes warmed. ICSI was applied on all survived oocytes. Fertilization rate was defined as the number of fertilized oocytes out of the number of oocytes survived. Cleavage rate, top cleavage rate, blastocyst rate and top quality blastocyst rate was defined as the number of cleavage embryos, top quality cleavage embryos, blastocysts and top quality blastocysts respectively out of the total number of mature (MII) oocytes.

3.13.2.3 Primary and secondary outcomes.

The primary endpoint of the study was the positive hCG rate per cycle. Secondary outcomes were clinical pregnancy rate per cycle, ongoing pregnancy rate, biochemical pregnancy rate, miscarriage rate, twinning rate and live birth rate. Secondary endpoints

were also the oocyte survival rate, the fertilization rate, the cleavage and top cleavage rate, the blastocyst and the top blastocyst rate.

3.13.2.4 Statistical Analysis

Continuous data are expressed as mean ± SD, while categorical data as percentages (%). Normality was assessed with Kolmogorov -Smirnov test. Independent sample t-test was used for parametric and Mann-Whitney test for non-parametric numeric variables. Categorical data were analyzed with Chi-square analysis. Statistical analysis was performed using the Statistical Package for Social Sciences version 17.0 (IBM/SPSS Inc., Chicago, IL, USA). Non-inferiority test was performed by using power calculator of sealed envelope™ at www.sealedenvelope.com.

3.13.2.5 Results

Data from 184 recipients were prospectively collected from January 2014 to December 2014. There was no case included in the study that was finally dropped-out due to survival, fertilization or blastocyst formation failure. The epidemiological characteristics of the included cases were similar between the two groups (Table 8).

A total of 2325 oocytes were retrieved, 1175 in group 1 and 1150 in group 2. 1966 of them were MII, 982 in the fresh group and 984 in the closed vitrification group (Table 9). There was no difference in the number of the allocated oocytes between the two groups (10.7 ± 2.1 vs 10.7 ± 2.0, P=.94), therefore confirming the initial hypothesis that our oocyte allocation method would not result in significantly different number of MII oocytes. The survival rate of the oocytes from the group 2 was 92.7% (912/984 oocytes).

No significant difference was observed regarding the fertilization rate, cleavage rate, top quality embryos on day 3 and blastocyst rate between two groups. Top quality blastocyst rate on day 5 was significantly higher in the fresh oocyte group (31.7% vs 26.1%, P=.003). Embryological parameters are presented in Table 2.

Ninety-two transfers were performed in each group. The mean number of embryos transferred was similar (2.0 ± 0.0 vs 1.97 ± 0.3, P=.25). No statistically significant difference was observed between two groups regarding pregnancy (b-hCG positive) rate per cycle (63.1% vs 60.9%, P=.76) and clinical pregnancy rate per cycle (55.4% vs 58.7%, P=.66). No significant differences were observed regarding all the other outcomes between the two groups. Regarding live births, 66 healthy babies were born from fresh group and 62 from vitrification group. No adverse outcome was reported by parents. Primary and secondary clinical outcomes of the present study are presented in Table 10.

Moreover it has been calculated the collected oocytes to baby ratio was 5.6% and the MII oocytes to baby ratio was 6.8% in the fresh group while in the vitrification group was 5.3% and 6.3% respectively (Table 11). The MII oocytes needed to achieve a pregnancy was 14.8 in fresh group and 15.8 in the vitrification group (Table 11).

Donor characteristics			
Age	28 ± 3.2		
BMI	22.6 ± 2.6		
Days of stimulation (range)	9.8 ± 1.1 (8-12)		
Total r FSH (IU)	2192 ± 407		
E2 (pg/ml)	2230 ± 640		

Recipient characteristics			
	Group 1 (fresh oocytes) *n=92*	**Group 2 (vitrified oocytes) *n=92***	**p values**
Age	43.2 ± 3.4	43.5 ± 3.4	.57
Endometrium thickness	10.9 ± 1.1	10.7 ± 0.9	.31
BMI	23.8 ± 3.5	23.6 ± 3.1	.61
Days of endometrial preparation	16.9± 1.1	16.8 ± 0.5	.42

Male partner characteristics			
	Group 1 (fresh oocytes) n=92	***Group 2 (vitrified oocytes) n=92***	***p values***
Age	*43.62 ± 5.6*	*42.92 ± 5.7*	*.41*
Sperm concentration (millions/per ml)	*49.2 ± 22.6*	*46.6 ± 22.4*	*.42*
Motility	*67.87 ± 11.8*	*67.5 ± 11.8*	*.85*
Forward motility	*56.1 ± 13.1*	*56.6 ± 14.3*	*.84*
Morphology	*16.52 ± 4.6*	*16.43 ± 6.4*	*.91*

Table 8: Baseline characteristics of donors, recipients and their male partners, expressed as mean ± SD

	Group 1 (fresh oocytes) *n=92*	**Group 2 (vitrified oocytes) *n=92***	**P value**
Retrieved oocytes	12.7 ± 2.4 (1175)	12.5 ± 2.1 (1150)	.86
MII oocytes	10.7 ± 2.1 (982)	10.7 ± 2.0 (984)	.94
Vitrified Oocytes	-	10.7 ± 2.0 (984)	-
Survived Oocytes	-	9.9 ± 2.1 (912)	-
Survival rate, %	-	92.7% (912/984)	-
Fertilized Oocytes	8.6 ± 1.8 (796)	8.1 ± 1.7 (744)	.23
Fertilization rate, %	81.1% (796/982)	81.6% (744/912)	.77
Cleaved Embryos (Day 3)	8.0 ± 1.6 (739)	7.5 ± 2.0 (692)	.29
Cleavage rate, %	75.2% (739/982)	75.8% (692/912)	.90
Top quality cleaved embryos	4.3 ± 2.0 (403)	4.1 ± 2.1 (382)	.37
Top quality cleavage rate, %	41.1% (403/982)	41.8% (382/912)	.80
Blastocysts	*5.2 ± 1.7 (484)*	*4.8 ± 2.1 (440)*	*.10*
Blastocyst rate, %	49.3% (484/982)	48.2% (440/912)	.51
Top quality Blastocyst	*3.4 ± 2.0 (312)*	*2.6 ± 1.6 (238)*	*.003*
Top quality blastocyst rate, %	31.7% (312/982)	26.1% (238/912)	.003
Embryos transferred	2.0 ± 0.0 (184)	1.96 ± 0.4 (180)	.25
Vitrified embryos	*2.76 ± 1.5 (254)*	*2.41 ±1.8 (222)*	*.16*

Table 9: Biological parameters expressed as mean ± SD (number).

	Group 1 (fresh oocytes) *n=92*	Group 2 (vitrified oocytes) *n=92*	P value
Positive b-hCG	63.1% (58/92)	60.9% (56/92)	.76
Clinical Pregnancy rate	55.4% (51/92)	58.7% (54/92)	.66
Ongoing pregnancy rate	51.1% (47/92)	52.2% (48/92)	.88
Implantation Rate	41.8% (77/184)	38.9% (70/180)	.57
Biochemical pregnancy rate	7.6% (7/92)	2.2% (2/92)	.05
Miscarriage rate	4.3% (4/92)	6.5% (6/92)	.52
Delivery rate	51.1% (47/92)	52.1% (48/92)	.88
Twin Pregnancy rate	40.4%(19/47)	29.2%(14/48)	.25
Live births	66	62	.23

Table 10: Number of embryos transferred (expressed in mean ± SD) and clinical parameters per cycle.

	Group 1 (fresh oocytes) *n=92*	Group 2 (vitrified oocytes) *n=92*
Collected oocytes to baby ratio	5.6%	5.3%
Collected oocytes used to achieve a live birth rate	17.8	18.5
MII oocytes to baby ratio	6.8%	6.3%
MII oocytes used to achieve a live birth rate	14.8	15.8

Table 11: Oocyte to baby ratio & oocytes used to achieve a pregnancy

3.14 Clinical Trial 3

One of the main issues in vitrification, which brings a debate between embryologists, is that vitrification/warming can provoke a hardening of the zona pellucida (ZP) and this could result to absence of hatching and thus no implantation. There are studies that support that possibility (121) and some others that claim the opposite (122). All of them were conducted on vitrification of cleaved embryos or blastocyts. However, oocytes have thicker ZPs and the potential hardening of the ZP could actually be a possibility. A recent review and meta – analysis (123) showed that results after oocytes vitrification show great heterogeinity among IVF clinics, thus highlighting that there is space for improving the clinical outcome. Zona hardening could be a parameter that could influence negatively the implantation and perhaps assisting hatching could improve the clinical outcome.

3.14.1 Study design, size, duration:

A prospective, randomized, comparative study was performed from January 2015 to December 2015. All cases were participating in oocyte donation program of Iakentro IVF Center. Two groups were under study. In group A we included cases where embryos derived from donated, vitrified oocytes and they were transferred without assisted hatching. In group B we include cases in which embryos derived from vitrified oocytes and they were hatched prior transfer using a laser system.

Seventy cases were included in each group. In all cases, recipients were donated oocytes which were vitrified using a closed system vitrification. The two under study groups were formed after a randomization procedure. We used a simple randomization software which automatically formed a randomized order of 140 cases using laser

hatching or not (www.sealedenvelope.com). The vitrification/ warming protocol was the same with the one used in clinical trial #2. After warming, oocytes were fertilized and cultured to day 5.

The laser hatching that was performed in group B embryos was done 2h prior transfer. The opening of the ZP was around the 1/6 of its perimeter (Figure 14).

All transfers were performed on day 5 (blastocyst stage). All embryos culture methods, data collection procedures and statistical analyses were similar to the ones we used in clinical trial #2.

3.14.2 Main results and the role of chance:

Data from 140 oocyte donation cases were collected prospectively. The comparison of the clinical data of the two under study groups are shown in table12. Pregnancy rate (48.5% vs 74.28%), clinical pregnancy rate (45.7% vs 68.5%) and ongoing pregnancy rate (40.2% vs 66.5%) were significantly higher in group B (laser hatching group). Furthermore implantation rate was significantly higher in group B (29.2% vs 58.8%). In fact the IR was doubled when laser assisted hatching was used (table12).

Figure 14: Laser Assisted Hatching of a blastocysts that derives from closed vitrified oocyte. Laser pulse was used 2 h prior transfer.

	Group A Control (n=70)	**Group B** Laser Hatching (n=70)	**p**
Positive b-hCG	**48.5% (34/70)**	**74.28% (52/70)**	**.02**
Clinical Pregnancy rate	**58.7% (32/70)**	**68.5% (48/70)**	**.06**
Ongoing pregnancy rate	**40.2% (28/70)**	**68.5% (48/70)**	**.01**
N of Sacs	**38**	**80**	**.01**
Embryos transferred	**130**	**136**	**.92**
Implantation Rate	**29.2% (38/130)**	**58.8% (80/136)**	**.01**

Table 13: Data of clinical trial 3

PART 4 – DISCUSSION & CONCLUSIONS

4.1 Discussion

The present thesis had as a main purpose to study the application of vitrification, a cryopreservation technique, in human oocytes. The general valuation of our research is that vitrification of oocytes is a technique that can preserve human oocytes and thus the female fertility. The vitrification can be successfully performed either with open or closed devices. The fact that closed system vitrification performs equally to open system was something that was proven in this research. This result is considered important because now we can propose the replacement of open vitrification with aseptic (closed) vitrification. Closed system vitrification can provide excellent biological and clinical results leaving behind any concern regarding the biosafety of the human tissue when the last one comes in touch with liquid nitrogen.

The first aim of our research was to compare open and closed vitrification system. Before doing that, we had to select the protocols for this comparison. For the open vitrification we used the Kitazato protocol which is a very popular protocol for vitrification of embryos and oocytes. As far as it concerns the closed system, we had to form it ourselves. Closed systems were not popular when this research started and there were not available media for closed vitrification. Using our knowledge in cryobiology and based on a previous protocol for closed vitrification of blastocycts we set up experimental part 1 in order to form the ideal protocol for vitrifying human oocytes. From these experiments besides the protocol we retrieved useful conclusions. Firstly, closed system vitrification is possible as long as we increase the concentration of cryoprotectants. This increased concentration of cryoprotectants is not toxic for the cells. Both outcomes are a very interesting because for years many scientists in the field of human reproduction were

claiming that closed system is not an efficient technique. They supported that in closed system cooling rates are very low and cannot protect the cells and if we increase the concentration of the cryoprotectants to overcome this problem then the toxicity would be lethal for the cell. On the contrary, in experimental part #1 we prove that with the right modifications on the protocol, closed system is sufficient.

The second step of our research was to apply clinically closed vitrification system and to compare it with open. Clinical trial #1 was designed in order to compare the two methods. This prospective randomized study has shown that in oocyte donation cycles, although the survival rate of vitrified oocytes was lower for the closed system, the fertilization rate, the developmental competence of the resulting embryos, b-HCG positive rate, the implantation, the clinical pregnancy and the live birth rates did not differ when the closed or the open system were used for vitrification.

In that trial, in order to eliminate confounding effects related to the ovarian stimulation we studied a population of oocyte donors and their recipients sharing sibling oocytes coming from the same donor. Additionally, recipients received oocytes in a random way, further minimizing statistical bias.

This direct comparison between closed and open vitrification showed that survival rate is significantly lower in the closed (82.9%) comparing to the open (90.9%). Similar results are reported in a study where closed vitrification (survival rate 57.9%) is compared with open vitrification (survival rate 82.8%) (124). A possible explanation for this difference could be the lower cooling rates that are developed in the closed vitrification devices (< 2.000oC /min) comparing to those that are developed in the open devices

(>20.000oC /min) (100). However, a high survival rate has been achieved for a closed system recently (125). Moreover, the survival rates in clinical trial #1 are comparable to survival rates reported to multicenter cohort studies (126) and other randomized control studies in which oocytes were cryopreserved with open vitrification system or slow freezing (127).

Another parameter that could affect the survival of the oocytes is the warming rate which is equally essential to the cooling rates. According to Mazur and Seki observations (Mazur et al 2012, Seki 2012) warming rates could be more important than the cooling rates. The Vitrisafe device guarantees warming rates similar to those used in open vitrification devices (eg Cryotop) because the tip is plunged directly into the warming medium and therefore provides high warming rates.

Another variable that was examined in trial #1 was the use of more steps of stepwise additions to achieve higher cryoprotectant concentrations (20%/20%) with closed system compared to the three step addition to a final lower concentration (15%/15%) of the open system. The protocol that is used in the closed system of this study was based in a cinematographic analysis (Vanderzwalmen 2010) showing in a tangible way the collapsing and swelling behavior of the oocytes in response to the osmotic stress. A similar protocol was proposed (128) where the vitrification procedure consisted of seven steps with increased CP concentration for each step. However the gold standard for the necessary steps has to be further studied. In terms of biological and clinical parameters we saw that multiple steps of equilibration solutions gave similar results to the open vitrification. On the contrary, a recent study (125) demonstrates that

closed vitrification can be achieved without the need to modify the cryoprotectant exposure to counteract reduced cooling rates.

It has been reported that the open system of vitrification is more successful than the closed system (124). In this report the survival rate, the fertilization rate, the cleavage rate and the clinical pregnancy rate were significantly lower in the closed vitrification system compared to the open. The findings in our first trial were different. There are several possible explanations for these findings. The Pafoni's study (2011) was not a randomized controlled study with oocyte donation patients. Additionally, the devices used for the vitrification procedure were different. Paffoni et al (2011) used the Cryotip for the closed protocol and the Cryotop for the open protocol. In our study the vitrisafe device was used for all cases. In open vitrification systems the oocytes are in direct contact with LN2 and therefore the devices used can be easily compared. In closed systems, when different devices are used they may have different thermo-insulation capacities due to different design, protective straw and carrier thickness and due to different materials used for their production. But the main difference between the two closed systems is that cryotip uses low warming rates while Vitrisafe very high warming rates. This could be a detrimental difference between the two devices and it could justify the differences found between the two trials. Another study that uses closed system device similar to the present has been published recently (125). This study has shown high survival (90.2%), fertilization (77.5%) and embryo development rates (61.6%).

The implantation rate between the two groups (closed: 13.8%, open: 10.1%) of our study had no difference. But when we compare these rates with the implantation rates of our

fresh oocyte donation program (129,130) we can see that they are significantly lower. This could be explained by the fact that a significant proportion of the vitrified oocytes don't have the same developmental competency as the fresh ones. This fact could explain the significant difference in the embryonic score between our fresh (2.06±0.86) (130) and vitrified (closed: 2.62 ±1.25, open: 2.59 ± 1.25) oocyte donation program. In a cohort multicentre study (126) it is summarized that vitrification is a safe and efficient method for cryopreservation of human MII oocytes and they report live birth rate of 29.4%. The live birth rate (36.0%) in the closed protocol that we found in our first trial was similar to the rates reported in their study. The period (2011-2013) that the first clinical trial was conducted the above biological and clinical rates were promising and scientifically acceptable. The superiority of vitrification over slow freezing has been well established but as we mentioned above, the rates that had been achieved were significantly lower compared to those that fresh oocytes were providing. At the same time a RCT from a Spanish group (106) was demonstrating clinical rates similar to fresh. That study was a proof that there was space for improving the vitrification method since open system has reached high biological and clinical rates; it was extremely challenging to explore the possibility to reach the same results with a closed system.

In order to improve the results, we had to critically evaluate the findings of our first clinical trial. Besides the survival rate, open and closed protocol in our hands had the same results. This lower rate and the lower embryonic score that we had in vitrified oocytes, compared to fresh oocytes, led as to the assumption that the protocol we used, especially in closed vitrified oocytes was provoking a stress to the cells. Many scientists claim that lower cooling rate in closed system vitrification is the reason for lower survival

rates and developmental competence (85). This theory has been declined by an important scientific group (90,131) that works in cryobiology for decades. Their work demonstrates that if you keep a high warming rate then a cooling rate around 2000^{o} (closed system), is still high enough in order to achieve vitrification, especially if you increase the concentration of the CPs (like in our protocol). Ice crystal formation is more possible to form during warming (this procedure is called devitrification) than during cooling. The Vitrisafe is a closed device that ensures extremely high warming rates (>20.000^{o}C). In experimental part 1 we have thoroughly examined and formed the best CP exposure protocol focusing in minimizing any kind of stress. Moreover, by understanding the importance of the warming procedure, we question ourselves whether there was a stressful factor during the rehydration process that could compromise our results. The comparison between the protocol we used for warming and the one that was used by Cobo et al, led us to the conclusion that our rehydration process involved a higher exposure time in hypertonic solutions (e.g. 4 min to 0,5M). This step possibly was pushing cells to remain shrinked for a longer time than it was necessary and as a consequence the osmotic stress was higher than a cell could handle. The warming protocol we were using was designed for blastocysts and perhaps these expanded embryos need more time to hypertonic solutions during rehydration while the oocytes do not.

In experimental part 2, the above hypothesis was confirmed. When the exposure time in 0.5M and 0.25M was diminished, the survival rate of the warmed oocytes and the overall efficiency of the resulting embryos increased significantly. The rates of developed embryos and top quality embryos were high and reached similar percentages to embryos

deriving from fresh oocytes. In IVF unit of IAKENTRO Advanced Medical Center, Thessaloniki, Greece this modified protocol was used and clinical results (unpublished data) similar to fresh oocytes were achieved.

In order to verify the above protocol and to study the possibility that aseptically vitrified oocytes can retain their sufficiency and functionality, we set up clinical trial #2. In this trial we decided to do the absolute comparison; we tested clinically donated sibling fresh vs closed vitrified oocytes. This prospective comparative study brought two important messages. First, fresh and aseptically vitrified sibling oocytes have similar developmental potential, implantation, pregnancy and live birth rates in oocyte donation cycles. Second, these findings eliminate the fear that reduced cooling rates, produced in vitrification closed systems due to thermo-isolation, could be harmful or lethal to the cells. In this study, in order to eliminate confounding effects related to female age and ovarian stimulation, a population of oocyte donors and their sibling recipients who shared the oocytes of the same donor were included.

The final proof of the competence of the vitrified oocyte would still be healthy, live birth rates similar to the fresh. Using the open vitrification technique in oocyte donation program (106) reported similar outcomes between fresh and vitrified oocytes. Later, (132) reported comparable results obtained with vitrified oocytes using the open technique. Clinical trial 2 has shown that the same outcome could be reached by using our closed vitrification device(120). Implantation, pregnancy, clinical pregnancy, and ongoing pregnancy rates were equivalent between vitrified and fresh sibling oocytes. This enables us to easily comply with the strict quality requirements of the European tissue

directive 2004/23, 2006/17/EG, and 2006/86/EG (European Parliament and the Council of the European Union, 2004, 2006) without affecting the final outcome.

The survival rate of the vitrification group was 92.7%. This result is similar to the ones reported in open vitrification system studies (106) and above the average reported in a recent systematic review and meta-analysis (123). Fertilization, cleavage, top cleavage and blastocyst rates were similar between vitrified oocytes and fresh groups and in accordance with recent studies which used vitrified donor oocytes (123) .

The current study revealed a statistically higher rate of top quality blastocyst in the fresh oocytes group compared to the vitrified group. Although this difference did not affect the clinical outcome, an equivalent statistically significant decrease in biochemical pregnancies were observed in vitrified oocytes compared to the fresh. Recently it was reported that embryos resulted from vitrified oocytes had a different pattern of early embryo development compared to the ones derived from fresh oocytes (133). Additionally, it's been highlighted (134) that vitrified oocytes have cytoplasmic insufficiencies which might fails to support fertilization and early embryonic development. Obviously these two proposals could be useful to explain our lower rate of top blastocyst in embryos derived from vitrified oocytes. Otherwise, we should assume that this difference could be a random finding that could disappear in a larger scale study or the morphology is not a reliable criterion for the blastocyst's competence.

Combining the lower rate of top quality blastocyst with the lower rate of biochemical pregnancy found in the vitrified group led us to the assumption that vitrification could be a useful filter to eliminate the doubtfully competent oocytes that are able to be fertilized

and reach the blastocyst stage embryos but they fail to implant or to develop a normal pregnancy. Like a stress test, vitrification of oocytes could block the development of embryos destined to result in a biochemical pregnancy. However further prospective studies are needed to establish this assumption.

To our knowledge, this work is the only prospective study with the following characteristics: i) prospective study with randomization, ii) sibling donated oocytes, iii) one fresh group and one vitrification group, iv) vitrification of oocytes was performed in a closed system (aseptic conditions), v) in both groups, we did blastocyst culture and transfer. Because of these characteristics, we consider these findings of high importance. Therefore, we conclude that closed vitrification system, is an efficient strategy for vitrifying oocytes in aseptic conditions, leaving behind any concern regarding the biosafety of vitrified human tissue.

Looking to improve even more our knowledge and further the clinical result we performed clinical trial #3. One of the main debates among embryologists is wheatear laser hatching is beneficial for implantation. Studies on laser assisted hatching (LAH) on vitrified embryos are controversial. Some support the positive impact of the technique (121) while some others do not (122).

In the third clinical trial we reported a very important result. The assisted hatching of embryos that derive from closed vitrified oocytes helped the clinical outcome in a very impressive way: the implantation rate was double in laser hatching group. Once again this results came from a study in which two similar groups of oocytes donation cases were compared, leaving aside any confounding factor and thus enhancing the result of the

study. The ongoing clinical rate that was achieved in the laser hatching group was extremely high (68.8%) and the fact that derives from a group of vitrified oocytes makes it very important.

The development of a reliable and safe aseptic (closed) vitrification protocol is of high importance for human tissue cryopreservation after the Bielanski's reports (135,136,93) that have raised skepticism on the use of open vitrification protocols. The guidelines of European Parliament (European Parliament and the Council of the European Union, 2004, 2006) has impelled scientist to look for solutions that would maintain vitrification in an aseptic status. e.g. liquid nitrogen sterilized by filtration (137) or UV irradiation (96) or the storage of samples in vapors of nitrogen (138,95) and the majority of them were focused on how to solve the problem using the open system devices. The closed systems were over sighted and considered as potentially harmful for the cells due to the lower cooling rates. The results of our study have shown that closed vitrification systems are sufficient and can be used as devices to store biological samples in the safest way.

Nowadays, the indications for oocyte cryopreservation could be enlarged. Social egg freezing and donor egg banks could be promoted and used frequently since the vitrified oocytes appear to have the same competence to fertilize and implant with the fresh ones. In social egg freezing and egg donor cryo-banking, we expect a longer storage period for these oocytes, ranging from some months to several years. In such prolonged conditions our main concern is to store the biological material in the safest way. A closed system device could guarantee the appropriate isolation from any detrimental factor, like toxic low-molecular-weight compounds found in liquid nitrogen (Yan et al., 2011), whose long

term action is not known. In our study we obtained very promising results by using the safest choice for the storage of the oocytes in liquid nitrogen.

Oocyte vitrification is a new trend in modern society and nowadays is more than a backup plan in medical emergencies. In a recent meta- analysis (123) we notice heterogeneity in the results among the studies included in the systematic review. This difference could be related to the divergence of the techniques and devices available for oocyte vitrification. They concluded that the better results come from the clinics expertise in cryopreservation techniques. So, it is very important to find an easy and universal vitrification protocol which could reassure good results in every clinic.

There is a new era coming in human reproduction. The introduction of new technologies which use stem cells to improve implantation or to induce the generation of gametes is a matter of time to pass in humans. All these technologies use lines of stem cells that are cryopreserved at some point. The application of vitrification in human stem cells is something that should be happening with strict criteria and safety. The knowledge of this thesis could help the application of a closed to vitrify this type of cells.

5.1 Conclusions

- Closed vitrification system can provide excellent biological and clinical results.
- Closed vitrification system is as efficient as open for human oocyte cryopreservation.
- Closed devices can replace open devices for human oocyte vitrification and leave behind any concern regarding the biosafety of the human tissue when the last one comes in touch with liquid nitrogen.
- In donation cycles, vitrified oocytes give similar biological and clinical results to fresh oocytes.
- A stepwise approach for CP and water movement during dehydration and rehydration is beneficial for the cells in terms of survival rate.
- Warming procedure is more important that cooling procedure.
- Vitrification-warming procedure induces hardening of the zona pellucid.
- The application of laser assisting hatching before transfer in blastocyst that derive from vitrified oocytes improved the clinical outcome.
- The birth of healthy infants after closed oocyte vitrification and warming, verify the safety of the technique.
- The indications for oocyte cryopreservation could be enlarged. Social egg freezing and donor egg banks could be promoted and used frequently since the vitrified oocytes appear to have the same competence to fertilize and implant with the fresh ones.

Περίληψη

Εισαγωγή: Η υαλοποίηση είναι μία τεχνική κρυοσυντήρησης. Η χρήση αυτής της τεχνικής για την κρυοσυντήρηση ανθρώπινων ωαρίων προσέφερε πολύ ελπιδοφόρα αποτελέσματα. Για πολλά χρόνια η βασική επιλογή για την κρυοσυντήρηση των ωαρίων ήταν αργή κατάψυξη. Τα αποτελέσματα με τη μέθοδο αυτή δεν ήταν ικανοποιητικά και έτσι η κρυοσυντήρηση ανθρώπινων ωαρίων θεωρήθηκε ως πειραματική τεχνική. Η εισαγωγή της υαλοποίησης στην υποβοηθούμενη αναπαραγωγή ώθησε τους επιστήμονες να επανεξετάζουν τις επιλογές τους για τη κρυοσυντήρηση των ωαρίων. Τελικά η αργή κατάψυξη εγκαταλείφθηκε λόγω περιορισμού στην επίτευξη ικανοποιητικών κλινικών αποτελεσμάτων και αντικαταστήθηκε από την υαλοποίηση. Η πλειοψηφία των κλινικών ιατρικώς υποβοηθούμενης αναπαραγωγής, χρησιμοποιούν ανοικτά συστήματα για την υαλοποίηση των ωάριων. Η άμεση επαφή του βιολογικού υλικού με το υγρό άζωτο εξασφαλίζει ένα πολύ υψηλό ρυθμό ψύξης, ο οποίος πιστεύεται ότι είναι το κλειδί της επιτυχίας για την επίτευξη της υαλοποίησης. Από την άλλη πλευρά, η ίδια επαφή του βιολογικού υλικού με το υγρό άζωτο είναι η αφορμή μιας αντιπαράθεσης για την ασφάλεια του ανθρώπινου υλικού, τόσο κατά τη διάρκεια της διαδικασίας όσο και κατά την επ'αόριστο διατήρηση του δείγματος μέσα στο υγρό άζωτο. Υπάρχουν επιστήμονες που προτείνουν τη χρήση κλειστού τύπου συσκευών προκειμένου να πραγματοποιηθεί η υαλοποίηση. Η πρότασή αυτή έχει επικριθεί λόγω των χαμηλότερων ρυθμών ψύξης που επιτυγχάνονται με τη χρήση κλειστών συσκευών, οι οποίες δυνητικά μπορεί να οδηγήσουν σε αρνητικές συνέπειες για το κύτταρο. Με βάση την θεωρία της κρυοσυντήρησης, μία τροποποίηση του κλειστού πρωτόκολλο υαλοποίησης (όπου χρησιμοποιείται αυξημένη συγκέντρωση κρυοπροστατευτικών) θα

μπορούσε να αντισταθμίσει το χαμηλότερο ρυθμό ψύξης και τέλος, να επιφέρει εξίσου καλά αποτελέσματα. Η διατριβή αυτή είχε σαν αρχικό στόχο να μελετήσει την εφαρμογή κλειστών και ανοιχτών συστημάτων υαλοποίησης στα ανθρώπινα ωάρια και να συγκρίνει τις τεχνικές μεταξύ τους. Επιπλέον, προκειμένου να εξεταστεί η κλινική αποτελεσματικότητα της υαλοποίησης των ωαρίων, συγκρίναμε με άμεσο τρόπο την επάρκεια των φρέσκων και υαλοποιημένων ωαρίων.

Υλικά και Μέθοδοι: Αυτή η διατριβή αποτελείται από δύο πειραματικά μέρη και από τρεις κλινικές μελέτες. Τα πειραματικά μέρη είχαν ως στόχο την ανεύρεση του καλύτερου πρωτόκολλου για την υαλοποίηση και αναθέρμανση των ανθρώπινων ωαρίων. Όταν το κατάλληλο πρωτόκολλο διαμορφώθηκε, οι κλινικές δοκιμές έλαβαν μέρος. Στην πρώτη κλινική μελέτη πραγματοποιήθηκε σύγκριση μεταξύ των κλειστών και ανοικτών συστημάτων υαλοποίησης. Προκειμένου να μεγιστοποιηθεί η σημαντικότητα των ευρημάτων μας, χρησιμοποιήσαμε αδελφά ωάρια από δωρεά που υαλοποιήθηκαν είτε με ανοιχτό είτε με κλειστό σύστημα. Η δεύτερη δόκιμη είχε ως σκοπό να διερευνήσει την κλινική αποτελεσματικότητα του ωαρίου υαλοποίησης. Μια παρόμοια μεθοδολογία με τη πρώτη μελέτη εγκρίθηκε και έτσι χρησιμοποιήθηκαν αδελφά ωάρια από την ίδια δότρια τα οποία χωρίστηκαν σε δύο ομάδες. Οι ασθενείς της πρώτης ομάδας έλαβαν τα φρέσκα ωάρια και της δεύτερης τα υαλοποιημένα. Τέλος, σε μια τρίτη δοκιμή εξετάστηκε κατά πόσον η υποβοηθουμένη εκκόλαψη με χρήση λέιζερ λίγο πριν την μεταφορά εμβρύων που προέρχονται από υαλοποιημένα ωάρια είναι ευεργετική. Έμμεσος σκοπός αυτής της μελέτης ήταν να διερευνήσει κατά πόσον η υαλοποίηση δημιουργεί σκλήρυνση της διαφανούς ζώνης, προκαλώντας δυσκολία στην επίτευξη εκκόλαψης, δημιουργώντας έτσι ένα σημαντικό εμπόδιο για την εμφύτευση του εμβρύου.

Αποτελέσματα: Η χρήση ενός πρωτοκόλλου με σταδιακή προσθήκη και απομάκρυνση των κρυοπροστατευτικών μέσα και έξω από τα κύτταρα είναι σημαντική και αυξάνει τη βιολογική και κλινική αποτελεσματικότητα των υαλοποιημένων ωαρίων. Η υψηλοί ρυθμοί αναθέρμανσης βρέθηκαν να είναι εξίσου σημαντικοί με αυτούς της ψύξης. ΟΙ κλειστού τύπου συσκευές για την υαλοποίηση ανθρώπινων ωαρίων αποδείχθηκε ότι είναι εξίσου αποτελεσματικές με τις ανοιχτού τύπου συσκευές. Τα υαλοποιημένα ωάρια, με κλειστού τύπου συσκευές, έδωσαν παρόμοια κλινικά αποτελέσματα με τα φρέσκα ωάρια. Τέλος, η υποβοηθούμενη εκκόλαψη με χρήση λέιζερ βρέθηκε να είναι επωφελής για τα κλινικά αποτελέσματα.

Συμπέρασμα: Η υαλοποίηση αποτελεί την χρυσή επιτομή στην κρυοσυντήρηση των ωαρίων. Τα κλινικά αποτελέσματα που επιτεύχθηκαν μετά από υαλοποίηση ωοκυττάρων ήταν συγκρίσιμα με εκείνα που εξασφαλίζουν τα φρέσκα ωοκύτταρα. Το αποτέλεσμα αυτής της διατριβής είναι πολύ ενθαρρυντικά και θα μπορούσαν να αποτελέσουν την απάντηση σε μια διαρκή αντιπαράθεση μεταξύ εκείνων που επιμένουν ότι η ανοιχτού τύπου υαλοποίηση είναι δυνητικά επιβλαβής για το βιολογικό υλικό και εκείνων που επικρίνουν το χαμηλό ποσοστό ψύξης του κλειστού συστήματος, θεωρώντας πως είναι θανατηφόρο για τα κύτταρα. Η κλειστή τύπου υαλοποίηση είναι μια πολύ αποτελεσματική τεχνική και μπορεί να αντικαταστήσει την ανοιχτού τύπου υαλοποίηση, αφήνοντας πίσω κάθε ανησυχία σχετικά με τη βιολογική ασφάλεια των ανθρώπινων ιστών.

Abstract

Introduction: Vitrification is a cryopreservation technique. The use of this technique for cryopreserving human oocytes attributed in very promising results. For many years the standard option for oocyte cryopreservation was slow freezing. The results were not satisfactory and thus, oocyte cryopreservation in humans was considered as an experimental technique. The introduction of vitrification in assisted reproduction made reproductive scientists review their choices for cryopreserving eggs and finally abandoned slow freezing due to clinical insufficiency. The majority of ART clinics use open systems for vitrifying the oocytes. The direct contact of the biological material with the liquid nitrogen ensures a very high cooling rate which is believed to be the key of success for achieving vitrification. On the other hand, the same contact of biological material with liquid nitrogen is a source of debate for the safety of the human material. There are scientists that propose closed devices in order to perform vitrification. Their suggestion has been criticized due to the lower cooling rates that are achieved using closed devices, which potentially could lead to detrimental effects for the cell. Based on cryopreservation theory, a modification of the closed vitrification protocol (in which increased concentration of cryoprotectants are used) could counterbalance the lower cooling rates and finally to generate good results. In this thesis we present our work on oocyte vitrification. Open and closed systems for human oocyte cryopreservation have been compared. Moreover, in order to examine the efficiency of vitrification on oocytes we compared clinically fresh and aseptically vitrified oocytes.

Material and Methods: In this project we included two experimental sets and three clinical trials. In the experimental sets we focused on determining the best protocol for

vitrifying and warming human oocytes. When the appropriate protocol was set up, the clinical trials were generated. In the first trial a comparison of closed and open vitrified oocytes was performed. In order to maximize the importance of our findings we used sibling donated oocytes which vitrified either with open or with closed system. The second trial had as a purpose to explore the clinical efficiency of oocyte vitrification. A similar set up was adopted and sibling oocytes from the same donors were separated in two groups. One group involved patients that used fresh oocytes for their cycles and the second group included patients that used vitrified oocytes. Finally, a third trial was performed in order to examine whether the use of laser assisting hatching technique prior embryo transfer is beneficial. The purpose of this trial was to explore whether vitrification/warming induces zona pellucida hardening and whether laser assisting hatching could overcome this important barrier to implantation.

Results: A stepwise protocol for addition and removal of the cryoprotectants in and out of the cells is important in order to increase the biological and clinical efficiency of oocyte vitrification. Warming process was found to be as important as cooling. Closed devices for oocyte vitrification were proven to be as efficient as open. When the correct protocol was acquired, closed vitrified oocytes gave similar results to fresh oocytes. Finally, laser assisted hatching was found to be beneficial for the clinical result.

Conclusion: Vitrification has been proven to be the gold standard technique for cryopreserving oocytes. The clinical results that were achieved after vitrification of oocytes were comparable to those deriving from fresh oocytes. The outcome of this research is very promising and could be used in order to give and answer to an everlasting debate between those that insist that open system vitrification is potentially harmful for

the biological material and those that criticize the low cooling rate of closed system is lethal for the cells. Closed vitrification is a highly efficient technique and can replace open devices for human oocyte vitrification, leaving behind any concern regarding the biosafety of the human tissue.

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