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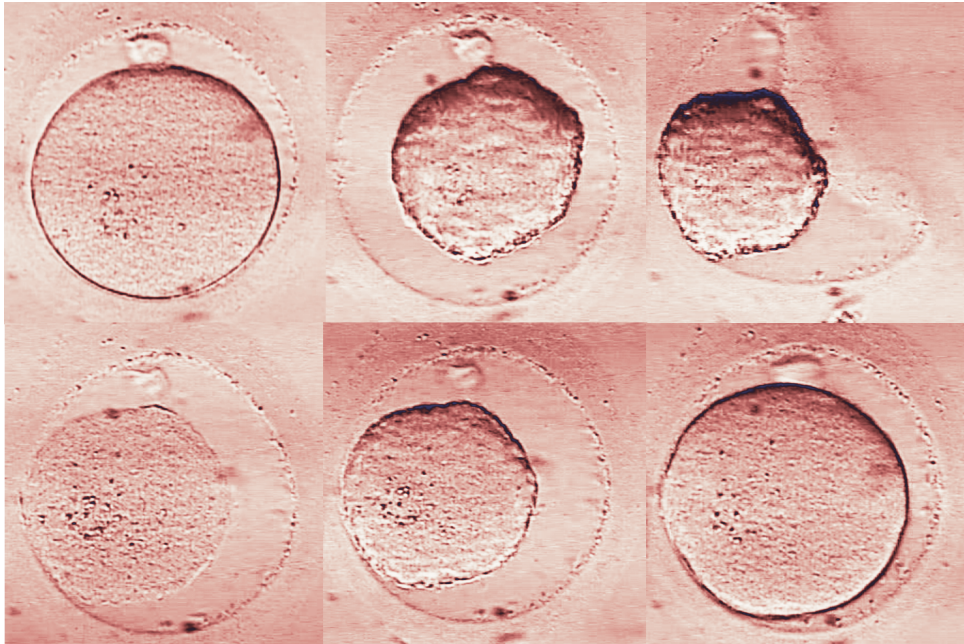
VITRIFICATION OF HUMAN OOCYTES EMPLOYING A CLOSED CARRIER WITH ENHANCED THERMAL EFFICIENCY AND SHORT TIMES OF EXPOSURE TO SYNTHETIC CRYOPROTECTANT SOLUTIONS

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TESIS DOCTORAL

VITRIFICATION OF HUMAN OOCYTES EMPLOYING A CLOSED CARRIER WITH ENHANCED THERMAL EFFICIENCY AND SHORT TIMES OF EXPOSURE TO SYNTHETIC CRYOPROTECTANT SOLUTIONS

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Prefacio

Desarrollar una tesis doctoral sobre criobiología en la Escuela Superior de Ingenieros de Sevilla, concretamente enfocada en la criopreservación de óvulos humanos, y compaginarla con el trabajo de embriólogo clínico suponía un gran desafío. El contexto del programa de doctorado donde se ha desarrollado este trabajo me ha obligado a abordar la cuestión desde una perspectiva centrada en las leyes de la física que regulan los procesos de criopreservación, en lugar de simplemente explicar el efecto de los mismos sobre las muestras biológicas sobre las que se aplican.

Sin embargo, esta situación que *a priori* juzgaba en mi contra, ha resultado ser un punto a favor, obligándome a salir de mi zona de confort científica, y adentrarme —o más bien mojar los pies en la orilla— en otras áreas de conocimiento. En mi perspectiva actual, después de esta inmersión de 4 años en el mundo de la criobiología, veo que mi situación no sólo no es inusitada, sino que es de hecho el recorrido natural de esta disciplina. Si se echa la vista hacia atrás, muchos de los criobiólogos que han investigado sobre la materia provienen de una formación en ciencias de la salud. Sirven como ejemplo el considerado padre de la criobiología moderna y recientemente fallecido Peter Mazur, así como uno de los actuales referentes en el área, Gregory Fahy. Ambos licenciados en biología.

Sin embargo, no es fácil encontrar un contexto de investigación interdisciplinar donde un profano en la materia pueda adquirir y profundizar en los conocimientos relevantes de biofísica de la criobiología, especialmente en el ámbito nacional. Es por ello que aprovecho para agradecer al Dr. Ramón Risco y a los Sres. Ingenieros de la comisión del programa de doctorado la oportunidad que se me ha brindado.

Sin más preámbulo, espero que los lectores de los diferentes gremios científicos puedan disfrutar de este trabajo.

Agradecimientos

Me gustaría agradecer a todos mis compañeros de laboratório de FIV de Ginemed Sevilla (Ana, Bea, Elena, Gema, Laura, María, Marta, Mercedes, Mónica, Mercedes...) el apoyo para llevar a cabo la tesis y lo que me han enseñado durante mis comienzos como embriólogo. Igualmente agradecer a mis compañeros del laboratorio de Ginemed Lisboa que me han acompañado en los dos últimos años: Lorena, Pedro, Ana, María, Joana, la Dra. Ana Paula y el resto del equipo. Y por supuesto a la dirección de Ginemed —Pascual, Fernando, Manolo, Eva— por su apoyo para desarrollar este proyecto.

Estoy profundamente agradecido y en deuda con la Dra. Christiani Amorim por acogerme en su grupo de investigación de la Universidad Católica de Louvain para realizar una estancia de investigación en la línea de investigación de criopreservación de tejido ovárico. Una experiencia formativa enorme en todos los sentidos y con un equipo de calidad humana y profesional muy grande, liderado por la Prof. M-M Dolmans, que me hicieron aprender mucho y pasármelo muy bien. Un abrazo para mi compañero Javier, el maestro cafetero Guillaume, para Emna, Diego, Luciana, Alice, Rosella, Costy, Parinaz, y por supuesto Olivier y Dolores sin los que no me hubiese salido ninguna inmuno. También agradezco a Samuel Ribeiro y Neelke de Munck su cercanía y disponibilidad para enseñarme. Agradecer a Mike y Pilar el periodo formativo importantísimo con su grupo de investigación durante mis prácticas universitarias, cuya calidad humana y profesional me han servido de referencia desde entonces.

Aprovecho también para dar las gracias a Ariadna Corral y a Jaime Sáenz, antiguos alumnos de la Universidad de Sevilla y expertos en Criobiología, por sus generosas contribuciones a esta tesis, así como a Juan Tubío, uno de los culpables de que me interesase por la vitrificación en primer lugar.

Agradecimientos sinceros para mis directores de tesis, los Dres. Luis Vilches y Fernando Sánchez y sobre todo al Dr. Ramón Risco, por enseñarme y animarme a aprender, espero que sea capaz de preservar y transmitir todo ese conocimiento durante mucho tiempo.

Dedicado a todos mis amigos, familia y pareja, por todas las horas que les he robado para dedicarme a esto. Los últimos y más importantes agradecimientos a mis padres Curro y Maribel, por ponérmelo tan fácil, a mi hermano Javier que siempre me obliga a esforzarme, y a Catarina por su apoyo incondicional, y a Nebraska y Nzinga por su compañía en los meses de escritura.

Lista de publicaciones

I. Gallardo, M., Hebles, M., Migueles, B., Dorado, M., Aguilera, L., González, M., ... & Risco, R. (2016). Thermal and clinical performance of a closed device designed for human oocyte vitrification based on the optimization of the warming rate. Cryobiology, 73(1), 40-46.
https://doi.org/10.1016/j.cryobiol.2016.06.001

II. Gallardo, M., Hebles, M., Migueles, B., Dorado, M., Aguilera, L., González, M., ... & Sánchez-Martín, F. (2017). Hydroxypropyl cellulose supplementation in vitrification solutions: a prospective study with donor oocytes. *Journal of assisted reproduction and genetics*, *34*(3), 417-422.
https://doi.org/10.1007/s10815-016-0841-y

Declaración de autoría de las publicaciones

En los dos artículos originales de investigación que constituyen el cuerpo principal de esta tesis, Miguel Gallardo ha llevado a cabo el trabajo experimental, la autoría intelectual y la redacción del manuscrito, bajo la supervisión y asistencia del autor de correspondencia de las publicaciones y director de tesis Dr. Ramón Risco, y los directores de tesis Dr. Luis Vilches y Dr. Fernando Sánchez. Los otros coautores participaron en los procedimientos clínicos y de embriología descritos en los manuscritos. Todos los coautores han confirmado esta contribución y estan conformes con la presentación de estos trabajos como tesis por compendio de publicaciones.

Ponencias en congresos

- M. Gallardo Molina et al. 2015. Recalentamiento ultra-rápido: altas tasas de recuperación de ovocitos vitrificados en el sistema cerrado safespeed. Un nuevo paradigma en criopreservacion. *Rev Asoc Est Biol Rep.* Noviembre 20(2);109.

- M. Gallardo Molina et al. 2016. Hydroxypropyl cellulose is an effective surfactant agent for vitrification solutions: a prospective study on donor oocytes. European Society of Human Reproduction and Embryology. *Human Reproduction.* 31(suppl1); 100-1.

- M. Gallardo Molina et al. 2016. Hidroxi Propil Celulosa como agente surfactante en las soluciones de vitrificación: estudio prospectivo en óvulos de donante. Sociedad Española de Fertilidad. *Medicina Reproductiva y Embriología Clínica.* 2016;3 (Supl1-52); 31.

Otras publicaciones no incluidas en la tesis

- Guerrero, J., Gallardo, M., Rodríguez-Arnedo, A., Ten, J., & Bernabeu, R. (2018). Comparison of two closed carriers for vitrification of human blastocysts in a donor program. *Cryobiology.* 81, 12-16.

- Corral, A., Clavero, M., Gallardo, M., Balcerzyk, M., Amorim, C. A., Parrado-Gallego, Á., ... & Risco, R. (2018). Ovarian tissue cryopreservation by stepped vitrification and monitored by X-ray computed tomography. *Cryobiology.* 81, 17-26.

Resumen en castellano

La criopreservación consiste en la reducción de la temperatura del organismo, con el objetivo de detener de por completo la cinética de las reacciones biológicas que mantienen la homeostasis celular, y que al mismo tiempo provocan el envejecimiento de las mismas. Al arrestar el metabolismo celular, se logra la preservación del material biológico por tiempo indefinido.

El principal problema asociado a la disminución de la temperatura de una célula para tal propósito es la tendencia a la cristalización del medio acuoso intracelular durante el enfriamiento. La configuración natural de los grupos de moléculas de agua dentro de las células es el estado desordenado propio del estado líquido. Sin embargo, el agua, al adquirir la estructura cristalina propia del hielo, se expande, dañando las estructuras biológicas y comprometiendo su viabilidad.

La vitrificación es una transición de fase de segundo orden alternativa a la cristalización, en la que la solución acuosa adquiere propiedades de un sólido manteniendo el desorden natural de las moléculas de agua y solutos presentes en el citosol. Debido a que no se produce la reconfiguración molecular propia de la formación de hielo, permite mantener el sistema biológico que se pretende preservar sin alteraciones. Por lo tanto, para la criopreservación de material biológico, concretamente óvulos humanos en el caso de esta tesis, es necesario que el medio intracelular alcance el estado vítreo y se evite la formación de hielo, si no por completo, al menos cantidades lesivas del mismo. Existen diferentes estrategias para alcanzar el estado vítreo, pero en esta tesis nos centraremos en la técnica de vitrificación ultrarápida. Dicha técnica consiste en realizar el enfriamiento, desde la temperatura ambiente hasta temperaturas criogénicas estables (normalmente los -196 ºC del nitrógeno líquido), y posteriormente el recalentamiento, a altas velocidades.

El gameto femenino —el óvulo— es la célula humana de mayor tamaño. Para su criopreservación en el contexto de la reproducción humana asistida, este se encuentra en un estado de arresto en la metafase de la segunda división meiótica, preparado para la fecundación. Su gran tamaño y la presencia de orgánulos muy sensibles hacen que su criopreservación sea compleja, comparada con la de embriones en los primeros días de desarrollo. Sólo mediante la técnica de vitrificación ultrarrápida, sobre la que se trabaja en esta tesis, se consigue su criopreservación de forma satisfactoria.

Durante un proceso de vitrificación ultrarrápida, que se produzca o no la indeseada formación de hielo dependerá de las velocidades de enfriamiento y recalentamiento alcanzadas, y de las propiedades de la solución —en este caso el citosol, la fracción líquida intracelular. Con la concentración de solutos y viscosidad del citosol de los óvulos en su estado natural, las velocidades de enfriamiento y recalentamiento necesarias para evitar la formación de hielo serían muy difíciles de obtener. Por este motivo, es necesario aumentar la tendencia a la vitrificación del citosol, con un protocolo de preparación. Esta fase de preparación consiste en aumentar la concentración intracelular de solutos y la viscosidad del citosol, hasta un punto crítico que permita alcanzar la transición vítrea, a las velocidades de enfriamiento y recalentamiento obtenidas por los soportes de vitrificación actualmente disponibles. Para ello, el óvulo se expone a una serie de soluciones hipertónicas, que provocan la permeación de solutos hacia el interior celular y la salida de agua. Una vez concluida la preparación, el óvulo se coloca en un soporte de vitrificación —pajuela— y se procede al enfriamiento, por lo general por inmersión en nitrógeno líquido.

En esta tesis se describen dos estudios prospectivos con óvulos de donante llevados a cabo en una clínica de fertilidad (Ginemed Sevilla), en los que se estudia la eficacia del sistema de vitrificación desarrollado por la spin-off

de la Universidad de Sevilla Safepreservation, bajo la dirección científica del Dr. Ramón Risco. En el primer estudio, se emplea un kit soluciones de vitrificación cuya novedad es su composición totalmente sintética, sin la presencia de proteínas de origen humano como la albúmina, sustituidas por el polímero hidroxipropil celulosa. Este tipo de moléculas son generalmente empleadas como agentes surfactantes y también desempeñan actividad osmótica. Se comparan los resultados clínicos de un grupo de óvulos vitrificados con soluciones sintéticas frente a otro grupo de óvulos, proveniente de la misma donante, no sometidos a la vitrificación. Se comprueba que la alternativa desarrollada por el grupo de Risco obtiene unos resultados satisfactorios, similares a los obtenidos con soluciones de vitrificación clásicas que incorporan en su formulación proteínas de origen humano o animal, sin las desventajas asociadas a las mismas, como lo son el riesgo de contaminación y una vida útil reducida.

En el segundo trabajo se emplea un diseño experimental similar al del primer estudio, para probar la eficacia del soporte cerrado de vitrificación SafeSpeed. El soporte está compuesto por un capilar ultrafino unido a una pajuela de resina ionomérica. El capilar donde se cargan los óvulos para la vitrificación permite maximizar las tasas de enfriamiento y recalentamiento, ya que la eficiencia térmica del mismo es ampliamente superior a la de otros soportes de vitrificación disponibles actualmente. Cabe resaltar también el hecho de que es un sistema cerrado, en el que las muestras biológicas no entran en contacto con el nitrógeno líquido empleado para el enfriamiento y la manipulación, minimizando el riesgo de contaminación por agentes patógenos presentes en el mismo. En este caso también comprobamos que las tasas de supervivencia ovocitaria al procedimiento de vitrificación con este soporte son excelentes, y que el desarrollo de los embriones resultantes de los óvulos vitrificados es similar al obtenido con óvulos que no han sido sometidos a la vitrificación.

Con estos resultados, queda patente que este sistema de vitrificación desarrollado en la Universidad de Sevilla es una herramienta muy efectiva que permite obtener resultados punteros en el contexto clínico. Sin embargo, los protocolos de vitrificación aún son susceptibles de mejora. A pesar de que el enfriamiento y el calentamiento de las muestras es ultrarápido, el procedimiento en conjunto requiere mucho tiempo, ya que el tiempo estándar para preparar cada grupo de hasta 3 óvulos para la vitrificación lleva de 10 a 15 minutos de exposición a soluciones hipertónicas. Es deseable una reducción de la duración de esta fase para mejorar el flujo de trabajo en el laboratorio de FIV y reducir el tiempo de exposición a crioprotectores potencialmente tóxicos.

Con el objetivo de explorar esta posibilidad, decidimos estudiar la dinámica de la permeación de crioproctectores en el ovocito humano. En primer lugar, con una aproximación *in silico*, mediante un programa desarrollado en MatLab para integrar las dos ecuaciones diferenciales que describen la permeabilidad de la membrana plasmática del óvulo según un modelo 2-P. Estas simulaciones fueron complementadas con observaciones *in vivo* del comportamiento osmótico de los ovocitos. En un protocolo de preparación de óvulos para la vitrificación clásico, en primer lugar se realiza una exposición prolongada (10-15 minutos) del óvulo a una solución con una concentración intermedia de crioprotectores (≈25% w/w), denominada solución no-vitrificante o solución de equilibrado, seguida de una segunda exposición corta a una solución vitrificante (≈45% w/w). Comparamos la actividad osmótica que se produce en el protocolo estándar contra la de un protocolo corto, basado en la deshidratación, en el que la duración de la exposición de los óvulos tanto a la solución no-vitrificante como a la vitrificante era limitada a 60 segundos.

Comprobamos que la deshidratación del óvulo tras la exposición a soluciones de vitrificación ocurre muy rápido; el punto de volumen mínimo de la curva de contracción y expansión resultante del gradiente

osmótico se alcanza dentro de los primeros 60 segundos. En ese punto, el contenido de agua intracelular es mínimo, la penetración de crioprotectores de bajo peso molecular es casi completa y, como resultado, la concentración total de soluto intracelular es alta. Por lo tanto, prolongar la primera fase de exposición hasta 15 minutos, según es recomendado en los protocolos actuales, no produce una mejora significativa de la tendencia a la vitrificación del citosol del ovocito, y no mejora presumiblemente sus posibilidades de vitrificar a determinadas tasas de enfriamiento y recalentamiento. Los resultados de las pruebas en óvulos humanos y embriones, no aptos para uso clínico y donados para la investigación, muestran que la tasa de supervivencia a la vitrificación no se ve comprometida por reducir los tiempos de exposición, confirmando que el efecto osmótico deseado se produce en un tiempo reducido.

Las innovaciones en las técnicas empleadas para vitrificar las células reproductivas se rigen por la premisa de que se debe mantener un compromiso entre la seguridad y la eficacia; la técnica debe ser lo más aséptica y efectiva posible. Se podría argumentar que SafeSpeed, como soporte cerrado de virificación, cumple con este criterio de mejorar la eficiencia —eficiencia térmica, al menos— sin comprometer la seguridad, y como tal podría considerarse como un avance en la dirección correcta. Igualmente, el uso del polímero sintético hidroxipropil celulosa también representa una alternativa más segura y más estable a proteínas derivadas de humano o animal. Por último, lo mismo podría decirse sobre nuestros intentos de acortar el protocolo de vitrificación: si los ovocitos y embriones demuestran ser igualmente competentes, reducir la duración de la exposición a crioprotectores potencialmente citotóxicos y a temperaturas subóptimas debería ser más seguro. En última instancia, para cada modificación relevante del procedimiento, se debe realizar una ruta de validación bien diseñada antes de su uso en el contexto clínico,

desde la etapa preclínica en modelos de mamíferos y material humano donado, hasta estudios clínicos prospectivos controlados.

Abstract

In the first part of this thesis, the effectiveness of a vitrification system, developed by the spin-off of the University of Seville, Safepreservation, is studied. Two prospective studies with donor eggs, carried out in a fertility clinic (Ginemed Sevilla) were conducted. In the first study, we test a vitrification solution of fully synthetic composition, free of proteins of human origin such as albumin, which are substituted by the polymer hydroxypropyl cellulose. This type of molecules are generally used as surfactants and also perform osmotic activity. The clinical results of a group of oocytes vitrified with these solutions are compared against another group of oocytes, coming from the same donor, not subjected to vitrification. The results show that the synthetic alternative developed by Risco's group provides satisfactory embriology outcomes, similar to those obtained with classic vitrification solutions that incorporate in their formulation proteins of human or animal origin. Yet, without the disadvantages associated to them, such as the risk of contamination and a reduced lifespan.

In the second work, an experimental design similar to that of the first study is used to test the effectiveness of the closed vitrification carrier "SafeSpeed". The carrier consists of an ultra-thin capillary attached to a straw of ionomeric resin. The capillary where the oocytes are loaded for vitrification allows to maximize the rates of cooling and warming, since its thermal efficiency is superior to that of other vitrification carriers currently available. It should also be noted that it is a closed carrier, in which biological samples do not come into contact with the liquid nitrogen used for cooling and storage, minimizing the risk of contamination by pathogens. The outcomes of this study show that the ovocytary survival rates to the vitrification procedure with this carrier meet and even exceed current standards, and that the development of the embryos resulting

from the vitrified eggs is similar to that of embryos from eggs that have not undergone vitrification.

The results of this two studies provide initial evidence that the vitrification system developed at the University of Seville is a very effective tool that allows obtaining benchmark results in the clinical context. However, the vitrification protocols are still susceptible to improvement: although the cooling and warming of the samples is ultra fast, the whole procedure is time consuming, since each group of up to 3 oocytes takes from 10 to 15 minutes of exposure to hypertonic solutions with cryoprotectants to be prepared for vitrification. A reduction in the duration of this phase is desirable to improve workflow in the IVF laboratory and reduce the exposure time to potentially toxic cryoprotectants.

In order to explore this possibility, we decided to study the dynamics of the permeation of cryoprocectors in the human oocyte. Firstly, with an *in silico* approach, through a program developed in MatLab to integrate the two differential equations that describe the permeability of the plasma membrane of the human Metaphase-II oocyte, according to a 2-P model. These simulations were complemented with *in vivo* observations of the osmotic behavior of the oocytes. The standard protocol for the preparation of oocytes for vitrification consists on a prolonged exposure (10-15 minutes) to a solution with an intermediate concentration of cryoprotectants ($\approx$25% w/w), called a non- vitrifying, or equilibration, solution. This is followed by a second short exposure to a vitrifying solution with higher CPA concentration ($\approx$45% w/w). We compared the osmotic activity that occurs in the standard protocol against that of a short protocol, based on dehydration, in which the duration of exposure of the oocytes to both the non-vitrifying and vitrifying solutions was limited to 60 seconds

We verified that the dehydration of the oocyte after exposure to vitrification solutions occurs very fast; the minimum volume point of the

contraction and expansion curve resulting from the osmotic gradient is reached within the first 60 seconds. At that point, the intracellular water content is minimal, the penetration of low molecular weight cryoprotectants is almost complete and, as a result, the total concentration of intracellular solute is high. Therefore, prolonging the first exposure phase up to 15 minutes, as recommended in the current protocols, would presumably not reduce the chance of lethal ice formation at certain rates of cooling and warming. The results of tests on human oocytes and embryos, unsuitable for clinical use and donated for research, show that the vitrification survival rate is not compromised by reducing the exposure times, confirming that the desired osmotic effect occurs in a reduced time.

Innovations in the techniques used to vitrify reproductive cells are governed by the premise that a compromise must be maintained between safety and efficacy: the technique must be as aseptic and effective as possible. It could be argued that SafeSpeed, as a closed support for virification, meets this criterion of improving efficiency —thermal efficiency, at least— without compromising safety, and as such could be considered a step in the right direction. Likewise, the use of the synthetic polymer hydroxypropyl cellulose also represents a safer and more stable alternative to proteins derived from human or animal. Finally, the same could be said about our attempts to shorten the vitrification protocol: if oocytes and embryos prove to be equally competent, reducing the duration of exposure to potentially cytotoxic cryoprotectants and at suboptimal temperatures should be safer. Ultimately, for each relevant modification of the procedure, a well designed validation route must be performed before its use in the clinical context, from the preclinical stage in models of mammals and donated human material, to prospective controlled clinical studies.

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1. Introduction

"Interestingly, most of the water in the universe may exist as glass rather than ice, as a result of vapor deposition on dust grains in the coldness of space."

Bryan Wowk

1.1 The vitreous state, and its potential for cryopreservation

Cooling a cell, tissue or organism can halt the ever-occurring myriad of chemical reactions that maintain homeostasis, effectively stopping life in time, and avoiding its otherwise unavoidable decay (Tsujimoto et al., 2016). However, the high content of water present in living organisms tends to turn into ice when their temperature is decreased, disrupting biological structures and damaging cells, making cryopreservation a challenge (Fahy et al., 1984).

However, upon cooling, liquids can also, on the other hand, undergo a second-order phase transition and become amorphous solids; in this state of the matter molecules are found in the disordered arrangement typical of the liquid state, but are rigid (Jenniskens & Blake, 1994; Wowk, 2010). The process of formation of glass from the liquid state by cooling is called vitrification. The potential of vitrification as a mean for cryopreservation of living organisms was first identified by Stiles in 1930: if the intracellular water could become an amorphous solid upon cooling, instead of ice, it should "give the original system again, without change", after warming (Stiles, 1930).

Soon, Luyet would start actively pursuing vitrification as a means of cryopreservation, pioneering research in the field and identifying critical factors to avoid the formation of intracellular ice (Luyet, 1937). However,

the conditions necessary for successful vitrification were seemingly either unattainable or incompatible with cellular life. That is until Polge (1949) and Smith (1950) described the cryoprotection ability of glycerol for cryopreservation of fowl spermatozoa and red cells. As early as 1963, cryobiologist Peter Mazur would establish the thermodynamical basis of the slow-freezing technique of cryopreservation, cooling cells in a slow, controlled fashion (Mazur, 1963). By 1980, the occurrence of intracellular vitrification was demonstrated by Rall and colleagues (Rall, Reid, & Polge, 1984), and soon, with Fahy, he would achieve succesful vitrification of mouse embryos by ultrafast cooling and warming (Rall & Fahy, 1985). This milestone would not go unnoticed in the blooming field of human embryology; the ability to produce embryos *in vitro* was perfectly complemented by the ability to cryopreserve them inexpensively and fast by vitrification (Edwards, Bavister & Steptoe; 1969). This sparked interest in the technique, and the rest is history: in 2015, more than 100,000 human oocytes and embryos were cryopreserved by vitrification in Spain alone, in the context of assisted reproduction treatments (Registro SEF, 2015).

1.2 Vitrification at a molecular level

Vitrification is a phase transition that only occurs under very specific conditions: the most common reaction of an aqueous solution upon cooling is the formation of ice, as the melting temperature is, in most cases, higher than the glass transition temperature (Figure 4). Ice starts to form by the stochastic process of ice nucleation, when a cluster of water molecules forms a structure of stable hydrogen bonds — a nucleus — of a critical size that serves as a base from where this sequence is repeated, resulting in ice growth (Pradzynski et al., 2012). This process is liable to happen when the solution reaches its heterogeneous melting temperature, meaning that it is always catalysed by a nucleating agent, usually particles

present in the solution or surfaces the solution has contact with (Fuller & Paynter, 2004).

As ice grows, the disordered water molecules in the liquid rearrange into the hexagonal shape of a solid crystal. When this process occurs in the intracellular liquid milieu —the cytosol— ice will rapidly grow to a size that is physically damaging for the biological ultrastructures and will compromise their viability, usually with lethal consequences (Leibo, McGrath, & Cravalho, 1978). The formation of more than trace amounts of ice in cells is a lethal event (Karlsson et al., 1993). Conversely, if the cytosol undergoes vitrification, the molecular arrangement, and consequently the biological structures, will be preserved (Leibo et al., 1978; Luyet & Gehenio, 1947; Mazur, 1963).

However, it is no coincidence that vitrification is a phenomenon predominant in outer space, as the lack of heat is the factor that creates the thermodynamic necessity of the vitreous state (Jenniskens & Blake, 1994; Fahy, *Vitrification in assisted reproduction*, 2015, p.8). At a molecular level, as an aqueous solution is cooled, the kinetic energy of water molecules, their constant agitation, is reduced, and hydrogen bonds become more stable, favoring the formation of the crystaline structure. However, if the translational and rotational degrees of freedom of the molecules in the liquid state are reduced by cooling to those of a solid, without ice forming in the process, the result is an amorphous rigid glass (Kauzmann; 1948) by a second-order phase transition: vitrification. The achievement of such a state, especially in the aqueous media within a cell, poses however a challenge for many reasons, that will be described below.

1.3 How to attain the vitreous state: techniques for the cryopreservation of living cells

As previously stated, lowering the temperature of living systems is a very effective mean to decelerate the rates at which biologic machinery is

working, as described by Arrhenius' equation. A practical example is induced hypothermia for short-term preservation (Fuller & Lee, 2007). However, to accomplish total suspension of life, temperatures must be reduced to the cryogenic range. When liquids present in biological organisms are cooled for that purpose, they will reach their melting temperatures and thermodynamically favour the transition to the solid state by crystallisation, unless certain strategies are adopted to avoid it (Wowk, 2010).
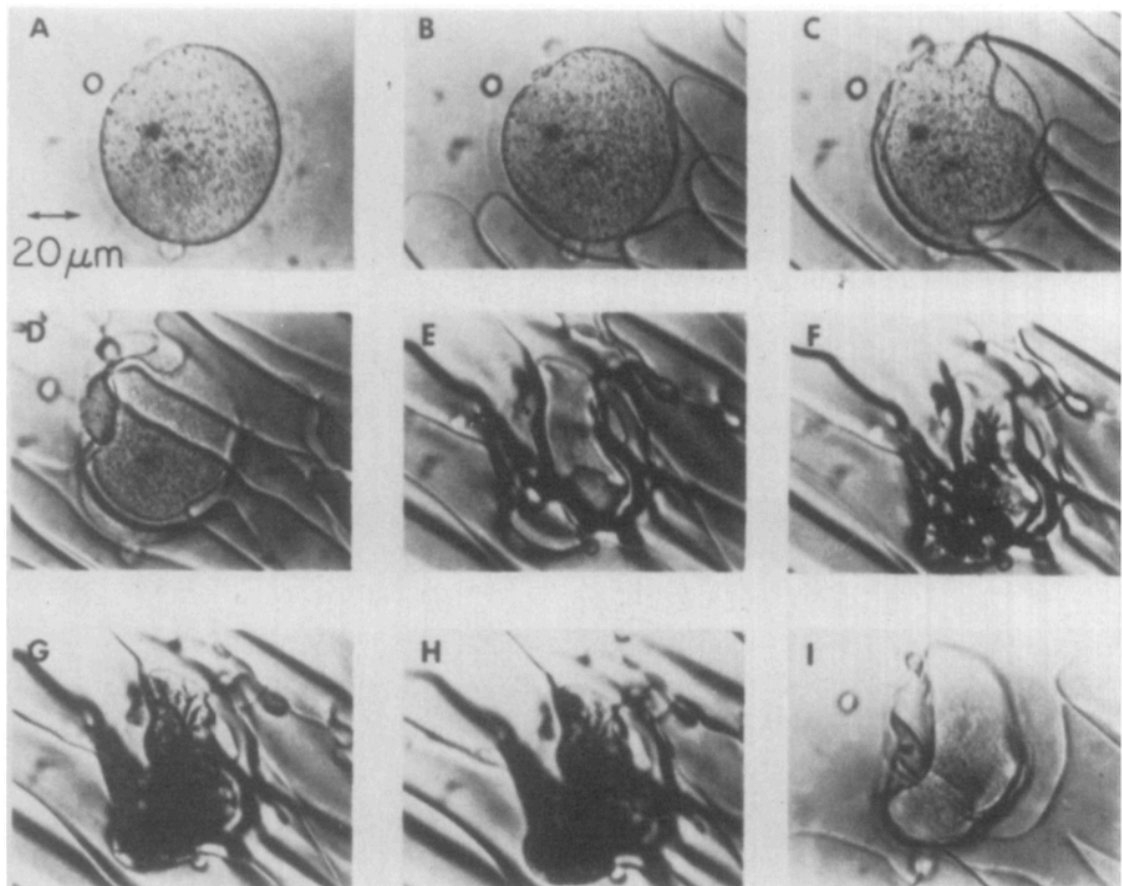
Figure 1. Mouse ovum cooled at 3 ºC/min to 0 (A), -3 (B), -5 [C], -20 (D), -28 (E), -48 (F), -50 (G), and -52 °C (H). The microscopic observations show that intracellular freezing generally occurred at about −40 to −45 °C. The sample was then warmed at 8 ºC/min and photographed as the ice melted at about -4 °C (I). Reproduced from Leibo et al. (1978).

Within the context of this thesis, we will be working within a very specific scenario, which is the cryopreservation of a single cell in the case of human eggs, or early stage embryos formed by a small number of cells with similar characteristics in terms of osmotic sensitivity and hydraulic conductivity. Tissues and organs, dense and with diverse cell types, are a different challenge and require different approaches outside the scope of this work (Amorim et al., 2011; Fahy et al., 2004; Pegg, Wang, & Vaughan, 2006).

1.3.1 Controlled rate freezing

The first cryopreservation technique that was applied to human embryos, and with very limited success to oocytes, was the controlled rate freezing (Whittingham, Leibo, & Mazur, 1972). In this technique, cells are suspended in a solution with a low initial concentration of cryoprotectant agents; in the case of human embryos, the most commonly employed cocktail is a combination of 1.5M of propylene glycol and 0.1M of sacarose. This suspension is loaded in a straw of 0,1-0,5 mL, which is cooled in a controlled, slow fashion with a programmable freezer: a common protocol is to employ a cooling rate of 2 °C/min until the seeding (manual induction of ice formation in the solution, usually by physical contact with a pre-cooled forceps) temperature around –7 °C. After seeding, a slower cooling rate of 0.3–1.0 °C/min is usually employed until temperatures around -70 °C are reached (Trounson & Mohr, 1983; Lassale et al., 1985). This technique is based in the gradual depression of the freezing point resulting of the increase in viscosity the solution experiences during cooling, and most importantly, the increase in solute concentration produced by extracellular ice formation. The temperature of the system is decreased just above the rate of depression of the freezing point, until the glass transition temperature is attained and the cytosol undergoes vitreous solidification.

1.3.2 Ultra-fast vitrification

The procedure of ultra-fast vitrification is a more radical approach, as the temperature is lowered below the melting point, all the way to the glass transition temperature. It relies on performing the cooling of the cells, and most importantly, the warming, at such high rates (usually 10^3 to 10^6 °C/min) that the water molecules in the cytosol, despite being below their melting point, do not have the necessary time to rearrange, form ice crystals and grow to a size that can produce cellular injury or death (Fahy et al., 1984). Before this happens, the solution reaches the glass transition temperature and becomes an amorphous solid, preserving the disordered molecular configuration of the living state.

However, for vitrification of human eggs and embryos, and most other cells for that matter, the liquid fraction of the cells' cytoplasm must acquire certain characteristics that will favour the glass transition, such as low free-water content, high viscosity and concentration of solutes. These factors will affect the coligative properties of the solution, decreasing the chances of ice formation.

1.4 Factors that determine the probability of vitrification

Ice nucleation and the kinetics of ice growth are complex physical phenomena. For simplicity's sake, it makes sense to separate the factors that determine the probability of ice formation during a vitrification procedure into two categories. On the one hand, the glass forming tendency of the liquid part of the system —the solution. On the other hand, the cooling and warming rates to which the system is subjected.
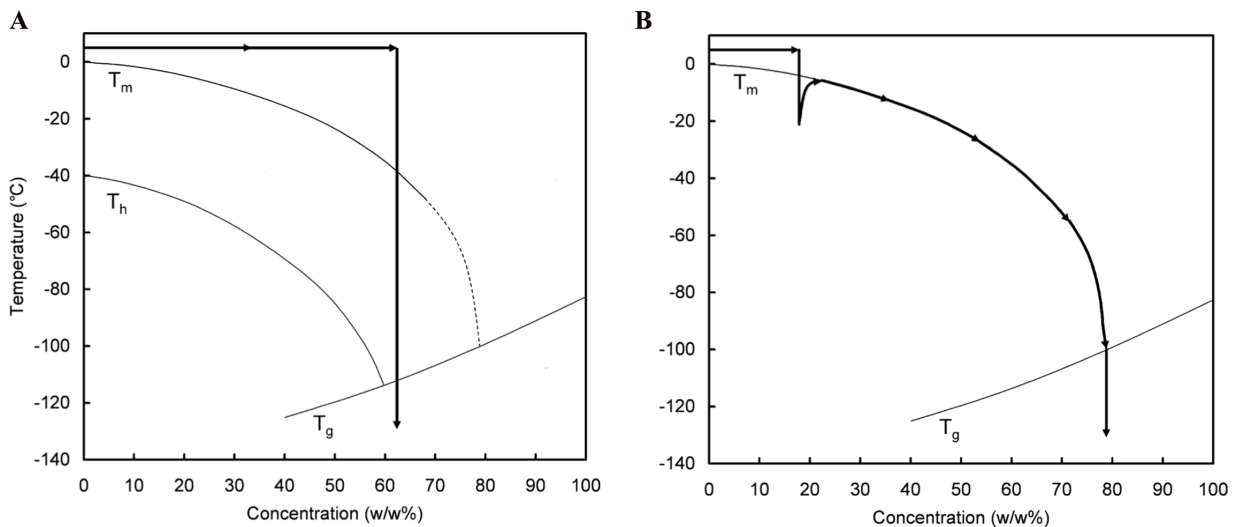
Figure 2. Hypothetical examples of the paths of the slow freezing and vitrification approaches on a glycerol–water phase diagram. A) Ultrafast Vitrification: cells are prepared for vitrification by increasing their solute concentration at room temperature, and afterwards are cooled rapidly to avoid ice formation. B) Slow freezing: the cell suspension, with a low initial cryoprotectant concentration, is supercooled until ice formation is induced and latent heat is released. From that point on, solutes concentrate as temperature decreases, so that the melting point of remaining unfrozen solution equals the solution temperature until vitrification occurs. Tm is the equilibrium melting temperature and Tg is the glass transition temperature. Both figures adapted from Wowk, 2010.

1.4.1 Cooling and warming rates

For cryopreservation, the solution in the system has to be cooled at high rates from Tm until Tg, in order to avoid ice formation. Similarly, the warming above Td until Tm must be carried out fast to avoid conversion from glass to ice and recrystallization (Rall, 1987). The thermal efficiency of the carrier, as well as the volume of solution containing the suspended cell that is loaded in it, will influence how fast the system can be cooled and warmed. For this reason, oocytes are usually suspended in a volume of ≈1 µL for loading in vitrification carriers. Also, the extracellular solution

must have a very specific composition, as this solution is in continuous osmotic exchange with the cell until cooling takes place (Shaw et al., 1997).

1.4.2 Glass forming tendency of the solution

The formation of ice is inhibited by the presence of solutes because their atoms directly interfere with the hydrogen bonding between water molecules necessary for the crystalline structure to form. If the solutes present also increase the viscosity of the solution, it reduces molecular agitation and furtherly challenges the union of water molecules. The type and quantity of solutes present and the viscosity of the solution will determine how easily the water molecules can re-arrange into ice crystals, determining how much time the solution can stay in the temperature range below Tm and above Tg without ice formation. Within a cell, the glass forming tendency of the cytosolic milieu will determine if ice will form at given cooling and warming rates (Fahy et al., 1984).

In conclussion, the rates of cooling and warming necessary to avoid ice formation are negatively correlated to the glass-forming tendency of the solution; a solution with higher concentration of solutes and higher viscosity can be cooled and warmed at slower rates and still vitrify and return to the liquid state without ice formation. Very dilute solutions, on the contrary, would require extremely high cooling and warming rates to avoid ice formation.

1.5 Mechanisms of ice formation during the temperature excursion of the vitrification procedure: the dominance of the warming rate

If the warming rate is the dominant variable determining the survival to the vitrification procedure (Seki & Mazur, 2009), it must be so because ice is more likely to reach lethal size during this step. When we examine the temperature excursion within the vitrification procedure in a hypothetical two-phase diagram, we can elucidate the reason.
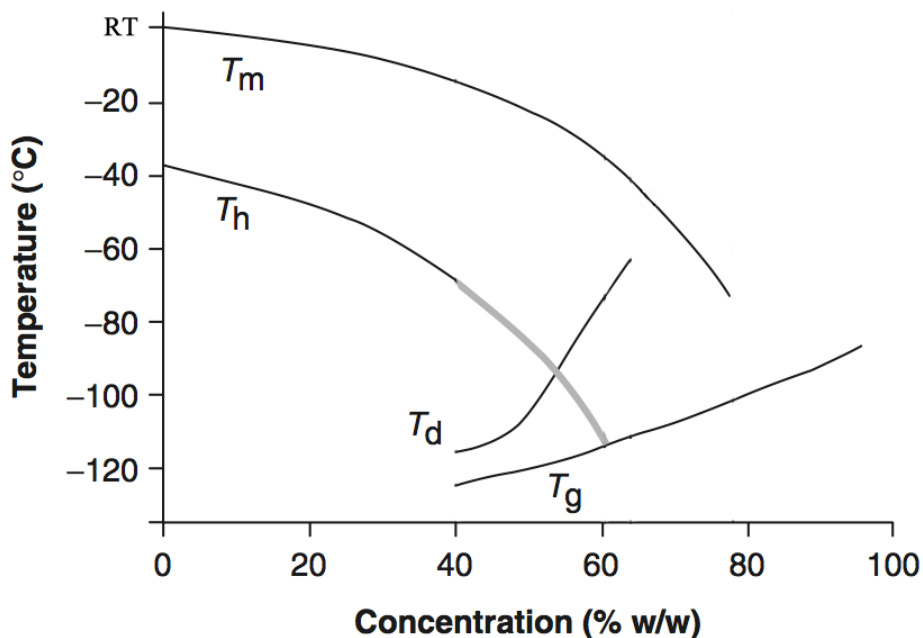
Figure 3. Hypothetical phase diagram of the cytosol of a human Metaphase-II oocyte during a vitrification procedure. The cell is loaded with cryoprotectants at room temperature (RT; 23-26 ºC) during the preparation phase. Then, the cell is cooled to -196 ºC at a given solute concentration. Vitrification occurs when the glass transition temperature (Tg) is reached. Ice may form below the heterogeneous (Tm) or homogeneous (Th) melting temperature. During warming, ice may form de novo above the devitrification temperature (Td) or may grow from previously formed ice crystals (recrystallization). The diagram is adapted from Fahy (Vitrification in assisted reproduction book, 2015, p.8).

At the intracellular solute concentration hypothetically attained in the cytosol of a Metaphase-II oocyte after it's been prepared for vitrification (approximately 45% of solutes per total mass of solution), ice still has a very high tendency to form during cooling. Ice formation starts being thermodinamically favoured below the melting point of the cytosol (Tm), until the solution reaches Tg. However, if ice does form during cooling, at far below 0 °C temperatures (-50 °C, for instance), the viscosity of the solution at those temperatures has increased to such an extent that the ince nucleus formed can hardly grow and may be innocuous. For that reason, lethal ice formation during cooling is less likely to happen in a vitrification procedure.

Whereas during warming, there is risk of *de novo* ice formation above the devitrification temperature (Td), but most importantly, there is the risk of growth of ice crystals previously formed during cooling, known as recrystallisation (Seki & Mazur, 2009). These previously formed ice nuclei now have a high tendency to grow, as temperature increases and viscosity decreases.

That is the reason why, as mentioned before, warming must take place at a faster rate than cooling: first to avoid *de novo* ice formation by glass to ice transition, but most importantly, to avoid recrystallisation. Therefore, deletereous ice growth during cooling will usually be limited because the nucleation process occurs at a temperature at which viscosity is high, hindering mobility of water molecules and the rate of ice formation. This, ice however, will grow to a lethal size if not warmed fast enough. Recent research points to the fact that the degree of dehydration of the cell, and the level of hydrogen bonding between present water and intracellular molecules seems to be the most relevant parameter, more than type or concentration of cryoprotectants (Jin & Mazur, 2015).

A

B
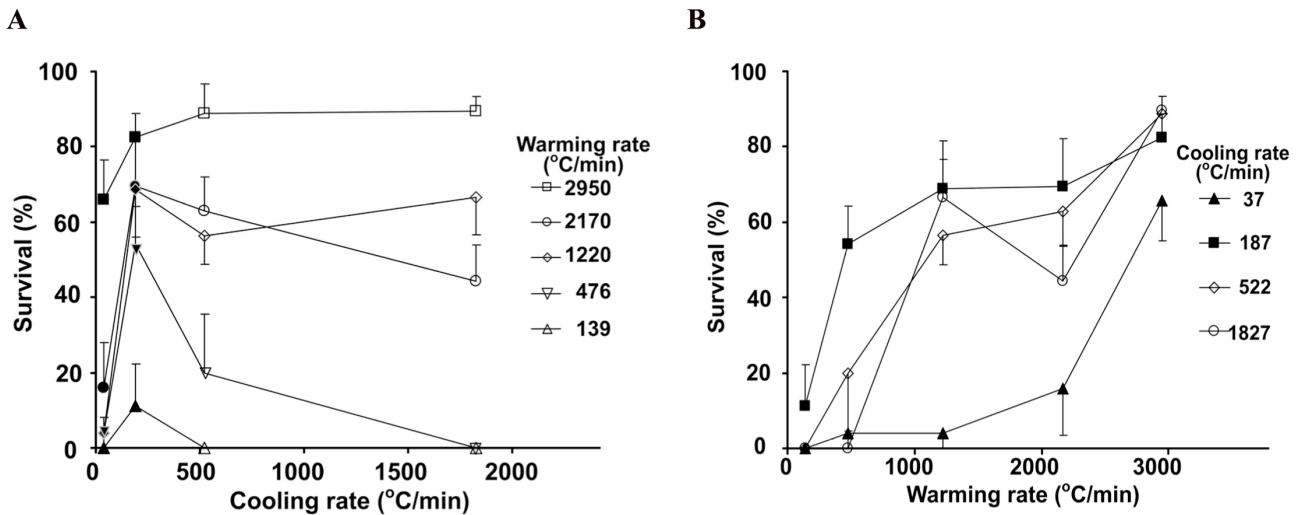
Figure 4. A) The survival of EAFS (ethylene glycol, acetamide, ficoll & sucrose) 10/10-vitrified oocytes warmed at 139, 476, 1220, 2170, 2950 °C/ min as a function of the cooling rate. (B) The survival of EAFS 10/10-vitrified oocytes cooled at 37, 187, 522, 1827°C/min as a function of the warming rate. The filled symbols depict conditions where the EAFS 10/10 solution froze; the open symbols depict where it vitrified. Figure from (Seki & Mazur, 2009)

1.6 Intracellular ice can be present in successful vitrification procedures: apparent vitrification.

Vitrification can be defined as the transition from liquid to glass, excluding ice formation (Luyet, 1937). However, in a vitrification procedure, as discussed above, the possibility of ice formation cannot be completely ruled out (Smith, 1954). In many cases, the literature defines vitrification as a procedure that yields a clear transparent medium after cooling below Tg, as opposed to an opaque sugar-like appearance (Vajta et al., 2009).

Table 1 Effect of warming rate on the survival of eight-cell mouse embryos cooled to -196

Container	Cooling rate (°C min^{-1})	Warming rate (°C min^{-1})	Total no. of embryos (replicate samples)	Survival (%)	s.e.m .
Embryos cooled in VS1					
Ministraw	2,500	2,500	343 (13)	87.8	1.9
Minitraw	20	2,500	110 (4)	83.6	3.9
Glass tube	500	300	171 (6)	80.7	4.0
Ministraw	2,500	10	63 (3)	0.0	—

Table 1. Murine embryos were gradually exposed to VS1 solution (Me2SO, acetamide, propylene glycol and polyethylene glycol, in Dulbecco's saline; total solute concentration of the solution = 13M), and cooled and warmed at different rates. Low warming rates resulted in lethal ice formation, whereas low cooling rates did not lower the survival rates of the embryos. Table adapted from Rall & Fahy, 1985.

There is no evidence however, in almost all published literature on the matter, regarding whether the formation of intracellular ice is completely avoided or not during a vitrification procedure. Ice formation can be excluded, for example, by thermal analysis of the presence or absence of latent heat of fusion (Rall et al., 1984; Seki & Mazur, 2009). Nevertheless, successful vitrification does not require that the whole system has completely achieved a glassy state: survival to a vitrification procedure does not exclude the formation of very small ice nuclei both inside and outside the cells. As the solute concentration decreases, the cooling and warming rates necessary to avoid ice formation increase dramatically (Toner et al., 1991).
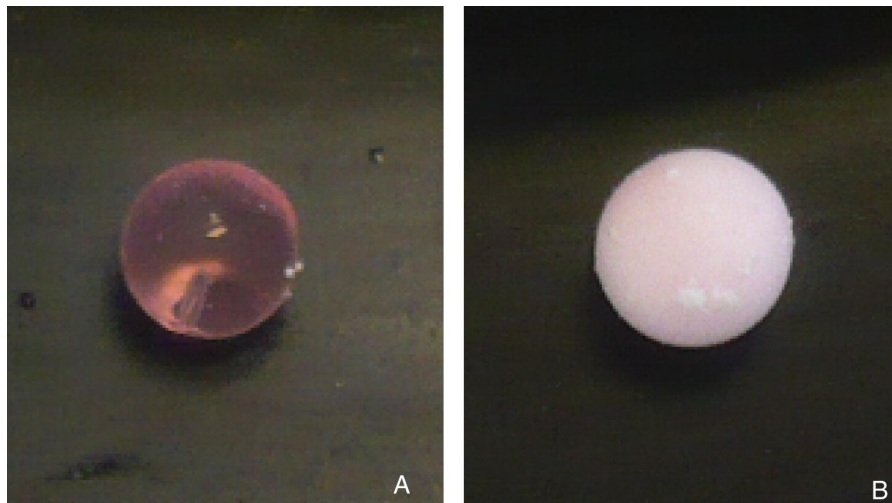
Figure 5. Solidified droplets after cooling by quenching in liquid nitrogen. A) Solution with high concentration of ethylene glycol (EG) and dimethyl sulphoxide (Me2SO), showing transparent, glass-like appearance. B) Low concentration of dimethyl sulphoxide, showing an opaque, crystallized look. Adapted from: (Amorim et al., 2011).

1. 7 What is, then, a successful vitrification procedure?

A successful vitrification procedure should not be defined as one that completely avoids any extent of ice nucleation, inside or outside the cells, first of all because it is unlikely, and secondly because it is challenging to observe experimentally. Instead, we say that a vitrification procedure is successful when the cell(s) subjected to it survive, without any apparent impairment of their functional and developmental ability. There are three main mechanisms by which a cell may be harmed during vitrification:

> **i) Ice formation:** the growth of ice deposits to a critical size that lethally disrupts the cell structures is the source of damage that is hardest to overcome. There are a number of mechanisms through which ice can be formed during the temperature excursion of the vitrification process. During cooling: a) crystallisation: *de novo* formation of ice from a liquid. During warming: b) devitrification: *de*

novo formation of ice from glass; and c) recrystallisation: ice growth from already existing glass nuclei.

ii) Osmotic damage: during the preparation and rehydration phases, cells are exposed to solutions of different solute concentration that will produce rapid outward and inward flows of water and penetrant solutes that affect cell mechanisms and produce stress. In addition, the resulting volumetric excursions, shrinking and swelling, might physically damage the biological structures, when above a certain threshold (Meryman, 2007).

iii) Cytotoxicity of cryoprotectants: In addition to the possible osmotic damage, permeating cryoprotectants can have a cytotoxic effect. This will depend on their concentration and duration, and the temperature at which the exposure is carried out (Fahy et al., 1984)

1.8 Phases of the vitrification procedure

Current ultra-fast vitrification protocols used in the context of human assisted reproduction for cryopreservation of oocytes and embryos can be divided into six phases:

i) Preparation of the cells for vitrification: The cytosol of oocytes and embryos' blastomers, due to their high water content, does not vitrify at the rates currently attainable by vitrification carriers, unlike spermatozoa. For this reason, the cells are subjected to a preparation protocol prior to cooling, to reach the critical cytosolic solute concentration necessary to achieve vitrification. This preparation consists of exposing cells to increasingly hypertonic solutions with permeant and non permeant cryoprotectants; the cells lose water by osmosis, and take cryoprotectants, increasing their glass forming tendency (Rall & Fahy, 1985).

ii) Loading of the cells in the vitrification carrier. The cells, usually suspended in a minimal volume of vitrification solution, are loaded in the vitrification carrier (Katayama et al., 2003). The total volume of the system and the thermal performance of the carrier will determine the rates of cooling and warming.

iii) Cooling: Once cells are loaded in a vitrification carrier, the system has to be cooled, usually by direct quenching in liquid nitrogen, despite there are other alternatives such as nitrogen vapors or slush nitrogen (Isachenko et al., 2018; Yavin et al., 2009). The angle, depth and speed of the quenching motion are relevant parameters that shouldn't be overlooked (He et al., 2008). The combination of the cooling rate and glass forming tendency of the solution will determine if the system can attain the glass transition temperature before ice forms.

iv) Storage of the system at cryogenic temperatures: This is essential for maintaining the vitreous state, and careful handling is required, to avoid accidental warming.

v) Warming: The vitrification carrier containing the cells must be warmed from -196 °C to 37 °C as fast as possible to avoid devitrification and recrystallisation (Rall, 1987; Seki & Mazur, 2009). The duration of the warming motion (time of flight) from the moment the carrier leaves the cold environment (usually a liquid nitrogen container) until it reaches the warming solution is therefore critical (Risco, et al., 2007).

vi) Washing and rehydration: Cryoprotectants are removed and cells rehydrate by osmosis during short periods of exposure to hypotonic solutions. The tonicity and times of exposure to these rehydration solutions are paramount, to avoid an excessive inward water flow and damage by overswelling (Hunter et al., 1992).

Human embryos, from the zygote stage to the expanded blastocyst, were being cryopreserved with a moderate degree of success by the slow freezing approach in the context of assisted reproduction treatments (Borini et al., 2006; Fabbri et al., 2001). The ultrafast vitrification strategy gained traction when it proved to be successful in cryopreserving mature human oöcytes at the Metaphase-II stage, very sensitive to chilling injury during the initial steps of the slow cooling process and for which very limited success had been achieved so far (Al-Hasani et al., 2007). Concerns regarding the use of higher cryoprotectant concentrations in the vitrification procedure were proved wrong when it was determined that the final intracellular cryoprotectant concentration was lower in oocytes subjected to a vitrification protocol than those subjected to a slow freezing protocol (Vanderzwalmen et al., 2013; Vanderzwalmen, et al., 2010a).

It was not until 2012, when it was already being used extensively, that the American Society of Reproductive Medicine removed the experimental label of the technique (P. C. o. A. S. f. R. Medicine & Technology, 2013). Despite both slow-freezing and vitrification techniques having their own advantages and challenges for embryo cryopreservation (Edgar & Gook, 2012), vitrification is now prevalent and more successful than slow freezing in the human assisted reproduction context, and is essentially the only effective cryopreservation technique for Metaphase-II oocytes (Rienzi et al., 2017). The procedure, however, may still be further improved (Vajta & Kuwayama, 2006). Indeed, the body of work in this thesis is focused on advancing the current state of the art of vitrification.

2. Ultrafast vitrification in the context of human assisted reproduction

Ultrafast vitrification is the most widely adopted method for cryopreservation of oocytes and embryos in fertility clinics. It is a key element of the clinical practice, one that has increased their efficiency and safety. It is so to such an extent that, currently, a large proportion of treatments do not even involve the transfer of fresh embryos: all the oocytes or embryos of a patient are often vitrified, such as in the case of oocyte banking, preimplantational genetic diagnosis and deferred transfer for optimal endometrial receptivity (Cobo et al; 2011; Devroey, Polyzos, & Blockeel, 2011; Schoolcraft et al., 2011).

Vitrification was pioneered in the clinical stage of assisted human reproduction in 1998, with cleavage-stage embryos (Mukaida et al., 1998), and soon was applied successfully to other developmental stages (Kuleshova et al,. 1999; Jelinkova et al., 2002). In general terms, current vitrification protocols rely on exposing cells to high concentrations of cryoprotectants, loading them suspended in a low volume of solution into a carrier, and cooling and warming them at ultrafast rates.

2.1 Biological features of the human Metaphase-II oocyte

Oocytes, the largest cells in the human body, present certain characteristics that make them more difficult to vitrify compared to cleaved embryos and blastocysts. Most importantly, their low surface to volume ratio results in inefficient water exchange, and hence slower dehydration and rehydration (Agca et al; 1998) . Water and and low molecular weight cryoprotectants only traverse the oocyte's lipid bilayer membrane by simple passive difussion (Paynter et al., 1999a; 1999b). Surrounding the oocyte there is a continuous glycoprotein coat, the zona pellucida. Its role in the osmotic behaviour of the oocyte is negligible. However, vitrification can produce zona hardening by promoting

exocytosis of the cortical granules, which may prevent sperm penetration and hinder natural fertilisation. Nonetheless, this drawback can be overcome by intracytoplasmic sperm injection. (Sathananthan, Trounson, & Freeman, 1987; Gook et al., 1995).

Additionally, oocytes in the Metaphase-II (M-II) stage possess very sensitive structural features, specially the meiotic spindle (Agca et al., 2000). These microtubules, attached to the chromosomes and lined up along the equatorial plate, are extremely sensitive to temperature changes. During vitrification, the oocyte is exposed to suboptimal temperatures and volumetric changes, causing the breakdown of the structure. It will however repolimerize and reassemble after the vitrification procedure if the oocyte is incubated at 37 ºC, allowing to maintain the developmental competence of the cell (Ciotti et al., 2009).

For these reasons, oocytes are arguably the most challenging cells to cryopreserve in the context of AHR. In this thesis, oocytes will be the model upon which we test the proposed advancements in the vitrification procedure. We do so under the premise that, what works for the oocyte will generally be applicable to embryos and blastocysts, as well as other cell types.

2.2 Solutes commonly employed in solutions for preparation of cells for vitrification: the cryoprotectants

As with most biological systems, the oocyte, as well as the embryo's blastomers and blastocyst's cells must be modified in terms of solute composition or concentration to achieve successful vitrification at currently attainable cooling and warming rates. Their osmotic response to an extracellular medium is our means to perform this manipulation, which is determined by i) the ratio of membrane surface area to cell water volume; ii) The stage-specific membrane permeability for water; iii) the

stage-specific membrane permeability for CPAs in relation to their respective activation energies (A. S. I. R. Medicine, 2012).

Achieving the necessary solute concentration for vitrification in a manner that does not kill the cells was initially regarded as an unsolvable problem (Luyet & Gehenio, 1940; Boutron & Kaufmann, 1979). Glycerol was the first molecule whose cryoprotective attributes were identified (Polge, Smith & Parkes, 1949). Since then, other cryoprotectant agents have been identified, such as Me_2SO (Lovelock and Bishop, 1959), ethylene glycol (EG; Kasai et al., 1981 & Kasai et al., 1992) and 1-2 propanediol (Mukaida et al., 1998), and are currently employed in vitrification solutions — solutions employed to prepare and carry the cells during vitrification (Rall & Fahy, 1985). The ability of CPAs to avoid ice formation increases with concentration, however so does their toxicity. CPAs can have a toxic effect on cells due to their intrinsic cytotoxic properties, but, equally or more importantly, will produce osmotic toxicity, which could be defined as the creation of an osmolarity that is not tolerated by the cells (Fahy, 2010). Both types of toxicity are dependent on temperature and the duration of the exposure, so these parameters are carefully controlled in vitrification protocols (Ali & Shelton, 1993).

Currently, most CPA cocktails employed for oocyte vitrification feature a synergic combination of permeating and non-permeating cryoprotectants (A. S. I. R. Medicine, 2012). Permeating cryoprotectants, small compounds that traverse easily the lipid bilayer, penetrate in the cytosol, replacing water in the cells and increasing the viscosity of the intracellular medium. These molecules also have specific capabilities of blockage of ice formation, generally by hydrogen bonding with water molecules. They also present low cellular toxicity and high solubility at low temperatures (A. S. I. R. Medicine, 2012).

For the role of non-permeating cryoprotectants, macromolecules such as disaccharides — sucrose and trehalose— ficoll and dextran are commonly

employed (A. S. I. R. Medicine, 2012). Aside from direct cryoprotection by increasing the viscosity of the vitrification solution and stabilising the cellular membrane, they act as osmotic agents, enhancing dehydration and the cellular intake of penetrant CPAs (Kuleshova, Shaw, & Trounson, 2001). Cellular macromolecules present in the cytosol of oocytes, such as proteins, polysaccharides, nucleic acids and other biopolymers, also play a cryoprotective role. For instance, when intracellular water is partially removed, the volumetric concentration rises and may be enough to avoid ice formation, albeit at very high rates of cooling and warming (Seki & Mazur, 2009). Vitrification solutions also feature surfactant agents that increase viscosity and prevent the oocytes from sticking to the surfaces of dishes and pipettes used during *in vitro* manipulation. Proteins from human or bovine serum albumin are the most common surfactant agents employed; yet our group, among others, has introduced the synthetic polymer hydroxypropyl cellulose as a safer and more stable alternative (Gallardo et al., 2015; 2016; 2017; Mori et al., 2015; Coello et al; 2016)

In conclusion, despite the considerable research involved, CPA toxicity has not been either avoided or neutralised. Instead, the synergic combinations of cryoprotectants and optimised exposure times have made it possible to surpass CPA's deleterious effects, enabling the success of these protocols (Fahy, 2010). However, to prepare oocytes for vitrification, long (8-15 minutes) exposures to high concentrations of cryoprotectants at suboptimal temperatures are still required.

2.2.1 Removal of cryoprotectants

So far we have discussed mostly the introduction of CPAs inside the cells and removing water so ice formation is avoided during cooling and warming. However, once this process has been completed, the cryoprotectants must be removed and the original water content restored. In order to do so in a controlled fashion, the oocyte is exposed to

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hypotonic solutions with decreasing concentrations (usually 1M followed by 0.5M) of non-penetrant cryoprotectants (usually sugars). The extracellular osmotic agents compensate the osmotic gradient, avoiding water rushing inside the oocyte producing lethal over-swelling. Water and CPAs are exchanged until the oocyte recovers its isotonic volume and original solute content (Shaw & Jones, 2003).

2.3 The evolution of vitrification carriers

The seemingly easiest approach, to cool a solution with suspended embryos —small droplets freely plunged into the liquid nitrogen (Landa & Teplá, 1990)— is a thermodynamically suboptimal and unpractical approach. For that reason, vitrification carriers were needed. A vitrification carrier could be defined as the physical support where the embryos are placed for cooling and storage, until warming. Cooling usually takes place by quenching in liquid nitrogen, and the warming in a volume of a solution at 37 °C where the embryos have to be recovered under the stereomicroscope.

As vitrification solutions evolved to increase cryoprotection and decrease toxicity, so did vitrification carriers. Initially, the evolution was completely driven towards increasing cooling rates. To achieve that goal, the volume of vitrification solution carrying the cells loaded in the carrier had to be reduced (Arav, 1992). Soon, scientists moved away from the 0.25 mL straws that limited the cooling rates to 2500 °C/min. (Mukaida et al., 1998). Placing a drop of a very small volume of solution (~1 µL) containing the embryos on a thermally efficient carrier was the solution to cool and warm the system faster. The initial array of carriers that appeared can be designated open carriers, as the biological sample has direct contact with liquid nitrogen during cooling and storage (Vajta, Rienzi, & Ubaldi, 2015).

2.3.1 Examples of open carriers:

- **Electron microscopy grids:** Were initially used as carriers, inspired by early cryofixation experiments in the field of electron microscopy (Nei, 1976). Inefficient handling and storage limited their application.

- **Open pulled straw:** A 0.25 mL cryostraw, manually heated and pulled to decrease its diameter and wall thickness, allowed to achieve warming rates of 20.000 ℃/min. Embryos were loaded by capillarity or aspiration. (Vajta, 1998; Kuleshova et al., 1999).

Figure 6: 8.5 cm *OPS* for general purpose. Image from: www.open-pulled-straw.com/

- **Cryoloop:** A nylon *cryoloop* is attached to the lid of a cryovial. The cells are loaded in the loop, coated with a thin film of vitrification solution, and quenched in the cryovial filled with liquid nitrogen. The vial is sealed for storage (Lane, Schoolcraft, & Gardner, 1999).
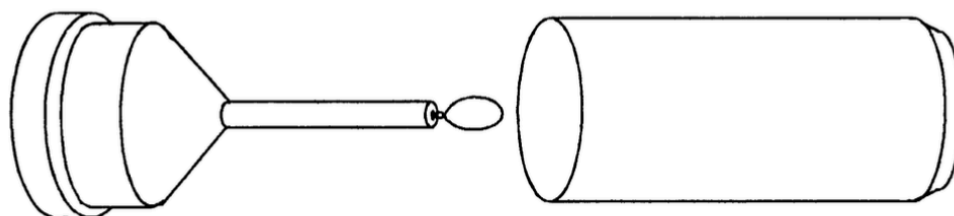
Figure 7: Illustration of the cryoloop. Image from (Lane et al., 1999)

- **Open Hemi-Straw:** consists of a standard 0.25 mL cryostraw, cleaved in length, to hold a small volume of vitrification solution containing the cells.
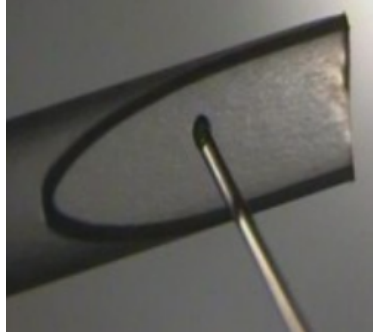
Figure 8: Picture of the tip of a HemiStraw, the cells are placed in a very small volume of cryoprotectant, avoiding the thermo-insulating effect of a conventional straw. The carrier, once quenched in liquid nitrogen, can be encapsulted inside a regular cryopreservation straw.

- **Flexi-pet denuding pipette:** Denuding pipettes with a diameter <250 μm are routinely used for human embryology. Oocytes and embryos can be loaded inside them with ~2 μL of vitrification solution and quenched in liquid nitrogen, and then introduced inside an external 0.25 ml regular straw for storage (Liebermann et al., 2002).

- **Cryotop:** Plastic handle with a fine, flat polypropylene strip attached to it, where embryos are loaded in a microdrop (Hamawaki, 1999).
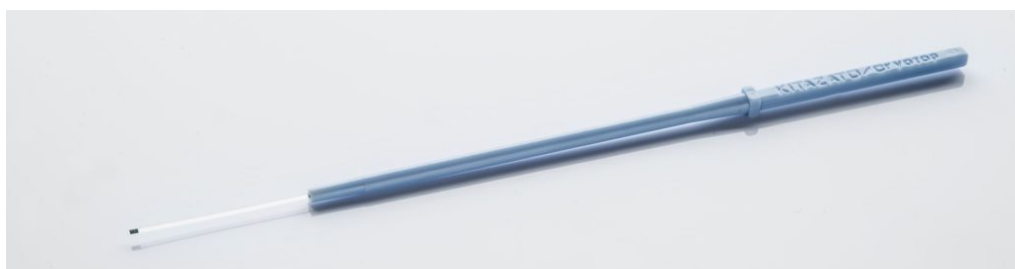
Figure 9: Most recent commercial version of the open cryotop carrier.

The *cryotop* concept, as a minimum drop size carrier with a long, easy to manipulate body and storage-efficient dimensions, gained very wide acceptation and eventually commercial success (Katayama et al., 2003; Kuwayama, 2007)

2.3.2 Closing the open systems: the risk of contamination

One drawback of the open-system approach is the hypothetical risk of contamination by infectious microorganisms present in the liquid nitrogen, and the risk of cross-contamination between patients' samples (Bielanski et al., 2000). Infectious agents may survive the cooling process and spread to other samples stored in open systems through the liquid or vapour-phase nitrogen used for cooling and storage (Grout & Morris, 2009). However, despite the intensive use of carriers allowing direct contact with the possible contamination vector, as yet there hasn't been any cryopreservation-related source of infection transmission reported in the field of AHR (Molina et al., 2016).

Whether it is preferable to use open or closed carriers is a hotly debated topic in the AHR field (De Munck & Vajta, 2017; Vajta et al., 2015; Youm et al., 2017). European directives do not impose the use of closed vitrification systems, but recommend laboratories to minimise the risk of contamination of tissues and cells (EPC, 2006). To deal with this concern, carriers have been developed that minimise or completely avoid exposure to LN$_2$ (Bielanski & Vajta, 2009). These are the semi-closed and closed systems. There are also alternatives in which liquid nitrogen is sterilised, but they remain very hard to implement in clinical practice (Isachenko et al., 2018).

2.3.3 Examples of closed carriers:

i) Hermetically sealed into a container carrier: The carrier, usually in the format of a thin-strip-attached-to-a-handle, is introduced inside an outer cryostraw, which is sealed before cooling. It is the most aseptic vitrification procedure, but results in very low thermal efficiency due to the insulation and leindefrost effect from the outer straw, with speeds of cooling around $\sim$2000 ºC/min (Isachenko et al., 2006; Kuleshova & Shaw, 2000; Vanderzwalmen et al., 2009). Alternatively, the outer straw can be precooled in liquid nitrogen to improve cooling rates, yet reducing asepsis by exposure to LN_2 vapors (Larman & Gardner, 2011; Vajta et al., 2015). Readiliy available consumables such a standard denudation pipette and an ionomeric resin 0.25 mL cryo-straw can be used as a closed-system kit (Schiewe et al., 2015; Schiewe et al., 2017)

Figure 10: The High Security Vitrification device is composed of a carrier straw (red), with a v-shaped tip in which the embryos are loaded with a minimum volume of vehicle solution (*). This carrier straw is then introduced in the outer straw, which is sealed before plunging in liquid nitrogen for cooling. For warming, the outer straw is cut open and the carrier straw extracted; the embryos are re-warmed by immersion of the tip of the carrier straw in 1 mL of warming solution Adapted from (Guerrero, et al., 2018).

ii) Capillary-based carriers: A capillary is attached to a main straw body, enabling the loading of the cells by aspiration and capillarity. The tip of the capillary and the back end of the straw can be sealed, hermetically closing the system before contact with LN2. The capillary, depending on its material and thickness, will have a certain thermal efficiency (Risco et al., 2007; Kuwayama et al., 2005). It also can be warmed in a bigger volume of water, prior to cutting and releasing the cells under the stereomicroscope, allowing maximizing warming rates.
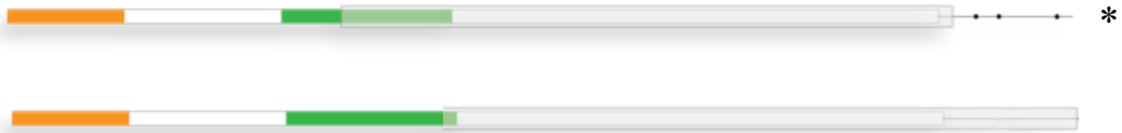
Figure 11: The SafeSpeed carrier (top) presents a capillary (*) where the embryos, while floating in the vehicle solution (vitrification volution), are loaded by aspiration. This capillary is sealed before plunging in liquid nitrogen and cut open before expelling the embryos in warming solution. The sealed capillary must be uncovered by the protective cap (top) during cooling in liquid nitrogen and warming in a 37 °C water bath. During storage and handling it is protected by a sliding cover, in grey (bottom). Adapted from (Guerrero et al., 2018).

2.4 Modelling the osmotic behaviour of the Metaphase-II oocyte

For a vitrification procedure to be successful at currently attainable cooling and warming rates, it requires preparing the cells in order to enhance the glass-forming tendency of the cytosol (Boutron & Kaufmann, 1978; Seki & Mazur, 2012). In order to do so, we osmotically manipulate the oocyte in a controlled fashion, subjecting it to exposures to hypertonic solutions made of various cryoprotectants and osmotic agents. The

osmometric behaviour of the oocyte depends on the permeability of the plasma membrane to water and each solute involved:

- **Permeability:** Determines the flow of a permeable substance through each unit of the cell surface as a function of time.
- **Activation Energy (E_A):** The required energy for the permeation process of a particular substance to occur. A lower activation energy implies higher permeability, as less energy is necessary for the process to happen. For instance, when exposed to a hypertonic solution with permeable cryoprotectants, dehydration is faster than CPA intake, as $E_A^{H2O} < E_A^{CPA}$ (Paynter et al., 1999a,b).

The two-parameter (2-P) transport formalism can be used to describe membrane permeability of the Metaphase-II oocyte in a solution of water, permeable and non-permeable solutes, as long as biophysical parameters of hydraulic conductivity (Lp), cryoprotectant permeability (P_{CPA}) and activation energies (E_A^{CPA}) are known (Kleinhans, 1998).

2.5 Determining permeability characteristics of the human oocyte

The permeability parameters of human M-II oocytes to CPAs can be determined by their response to hypertonic solutions with known molarity of these solutes (Paynter et al., 1999a,b). By recording the volumetric excursion of an oocyte exposed to a certain solution at a certain temperature, its volume can be determined by measuring the bidimensional area, radius, or diameter over time. Volume can then be calculated from these measurements, assuming that the oocyte remains in perfect spherical shape during the process. Once the volumetric data has been determined, the permeability variables can be cleared from the equations described by fitting with this experimental data through permeability models (Mullen et al; 2008; Paynter et al., 1999a,b). The permeability parameters of the oocyte's plasma membrane for relevant cryoprotectants such as Me_2SO and EG have already been determined

using this approach, as shown in Table 2 (Mullen et al., 2008; Paynter, Cooper, et al., 1999; Paynter, Fuller, et al., 1999).
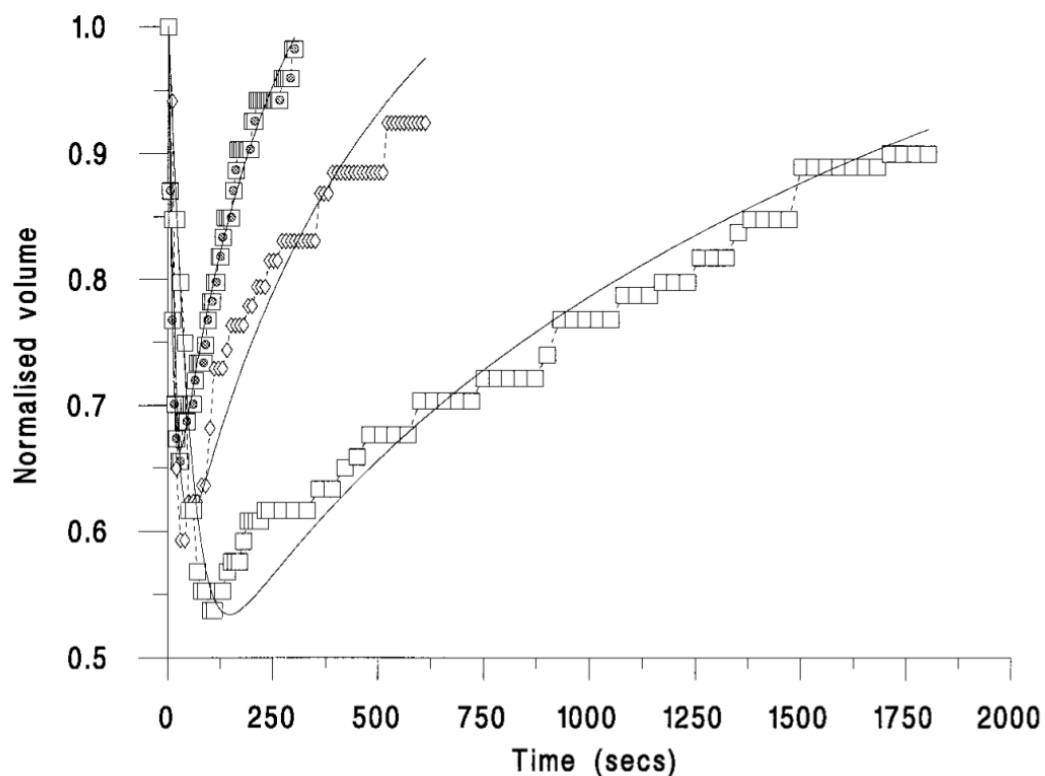
Figure 12: Typical osmotic response of mature mouse oocyte exposed to 1.5 mol/liter Me_2SO. Oocytes were exposed at 30 ºC (¤), 23 ºC (♦) and 10 ºC (□). The solid line for each case represents the predicted osmotic response. Adapted from Paynter et al., 1997.

2.5.1 The shrink-swell curve

As shown in Figure 12, when the oocyte's normalized volume is plotted on a graph against time of exposure to a hypertonic solution, we observe a very distinctly shaped curve, known as the shrink-swell curve. Oocytes quickly shrink to a minimum volume, as water leaves the cells faster than cryoprotectants permeate them, as the hydraulic conductivity is higher than the permeability to low molecular weight cryoprotectants. They

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subsequently swell slowly towards their isotonic volume, describing an asymptotic curve, as cryoprotectant permeates and water re-enter the cell.

Once these parameters have been established, the process can be reversed and the transport model can then be used to simulate the osmotic response of oocytes under theoretical conditions, by integrating the differential equations above. Later, we describe the use of this approach to analyse and optimise current protocols of preparation of oocytes for vitrification.

3. Aims and goals of the thesis

Our main goal, during the four years of research involved in the preparation of this thesis, has been to take a step back, analyse the state of the art of ultrafast vitrification as applied in the AHR field, and develop and test ways of improving the procedure. Considering that cryopreservation of human oocytes and embryos is a well-established technique, that has been the object of ever increasing scientific attention since its inception, it has posed a challenge to find ways to improve current protocols and defy the *statu quo* of vitrification; many would consider that progress had already peaked and that the technique had reached its efficiency limits. As our group and others have been proving, the safety, duration and repeatability of vitrification procedures may still be optimised.

The work in this thesis was developed as a member of the CryoBioTech research group, in the Advanced School of Engineering of Seville (University of Seville, Spain), founded back in 2004 by Prof Ramón Risco, with the goal of achieving a better understanding of the physical laws behind the cryobiology field and applying them to improve the different strategies of achieving the long-term cryostorage of biological material. This research group led by Prof. Risco had already explored, in collaboration with a team at The Centre for Engineering in Medicine (Harvard Medical School, Massachusetts, USA), the enhancement of thermal rates by using quartz capillaries as vitrification carriers and slush nitrogen as a cooling agent (Risco et al., 2007).

At the time of my inclusion in the research group, this line of work had already developed into a project that sought to bring these ideas into a versatile, economically feasible vitrification carrier, to be commercially exploited by university spin-off company SafePreservation. The original fragile and unpractical quartz carriers were substituted by a flexible polycarbonate capillary, attached to a main straw for easy handling, sacrificing as little thermal conductivity as possible

(PCT/ES2013/000228). The exact features of this vitrification carrier, as well as those of a set of solutions to prepare and recover human oocytes and embryos from the vitrification procedure are further described in the original research articles that constitute this thesis' main body of work.

As this vitrification system of carrier and solutions, commercially labelled SafeSpeed, was already at a very advanced stage of development and had already cleared the pre-clinical validation phase, I should not be attributed any intellectual merit on their design. I was, however, very interested in adapting the initial working prototypes into something that the clinical embryologist not only could use, but also would like to use. That goal was only partially achieved, as some of my feedback was not shared by the intellectual owners of the technology or was not financially feasible for a start-up project.

The initial research described in this thesis has consisted mainly on designing and conducting the studies to test how this vitrification system, with the innovative features it included, namely a thermally efficient and aseptic capillary-based carrier and a protein-free, fully synthetic set of vitrification solutions, performed on the clinical stage in the context of AHR. Firstly, the two original research articles published in the context of this thesis are reproduced and their scope and impact discussed. First, the SafeSpeed solutions are used to vitrify top quality donor eggs in a standard vitrification carrier, and the outcomes are compared with fresh sibling donor oocytes. Then, the same controlled experimental setting is repeated, now employing both SafeSpeed set of vitrification solutions and the SafeSpeed carrier, as well as analysing the thermal performance of the latter. Secondly, we explore further possibilities to optimise the vitrification procedure with this vitrification kit. We studied *in silico* and *in vivo* osmotic behaviour of human metaphase-II oocytes, and propose a method to significantly reduce the time needed to prepare human M-II oocytes for vitrification, with very promising results.

4. Main results

In this chapter, the reader can find a brief summary of two original research articles published in peer review specialized journals. The full papers are included at the end of the thesis as supplemental material for further details on methodology and results. The section also includes ongoing unpublished research in the section 4.2.

4.1 Validation of SafeSpeed carrier and solutions.

4.1.1 Summary of original research article 1: Hydroxypropyl cellulose supplementation in vitrification solutions: a prospective study with donor oocytes.

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The solutions employed for CPA loading of human oocytes prior to vitrification, and dilution after warming, require in their formulation the presence a surfactant agent to enable in vitro manipulation and prevent sticking of the cells to the surface of dishes and pipettes. Most commonly, human-derived protein supplementation is used for this task, for example a combination of albumin and globulins. However, as a human-source material, it presents risks of contamination and production variability associated with it (Shaw et al., 1997), so a replacement synthetic component has been sought.

The set of solutions for vitrification and warming developed by Prof. Risco's in the University of Seville (PCT/ES2013/000228) are free of these human or animal components, as encouraged by international regulatory policies. These macromolecules are substituted by hydroxypropyl cellulose (HPC), a variable length polysaccharide that is listed as pharmacopeia and is a common food additive and drug excipient. SafeSpeed (SafePreservation, Spain) vitrification solutions are supplemented with a range of 0.06–0.125 mg/mL of hydroxypropyl cellulose (pharmaceutical standard, 80,000 Da average molecular weight, Sigma Aldrich).

In order to test the efficacy of these solutions in the clinical setting, we designed a prospective study with donor oocytes in Clínicas Ginemed Seville's IVF Unit. We compared the outcomes of two groups of oocytes from the same donor; in the control group, oocytes were not subjected to vitrification, whereas in the experimental group, oocytes were vitrified using HPC-supplemented solutions and a standard open carrier. Vitrification of the experimental group oocytes was performed at laboratory room temperature (RT). The vitrification kit consists of three solutions: washing solution (WS, no CPAs), equilibration solution (ES 7.5% ethylene glycol and 7.5% dimethyl sulfoxide) and vitrification solution (VS 15% ethylene glycol; 15% dimethyl sulfoxide; 0.5M sucrose).

All solutions were exposed at least for an hour to laboratory RT. Oocytes were gradually exposed to the cryoprotective agents by placing them in a first droplet of 50 μl of WS, which was joined with the tip of the pipette to another droplet of 50 μl of ES. After 2 min, both droplets were joined with a third ES drop and allowed an additional 2 min of CPA diffusion to the primary droplet where the oocytes were located. Afterwards, oocytes were transferred to 100 μl of ES, where they remained up to 10 min, until the oocytes appeared to be fully re-expanded. Once the equilibration was

complete, oocytes were transferred to a 200-µl droplet of VS and washed at least three times to eliminate any leftover ES. Then the oocytes were placed on the thin plastic strip of a Cryotop cryodevice, as described by Kuwayama et al. (2005), and plunged vertically in liquid nitrogen. The amount of time the oocytes remained in VS for washing and loading purposes before plunging in liquid nitrogen was approximately 60 s.

SafeSpeed warming media consists in three solutions: thawing solution (TS) (1 M sucrose), dilution solution (DS) (0.5 M sucrose), and WS (no CPAs) [30]. For at least 1 h., closed vials of DS and WS were exposed to laboratory RT, and TS vial was placed in the incubator at 37 °C. The vitrification straw containing the oocytes was transferred from a styrofoam box with liquid nitrogen to a double-well dish (Becton-Dickinson, 60 × 15 mm Falcon Center-Well Organ Culture Dish) containing 1 ml of TS at 37 °C. The liquid nitrogen container and the double-well dish used for warming were placed as close as possible (10–20 cm) to allow for a fast transfer (less than a second), to facilitate a high warming rate. After 1 min, oocytes were carefully moved to a 200-µl droplet of DS, where they remained 3 min. Two washing steps of 5 and 1 min., respectively, were performed in 200 µl droplets of WS. Warmed oocytes remained in culture for 2 h prior to intracytoplasmic sperm injection (ICSI).

We observed benchmark survival rates of the oocytes to the procedure, and no differences in between groups in the primary outcome of the study, the rates of fertilization after ICSI. Additionally, the embryos originated from both groups of oocytes developed with similar quality, and the implantation and ongoing pregnancy outcomes were satisfactory irrespectively if embryos from fresh or vitrified oocytes were transferred. This design allowed us to evaluate the impact of vitrification on the developmental abilities of donor oocytes and assess the performance of an HPC-based vitrification formulation for its use in daily laboratory practice.

The advantages that HPC-supplemented, protein-free solutions provide are a reduced production cost and lot-to-lot variability, and the elimination of the hypothetical risk of viral contamination associated with protein purified from blood (Shaw et al,. 1997). The addition of hydroxypropyl cellulose instead of serum to vitrification solutions can provide the same viscosity that enables in vitro manipulation and enhances the glass-forming tendency of the solution, without the aforementioned drawbacks (Coello et al., 2016).

The prolonged use of HPC supplementation of vitrification solutions in a controlled setting is necessary to further guarantee the effectiveness of this approach, yet the results reported in our work provide initial evidence documenting the efficacy of the use of fully synthetic, protein-free vitrification solutions.

4.1.2. Summary of original research article 2: Thermal and clinical performance of a closed device designed for human oocyte vitrification based on the optimization of the warming rate.

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Although it was qualitatively pointed out by Fahy et al. (1984), the key role of the warming rates in non-equillibrium vitrification has only recently been quantitatively established for murine oocytes by Mazur and Seki (2011). The SafeSpeed vitrification carrier, composed of a thin polycarbonate capillary attached to a main straw was developed with the goal of maximizing thermal efficiency, specially towards optimizing the warming rate (PCT/ES2013/000228). The capillary and rear-end of the straw are hermetically sealed, completely isolating samples from contact with liquid nitrogen and any pathogens that may be present in it.

The geometry of the capillary and the thermal conductivity of its material enables very fast cooling upon quenching in liquid nitrogen (Risco et al., 2007). Additionally, the closed capillary allows carrying the warming in a large volume of water at 37 ºC, maximizing warming rates. Once the sample has warmed, the capillary is cut and the samples expelled in solutions for CPA dilution.

We measured the warming rate using an ultra-thin thermocouple probe (COCO-001; OMEGA Engineering Inc., USA). Its signal was amplified and introduced in a DAC converter (USB.1208LS; Measurement Computing

Inc., USA). Then, a program written in Labview 6.0 (reading rate 600 Hz) gives us the whole thermal history of the thermocouple junction. The process was recorded to determine with a temporal resolutions of milliseconds the duration of the time of flight: from the exit of the liquid nitrogen to the entrance in the warming water bath. Interestingly, there is an exponential increase in the warming rate as the time of flight is reduced, with a critical point around 300 ms (Figure X, Gallardo et al., 2017). The correlation between warming rates and time of flight between the liquid nitrogen and the warm water bath fits in our experiments to a potential curve of the form: $B(ºC/min) \approx 10^8 t^{-1}$, where B is the warming rate in (ºC/min) and t is the time of flight, in milliseconds (ms). The exponential increase of the warming rate (B) with the reduction of the duration of the warming motion (t), shows the relevancy of performing a fast rewarming of the vitrification carrier, as described in the section warming of this work.

We found that the warming motion must be completed very fast to maximize warming rates (A. S. I. R. Medicine, 2012). The carrier, when loaded with vitrification solution, obtained an average rate of warming of 200.000 ºC/min, very superior rates to other commercially available vitrification carriers.

The performance of this vitrification carrier, was assessed in a prospective study with a similar setting as the one used for the HPC solutions: a matched pair analysis with donor oocytes. In this case, a cohort of 143 donor MII sibling human oocytes was split into two groups: control (fresh) and vitrified with SafeSpeed device. The survival rate obtained was in the excellent range: 97.1 % of the oocytes survived vitrification. Similar results were found in both groups in terms of fertilization after ICSI (74.7% in control vs. 77.3% in vitrified) and good quality embryos at day three of development (54.3% in control vs. 58.1% in vitrified). The

pregnancy outcomes of embryos originated from vitrified oocytes were also satisfactory (ESHRE, 2017).
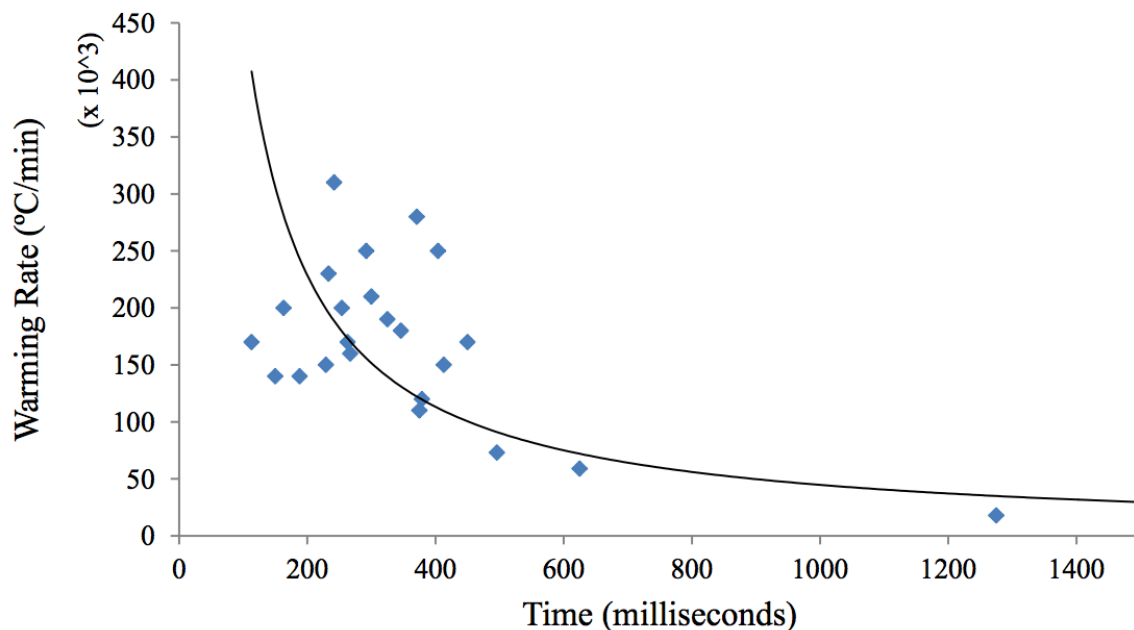
Figure 13. Relevance of the re-warming step. The graph shows the typical behavior of the warming rate (ºC/min) vs. the time of flight (the time to complete the warming motion from liquid nitrogen to the 37 C water bath) for VS vitrification solution. There is an exponential increase of the warming rate as the time of flight is reduced. A plateau seems to be reached for time of flights below 300 ms; in this area, the average warming rate for VS vitrification solution is 200.000 ºC/min. Figure from Gallardo et al., 2017.

In conclusion, in the SafeSpeed carrier the warming rate is maximized by the following factors: the warming is performed in a significant volume of warm water, in a very fast motion, and the capillary yields excellent thermal performance (Risco et al., 2007). The fact that the oocytes are encapsulated inside a capillary is not a drawback, but on the contrary, it enables this improvement in the warming rate by allowing the possibility of warming in bigger volumes of warm media/water without the risk of

losing the oocytes/embryos. It also minimizes the risk of contamination associated with direct contact with liquid nitrogen.

4.1.3 Summary of results

In the two original articles that comprise the first part of this thesis, we report on the outcomes of the SafeSpeed vitrification carrier —and a set of vitrification solutions developed in parallel— that was designed to achieve rates of cooling and warming far superior to those of other commercially available carriers. It proved to be an efficient design, yielding benchmark results for oocyte cryopreservation in the clinical context. However, the procedure *per se* was not improved: the SafeSpeed technique described has a similar duration, safety and efficiency to other available vitrification protocols.

However, with this optimized tool at hand we could now study introducing advantageous protocol modifications that would provide practical benefits in the bench. With SafeSpeed's high cooling and warming rates, ice formation could be theoretically avoided with lower intracellular solute concentrations, just as Jin & Mazur proved in 2014 with a laser-assisted warming procedure, but in a lower scale. With this is mind we analyzed one of the most time-consuming parts of the vitrification protocol: the preparation of the cells for vitrification by exposure to hypertonic solutions. Oocytes are dehydrated and permeated by cryoprotectant agents to reach the necessary intracellular solute concentration for vitrification. However current protocols feature long exposure to these hypertonic mediums, which may be inefficient, as developed in the following chapter.

4.2 A two minute protocol to prepare human oocytes and embryos for ultrafast vitrification

4.2.1 Introduction

The vitrification procedure is a key element of *in vitro* fertilisation units' daily clinical practice: over one hundred thousand embryos and thirty thousand oocytes originated in AHR treatments were cryopreserved in Spain in 2015 (CNRHA, 2015; (Rienzi et al., 2017). These cells are cryopreserved in vitrification carriers that, according to their design, will have variable thermal exchange rates (Vajta et al., 2015).

The cytosol of oocytes and embryos' blastomers, due to their large size and water content, does not vitrify at the rates currently attainable by those vitrification carriers, unlike spermatozoa (Isachenko et al., 2005). For that reason, the cells are subjected to a preparation protocol prior to cooling in which they lose water and are permeated by low molecular weight cryoprotectants, thereby increasing the cytosol's solute concentration, and hence, its glass-forming tendency. As a result, the cooling and warming rates necessary to avoid deleterious ice formation are lower. Regardless of the chosen vitrification carrier, most preparation protocols to ready oocytes and embryos for vitrification are very similar. They usually feature two or more exposure phases —to decrease the osmotic shock— to increasingly hypertonic solutions composed of permeating and non-permeating cryoprotectants. The composition of these solutions and the duration of the oocyte's exposure to them are critical, as they can result in osmotic injury by excessive shrinking and swelling and cryoprotectant toxicity (A. S. I. R. Medicine, 2012).

Even though shorter protocols have already been proposed (Jin & Mazur, 2015; Karlsson et al., 2014; Vanderzwalmen et al., 2009), most of them feature an initial, long exposure phase lasting from 10 to 15 minutes to a non-vitrifying solution (with a concentration of solutes that would not

vitrify if cooled with the intended carrier). Cells initially lose water and shrink to a minimum volume very rapidly. After that point, they slowly swell back towards their isotonic volume, as concentrations inside and outside the cells tend to osmotic equilibrium (Kleinhans, 1998). For this reason, this phase is usually termed equilibration or partial equilibration. This is followed by a short exposure to a more concentrated solution, that vitrifies upon cooling (vitrification solution) (Fahy, Levy, & Ali, 1987). It is in this solution that the cells are suspended, loaded in the carrier device, and cooled. This second exposure is short, and cells are not allowed to equilibrate; they shrink, becoming even more dehydrated and permeated by cryoprotectants. The duration of the exposure has to be carefully controlled and limited to 60 seconds to avoid an excessive osmotic effect (Fahy et al., 1984; Rall, 1987).

The equilibration, complete or partial, of the cells with non-vitrifying solutions during their preparation for vitrification is what accounts for most of the procedure's duration and makes current protocols so time-consuming. However, shorter protocols of preparation of embryos and oocytes for vitrification are feasible: the critical cytosolic solute content necessary for successful vitrification at currently attainable cooling and warming rates can be achieved faster. In this work, using *in silico* data from a biophysical permeability model of human oocytes and *in vivo* osmotic observations, we propose a short, dehydration-based protocol. Using a standard set of vitrification solutions, we limit the duration of the exposure to one minute, when cells are close to the minimum volume point of their shrink-swell curve. We analyse their molarity compared to a standard protocol, and test the viability of discarded human oocytes and abnormally fertilized zygotes subjected to it.

4.2.2 Materials and Methods

4.2.2.1 Two-parameter transport formalism model

The two-parameter transport formalism (2P) model has been used in this work to perform the necessary simulations to analyse the oocyte mass transfer dynamics in the presence of permeable and non-permeable solutes (Kleinhans, 1998). This model evaluates the flow of water and solutes through the cell membrane with a pair of coupled linear ordinary differential equations, assuming that there is no intermembrane interaction between water and the permeable solutes. Nevertheless, there are publications (Paynter 1999, Agca 1999) that have shown that the interaction factor or reflection coefficient (σ) of the 3-parameter Kedem-Katchalsky formalism is insignificant in many membranes, oocytes included.

The 2-P model characterises the permeability of the membrane to water with the parameter *Lp* (hydraulic conductivity), and the permeability of the membrane to permeant solutes with the parameter *Ps*, namely solute permeability. It is well known that permeability depends on temperature, and it is very common to define this relationship through an Arrhenius equation:

$$\text{Eq. 1:} \ L = L_0 \, e^{\frac{-Ea}{R}\left(\frac{1}{Tref} - \frac{1}{T}\right)}$$

The water transfer dynamics is defined by the equation

$$\text{Eq. 2:} \ \frac{dVw}{dt} = -L_p \bullet A \bullet R \bullet T \bullet \left(M^e - M^i\right)$$

The permeable solute transfer dynamics is defined by the following equation:

$$\text{Eq. 3: } \frac{dNs}{dt} = Ps \bullet A \bullet \left(M_s^e - M_s^i \right)$$

In our particular case, we will need to define two solute equations, one for Me_2SO and another for EG. This means that we will have to solve a system of three linear ordinary differential equations, with three parameters, Vw osmotically intracellular water, N_{DMSO} intracellular osmol of DMSO, and N_{EG} intracellular osmol of EG. In order to solve the system of differential equations we developed specific software in MATLAB version R2014A. In particular we employ the built-in function ODE45 based on the Runge-Kutta method.

The oocyte was assumed to be of perfect spherical shape, with a radius of 63 μm, and the osmotically inactive volume of the oocyte was considered to be 19% of its initial volume (Newton et al; 1999). Values for the permeability of the cell membrane to water (hydraulic permeability; Lp), and to permeating cryoprotectants Me_2SO and EG, and their respective activation energy (Ea), were used as estimated by Paynter et al. (1998a,b) (Supplementary Table 1).

As in previous cell dynamic modelling studies (Mullen et al., 2008; Newton et al., 1999; Saenz, Toner, & Risco, 2009), some simplifying assumptions were made: i) the oocyte remained in perfect spherical shape during shrinking and swelling, with an area determined by the cell radius ($A = 4 * \pi * r^2$); ii) the solutions are ideal; iii) hydrostatic pressure is zero; iv) the osmotic coefficient for solutes are equal to 1, except for common salt (NaCl), with molalities and osmolalities equivalent and linear

additivity of solute's osmolalities. The cytosol was considered to contain only water and common salt (NaCl).

4.2.2.3 Permeability Modeling

First, a simulation of a standard vitrification preparation protocol, in which cell are left to equilibrate in the first solution (equilibration protocol, EP) was run. In this simulation, the oocyte is directly exposed to a 7.5% v/v EG and 7.5% v/v Me_2SO phosphate buffered saline (PBS) solution (solution 1, S1) for 10 minutes. Next, it was directly exposed to a 15% v/v EG, 15% v/v Me_2SO and 0.5M sucrose PBS solution (Solution 2, S2) with a duration of 1 minute. A second simulation was run with a shorter preparation protocol (short protocol, SP), for which the exposure of the oocyte to both solutions S1 and S2 is limited to 1 minute each. Key parameters for oocyte survival to vitrification, such as the volumetric excursion of the oocyte, intracellular active water content, and total cytosolic solute concentration were determined and compared between the two approaches.

4.2.2.4 In vivo Oocyte Osmotic Behaviour

To establish whether the model predictions were accurate, the *in silico* dehydration profiles obtained with the theoretical models were reproduced with *in vivo* observations of the osmotic behaviour of human oocytes exposed to the standard and short preparation protocols. Metaphase-II human oocytes retrieved in a private IVF setting by ovarian hyperstimulation, discarded for clinical practice due to failed fertilisation by ICSI and donated for research, were employed for the experiment.

Symbol	Meaning
w, s, sol, cpa	Subscripts (w, water; s, solutes; sol, cellular solutes; cpa, permeating cryoprotectants)
e, i,	superscripts; extracellular, intracellular
d	derivative of the function
V	Volume
A	Cell area
R	Universal gas constant
T	Absolute temperature
T_{REF}	Reference temperature
t	Time
L_p	Water permeability (hydraulic conductivity)
L_O	Permeability at a reference temperature
M	Osmolality
n	Number of moles
$\overline{V}$	Partial molar volume
N	Osmoles of solute
P_S	Solute permeability

Table 2. Definition of symbols employed in equations 1-3.

Perfusion with CPA solutions

Drops of 2 μL of iso-osmotic PBS, 200 μL of S1 and 2 mL of S2 were placed near-contact in a 60 mm Petri dish. The Petri dish was placed on a heated stage at 25 °C (INUG2H-TIZSH, Tokai, Japan) on an inverted microscope (Ti-U, Nikon, Japan) with an assembled micromanipulator (Narishige, Japan). For each experiment, an oocyte was placed in the PBS drop with a capillary, and secured by suction of the zona pellucida by a holding pipette

(MPH-MED-30, Origio, Denmark). To avoid dissecation, time between oocyte placement and perfusion of the solutions was minimised. The PBS drop and adjacent DS1 drop were merged with the tip of the capillary to trigger the osmotic process. Once the exposure time to DS1 was complete, the drop was merged with the adjacent DS2 drop. EP and DP protocols were replicated until we recorded five oocytes that retained a spherical morphology and presented fewer folds and creases during shrinking. Out of a total of 22 oocytes subjected to the experiment, 5 replicates of each protocol were selected for analysis and calculation of the cytoplasmic area.

4.2.2.5 Image acquisition and quantitative analysis of dehydration

The process was recorded on a PC (Software X) with a video acquisition system connected to the inverted microscope (Sony, Japan), with 400X magnification. A video frame of every ten seconds for the first minute of exposure on DS1 and DS2, and every 60 seconds after the first minute for the 10-minute exposure to DS1 was extracted and analysed to determine the volumetric excursion of the oocyte relative to the initial isotonic volume. Still images of the oocytes were processed to determine their area for volume calculation, as described by Mullen et al., (2008). To reduce skewing by irregularities in the spherical shape of the oocyte during shrinking, the radius was calculated from the oocyte's bidimensional area, and then used to determine the oocyte's volume, assuming that a perfect spherical shape is maintained throughout the process.

Parameter	value; uds; 25 °C; 1 atm	Reference.
Lp	0,69 μm/min	(Hunter et al., 1992)
E_A^{H2O}	14,42 kcal/mol	
P_{Me2SO}	15 μm/min	(Paynter, et al., 1999a)
E_A^{Me2SO}	23,52 kcal/mol	
P_{EG}	9,16 μm/min	(Mullen et al., 2008)
E_A^{EG}	21,20 kcal/mol	
$\bar{V}_{H2O}$	18,61 cm³/mol	(Markarian, Asatryan, & Zatikyan, 2005).
$\bar{V}_{NaCl}$	16,68 cm³/mol	(Pitzer, Peiper, & Busey, 1984).
$\bar{V}_{Me2SO}$	64,323 cm³/mol	(Markarian, Asatryan, & Zatikyan, 2005).
$\bar{V}_{EG}$	55,31 cm³/mol	(Ambrosone et al., 1996).

Table 3. Relevant parameters for permeability modeling of human Metaphase-II oocytes

4.2.2.6 Survival and competence of human zygotes after dehydration-based protocol.

The dehydration protocol was tested on discarded unfertilized human oocytes, and abnormally fertilised human zygotes, showing ≥2 pronuclei at the fertilisation check 17±2 hours after *in vitro* fertilisation. These oocytes and zygotes were discarded from clinical practice and donated for

research. In order to detect, with 80% power and an alpha level of 0.05, a 15% one-sided difference from a benchmark survival rate to vitrification of 95% (A. S. I. R. Medicine, 2012), 30 oocytes and 27 zygotes were included in the study.

Oocytes and zygotes were prepared for vitrification following the short protocol (SP) and loaded in a closed vitrification carrier (Safepreservation, Spain), which was hermetically sealed at both ends prior to a fast plunge in liquid nitrogen. Note that the loading, sealing and cooling of the vitrification device were performed during the 60 seconds of exposure to VS, and not afterwards. The process of loading the vitrification carrier is described thoroughly elsewhere (Gallardo et al., 2016). For re-warming, the capillary was transferred to a 37 °C water bath, stirred for two seconds, dried in sterile cloth and cut, releasing the oocytes in a 1 M sucrose solution. After 60 seconds, oocytes were transferred to a 0.5 M sucrose solution for three minutes and eventually placed for 5 minutes in a CPA-free solution. According to previous published measurements employing the same closed vitrification carrier, the zygotes were subjected to warming rates of 200,000 °C/min (Gallardo et al., 2016).

The survival of the oocytes and zygotes was assessed immediately after completing the warming and rehydration process: those whose morphological appearance remained similar to fresh after vitrification were considered positive for survival (A. S. I. R. Medicine, 2012). As an additional viability indicator, post-warming mitosis resumption and cleavage were assessed after 24 hours of culture. Results were compared with a control group of 27 abnormal zygotes not subjected to vitrification. Embryo culture was carried out in pre-equilibrated embryo-tested 60 mm Petri dishes, containing 30 μL drops of G1-Plus medium (Vitrolife, Sweden) with a mineral oil overlay at 37 °C and 6% CO_2.

4.2.3 Results

4.2.3.1 Modeling of the osmotic behaviour of the human Metaphase-II oocyte

The predicted relative volume and total intracellular solute concentration of oocytes subjected to two preparation protocols, the standard equilibration protocol (EP) and a short dehydration protocol (DP), as a function of time is shown in Figure 1. Both protocols feature exposure to a non-vitrifying solution (Sol1; 7.5% v/v Me_2SO and 7.5% v/v EG) and a vitrifying solution (Sol2; 15% v/v Me_2SO, 15% v/v EG and 0,5M sucrose). In EP, the duration of exposure to Sol1 is 10 minutes, and the duration of exposure to Sol2 is 1 minute. In DP, the duration of the exposure to both Sol1 and Sol2 is 1 minute each.

In both protocols, model predictions show that after exposure to Sol1, oocytes experience fast dehydration, shrinking to a minimum of 44.8% of their isotonic volume after 29.4 seconds of exposure. At the end of the exposure to Sol1 in DP, after 1 minute, equilibration has barely started, with the volume increasing to 48.6%. In the EP, exposure is prolonged to 10 minutes to allow for equilibration, and the oocyte swells further, back to 79.4% of its isotonic volume. Intracellular osmolarity, however, is very similar after either 1 or 10 minutes of exposure to Sol1 (Table 1 & Figure 1).

Models show the oocyte subjected to the EP shrinking to 49.9% of its isotonic volume after 1 minute of exposure to the Sol2, whereas oocytes subjected to DP, with a previous shorter exposure to Sol1, are expected to shrink to 39.2%. A similar total intracellular molarity is reached at the end of EP and DP, with 5.40 and 5.37 mol/L, respectively. However, in the EP, Me_2SO and EG contribute to 92.5% of the intracellular molarity, vs. 88.5% in the DP protocol. The normalised water volume, on the other hand, is lower after DP compared to EP (17.7 vs. 26.6, respectively) (Table 1).

		a)	b)	c)	d)	e)
		Duration of exposure	Intracellular Molarity	CPAs' contribution to total intracellular molarity	Relative Volume	Normalized water volume
		(min)	mol/L	(%)	(%)	(%)
Equilibration Protocol	Sol1	10	2.61	92.1	79.4	63.7
	Sol2	1	5.40	92.5	49.9	26.6
Dehydration Protocol	Sol1	1	2.56	83.5	48.6	31.6
	Sol2	1	5.37	88.5	39.2	17.7

Table 4. Comparison of the results of the 2-P model for relevant osmotic parameters between equilibration and dehydration protocols. a) Time elapsed since oocytes are initially exposed to each solution; b) Intracellular molarity of the oocyte; c) Contribution of CPA permeation to the total intracellular molarity; d) Relative volume of the oocyte as a ratio of the isotonic total cell volume; e) Water volume in the oocyte as a fraction of the initial water volume of the oocyte. The molarity of Sol1 and Sol2 is 2,4 mol/L and 5,3 mol/L, respectively.

4.2.3.2 In Vivo Oocyte Osmotic Behaviour

The *in vivo* recordings of human oocytes exposed to EP and DP show a milder initial osmotic response of the oocytes to Sol1. The minimum relative volume attained was of ~60% in EP and DP, and it took slightly longer than predicted to do so (~40 seconds of exposure to Sol1). Oocytes subjected to EP recovered ~80% of their initial isotonic volume after 10 minutes of exposure to Sol1, and then shrank to ~65% after 1 minute of exposure to Sol2. Oocytes subjected to DP shrank to ~50% after 1 minute of exposure to Sol2.
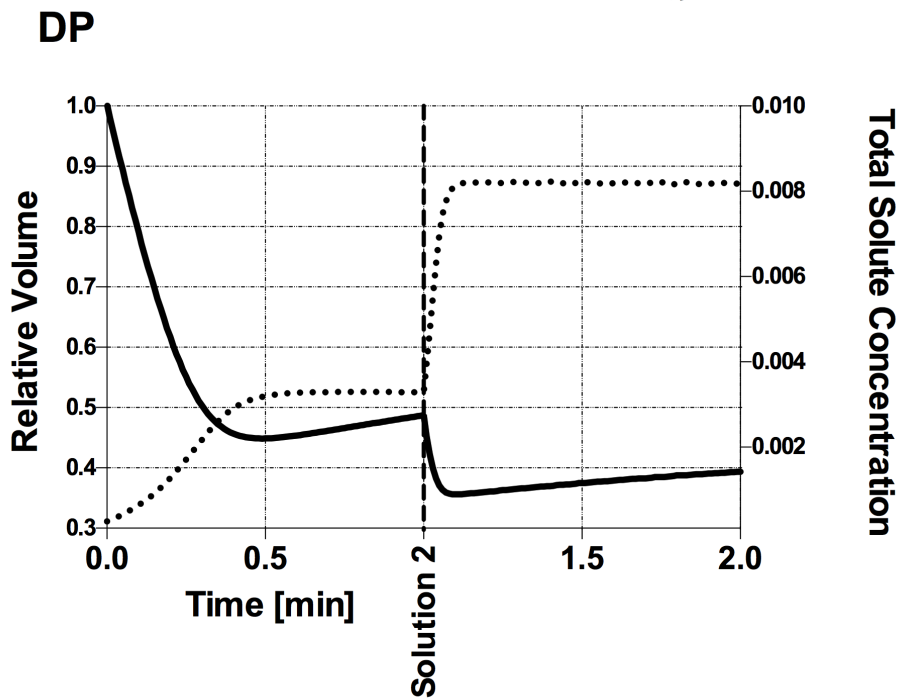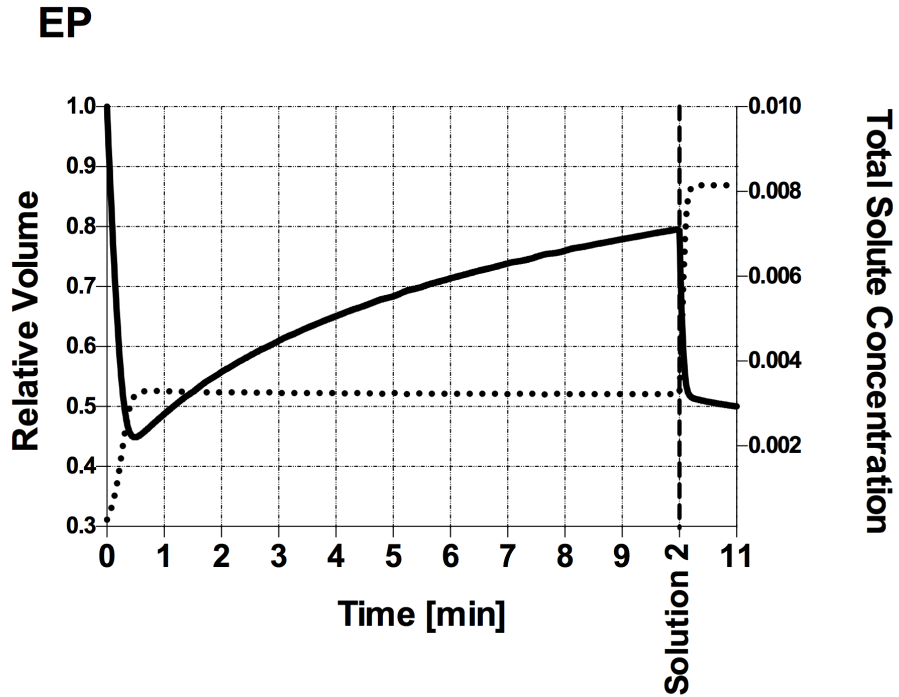
Figure 14: Simulation of the relative volume and intracellular solute concentration of human M-II oocytes with EP and DP. Relative volume as solid line, intracellular solute concentration as dotted line. Exposure to solution 1 (S1; 7.5% v/v Me2SO and 7.5% v/v EG) starts at t=0, and the change to solution 2 (S2; 15% v/v Me2SO, 15% v/v EG and 0,5M sacarose) is demarcated in the X axis by a vertical striped line.

4.2.3.3 Survival and viability of human oocytes and zygotes to vitrification and warming with SafeSpeed carrier after the short preparation protocol

As shown in Table 2, all oocytes and zygotes subjected to the experimental vitrification protocol DP were positive for survival (30/30 and 27/27, respectively). The observed survival of the oocytes was compared to the expected benchmark outcome of 95% survival in a normal population of oocytes, based on recent consensus (A. S. I. R. Medicine, 2012). Of 27 tripronuclear zygotes subjected to vitrification with DP, 25 resumed development and had cleaved after 24 hours of culture, compared to 24 of 27 fresh tripronuclear zygotes used as a control ($p=1$).

4.2.4 Discussion:

The survival and viability of unfertilised human oocytes prepared for vitrification with the dehydration protocol shows that their intracellular solute concentration was sufficiently high for the vitrification procedure to be successful at the cooling and warming rates obtained.

Despite non-fertilized human oocytes, that have remained in *in vitro* incubation for >24 hours, are reported to be more resilient to a vitrification procedure than their fresh counterparts (Vajta et al., 2009), the survival and mitosis resumption of the abnormally fertilised zygotes is reassuring regarding the effectivity of the approach. This outcome could to some extent be expected, as according to the 2-P model used to predict the osmotic behaviour of human oocytes, the intracellular molarity after the standard EP, with 11 minutes of duration, and the DP, with a duration of 2 minutes, was similar.
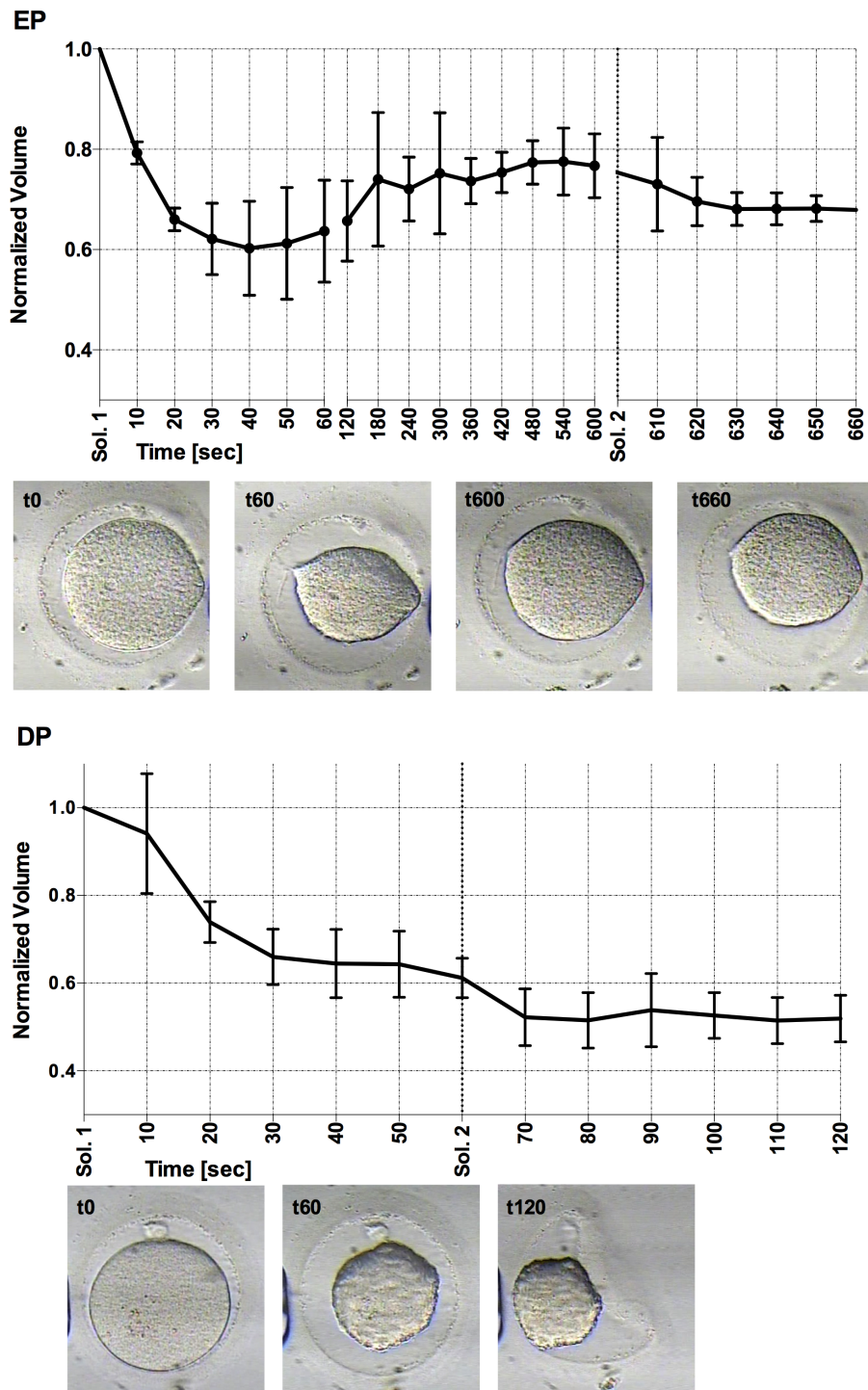
Figure 15: In vivo recording of the volumetric excursion of human M-II oocytes subjected to: Dehydration protocol (DP); Equilibration Protocol (EP) of preparation for vitrification. Exposure to S1 starts at t=0, the change to S2 is demarcated at the X axis. An example of the oocyte's morphology at relevant timeframes is shown below each graphic.

No. of oocytes positive for survival after 2 hours / No. of oocytes subjected to vitrification		30/30 (100)
No. of zygotes positive for survival after 2 hours / No. tripronuclear zygotes subjected to vitrification		27/27 (100)
No. of cleaved embryos after 24 hours / No. tripronuclear zygotes subjected to vitrification		24/27 (88,8)
No. of cleaved embryos after 24 hours / No. of fresh tripronuclear zygotes		25/27 (92,6)

Table 5. Survival and viability of human oocytes and zygotes after preparation for vitrification with the short protocol and vitrification with the SafeSpeed carrier.

However, the exact composition of the cytosolic medium after each preparation protocol may differ: in DP the contribution of permeating cryoprotectants Me_2SO and EG to the final cytosolic solute concentration was slightly lower than in the EP. On the other hand, SP resulted in lower intracellular active water content in the oocyte compared to EP. Relevant parameters that determine the glass-forming tendency of the cytosol, such as the viscosity and the degree of hydrogen bonding between present water and intracellular macromolecules, could for this reason also differ between oocytes prepared to vitrification with each protocol (Jin & Mazur, 2014; Rall, 1987). It should also be considered that we employed a carrier that is reported to produce higher rates of cooling and warming than others (Castelló et al., 2018; Gallardo et al., 2016; Kuwayama et al., 2005; Larman & Gardner, 2011; Risco et al., 2007; Schiewe et al., 2015;

Vanderzwalmen et al., 2009), which may compensate a possible lower intracellular glass forming tendency in the cells after DP (Yavin & Arav, 2007; Jin & Mazur, 2015).

The osmotic pressure and ensuing volumetric excursions that cells undergo during preparation for vitrification might also be higher during DP. The oocyte is not given time to equilibrate and regain volume, and as a result the total relative volume of the oocyte at the moment of cooling is 10% lower, compared to a standard equilibration protocol. Even if cell shrinkage is indeed desirable as it results in a more stable cytoplasm (Fahy et al., 1984), it is well known that osmotic stress can seriously jeopardise the developmental potential of oocytes, causing disruption in cytoskeleton structures, especially depolimerisation of the meiotic spindle (Agca et al., 2000; Hotamisligil, Toner, & Powers, 1996; Mullen et al., 2004; Vincent & Johnson, 1992). However, the exact osmotic tolerance limit of oocytes is unclear (McWilliams, Gibbons, & Leibo, 1995) and it has been conservatively estimated in previous attempts at optimisation of preparation protocols (Karlsson et al., 2014; Mullen et al., 2004; Vanderzwalmen et al., 2013). On the other hand, the reduced duration of the exposure to cryoprotectants in DP reduces the risks of CPA's chemical toxicity (Fahy et al., 1987; Fahy et al., 1984). Ultimately, the developmental ability of oocytes and embryos is the indicator of the safety and efficiency of a vitrification procedure (Agca et al., 2000; Castelló et al., 2018; De Munck et al., 2015), and such tests would be necessary to confirm the validity of the proposed protocol for human oocyte and embryo cryopreservation.

When the relative volume of an oocyte is calculated from a bidimensional framegrab of the shrinking-swelling process, several assumptions are made, so the permeability parameters inferred from these observations might not be completely accurate (Mullen et al., 2008; Paynter, et al; 1999a,b). Also, the biophysical parameters of oocytes seem to present high

intervariability, potentially associated with many factors (Paynter et al., 2005; Hunter et al., 1992). For these reasons, permeability models might not reflect in an entirely accurate fashion the biological reality of the process. In spite of that, we found a satisfactory agreement from our *in silico* and *in vitro* results, revealing that the point of minimum volume of the shrink-swell curve is attained within one minute of exposure to the hypertonic solutions employed (Gallardo et al., 2017). Interestingly, at this point, the osmotic effect of water loss and CPA intake was close to completion. Hence, limiting the exposure of the cells to the non-vitrifying and vitrifying hypertonic solutions to this point of minimum volume could be a more efficient approach to prepare them for vitrification, compared to a prolonged exposure to allow for partial osmotic equilibration. Under this premise, we were able to successfully reduce the necessary time to prepare human oocytes and zygotes for vitrification to a mere two minutes. Similar time-efficient dehydration-based protocols comprising short sequential exposures to increasingly hypertonic solutions could be fine-tuned for its application to other mammalian gametes and embryos as well as more complex models.

5. Main findings of this thesis

Over the last ten years, the late Peter Mazur's group developed a research line that progressively established the relevance and dominance of the warming rate as the most critical variable determining survival of cells to vitrification (Mazur & Paredes, 2016; Seki, Jin, & Mazur, 2014; Seki & Mazur, 2009, 2012). Their research gradually shifted the attention of cryobiologists in the context of AHR away from the cooling rate. It would not be fair to say, though, that cryobiologists working in AHR neglected the importance of warming rates; Rall and Fahy (Rall, 1987; Rall & Fahy, 1985) suggested their importance right from the technique's inception. In spite of that, this was barely acknowledged in subsequent manuscripts describing vitrification procedures, whereas the cooling rate was carefully examined.

However, one could argue that the change of paradigm towards the dominance of warming rates has limited to a theoretical shift. The way vitrification is carried out —the protocols of preparation of cells, the carriers— have not experimented much change, at least at the bench. If anything, Seki and Mazur's research has provided a more thorough explanation as to why some already existing vitrification carriers work. On one hand, we find that the success of open carriers could be put down to the fact that they were already more efficient in warming than in cooling (≈40,000 and ≈20,000 °C/min, respectively). On the other hand, the use of carriers hermetically sealed into a container is plausible because their warming rates compensate for the impaired cooling (≈2,000 °C/min): the carrier is insulated during cooling, but is warmed in a similar fashion to open systems, by direct transfer of the carrier to a volume (1-4 mL) of "warming solution" (Vajta et al., 2015).

Whether open and hermetically closed approaches, with their different warming rates, provide equivalent clinical results is a matter of controversy: a recent meta-analysis shows that despite similar survival, the ability to implant and develop a healthy pregnancy of embryos vitrified with systems with lower cooling rates seems to be impaired (Youm et al., 2017). This could be interpreted as the warming rates not being fast enough to fully compensate for the impaired cooling. The resulting damage, even if it is subtle enough not to reduce survival rates, may undermine the embryo's developmental potential at a late stage. That said, the safety of closed vitrification carriers has been extensively studied (De Munck, Belva, Van de Velde, Verheyen, & Stoop, 2016; De Munck et al., 2015; De Munck, Verheyen, Van Landuyt, Stoop, & Van de Velde, 2013; Panagiotidis et al., 2013; Papatheodorou et al., 2016; Stoop et al., 2012; Vanderzwalmen, et al., 2010a)

The capillary-based SafeSpeed is a vitrification carrier that not only provides higher warming rates (200,000 °C/min), but also higher cooling rates (120,000 °C/min) than other carriers, including previous capillary-based carriers (Kuwayama, 2007; Kuwayama et al., 2005). It could thus be assumed that for a given cytosolic solute concentration, the SafeSpeed is operating more safely (no pun intended) than other carriers, well above the critical cooling and warming threshold for lethal ice formation. It is from this conceptual point, and motivated by Jin & Mazur's success with laser-assisted warming, that we decided to explore the opportunities that these optimised rates of cooling and warming provided.

Another finding of Mazur that has not been exploited for current vitrification protocols was the role of free, unbound, water content inside the cell. Jin & Mazur (2014) were able to attain a cytosolic solute concentration in murine oocytes adequate for vitrification by using the non-permeant osmotic agent sucrose to dehydrate the oocytes, proving that survival to vitrification depended more on the active water content

prior to cooling and warming than the type and concentration of cryoprotectants in the cell interior. They, once again, shifted the focus from permeating cryoprotectants to decreasing water content. Survival rates to vitrification were only satisfactory, however, when oocytes were subjected to a laser pulse that produced warming rates of 10^7 °C/min, unattainable with vitrification carriers appropriate for use in the human assisted reproduction context. Other authors have optimised protocols of preparation of human oocytes and embryos for vitrification, yet focused on CPA permeation rather than dehydration, trying to minimise water loss and volumetric changes to avoid osmotic harm (Karlsson et al., 2014; Mullen et al., 2008; Vanderzwalmen et al., 2009)

As a clinical embryologist, I knew first hand that a desirable improvement of the vitrification protocols could be to reduce the total time the procedure takes, from the moment the embryos are removed from the culture plate to the time the carrier is stored. The number of embryos and oocytes that are cryopreserved rises each year, and the duration of current protocols limits the efficiency and scalability of the technique. Furthermore, the preparation of the cells to vitrification by exposure to the hypertonic vitrification solutions is very time consuming and delicate, as embryos are exposed to suboptimal temperatures (23 - 25 °C) and a potentially cytotoxic medium. After predicting the osmotic response of human Metaphase-II oocytes with a two parameter permeability model, and reproducing the *in silico* predictions by in vivo recording the osmometric behaviour of human oocytes, we came to the conclusion that long exposure times to hypertonic solutions may be unnecessary and inefficient.

Most of the sought-after osmotic effect (dehydration, CPA permeation) upon exposure of a cell to a hypertonic solution for its preparation for vitrification happens during the first seconds of exposure, ending when the cell reaches the minimum volume point of the shrink-swell curve. We

propose that a series of exposure to hypertonic with such duration would be the most time-efficient way to prepare a cell for vitrification. In the case of human oocytes, we test a protocol based on this premise with a total duration of two minutes: a >80% reduction from the average 12 minutes' duration that it takes to prepare oocytes for vitrification. This procedure was intellectually protected, and may be successfully exploited in diverse areas.

In the foreseeable future, the need for cryopreservation of human oocytes and embryos will persist. During fertility treatment, a woman is subjected to hormone stimulation in order to induce superovulation and recover a high number of mature oocytes. This high number of oocytes is necessary because only a few of those will get fertilised, and then a fraction of those will develop into good quality embryos. Then again, from that cohort of embryos, ideally only one is transferred into the uterus to avoid the risk of a multiple pregnancy. The other embryos of the cohort must be preserved, not only for ethical reasons but because we are unable to predict if the chosen embryo will implant, produce a pregnancy and develop to term. As long as AHR remains burdened with such inefficiencies, vitrification will remain a key element of fertility programmes, and for that reason it must be continually critically analysed and improved to guarantee is safety and effectiveness.

6. Future Perspectives

6.1 Automatization of the procedure

The vitrification of human oocytes and embryos is a manual procedure, with several operator-dependent parameters that can hardly be standardised. The care in the pipetting of the cells in the solutions, the meticulousness with which they are rinsed from non-vitrifying solutions prior to loading in the vitrification carrier, the volume of solution in which embryos are suspended in the carrier, their disposition, and even the quenching motion and storage-related handling are all factors that may affect the outcome of a vitrification procedure. All these aspects require a steep learning curve, and even though learning resources are abundant these days, not all centres of assisted reproduction can rely on their vitrification programmes. Even for experienced embryologists, there might be inter-procedure variability. Considering the hundreds or thousands of times the procedure is carried out, absolute reproducibility of the protocol is not assured.

Another factor that makes automated vitrification enticing is the time consuming nature of the process. As previously stated, it must be carried out in a controlled setting, and with batches of a limited number of samples, always by a single operator, to avoid putting the efficiency of the procedure at risk through manipulation mistakes and to guarantee traceability. As a result, for a vitrification machine to be a feasible and attractive alternative to the current state of the art, it must offer a high degree of automation, considerably reducing the manual intervention of the operator; improve the workflow in the IVF laboratory; increase the number of samples that can be cryopreserved within a certain workspace and time; and do so without impairing the outcomes.

Currently, there are two efforts towards automation in an advanced state of development. The "Gavi" device (Genea Biomedics, Australia) employs microfluidics to automate the exposure of the samples to CPA solutions, yet the

operations of quenching and warming are still completely manual, so it can only be regarded as a semi-automation. The advantages it offers time-wise per cryopreservation batch are consequently limited, and the cassette-format vitrification carrier is incompatible with many conventional cryogenic storage setups. However, outcomes of blastocyst vitrification with this system seem to be comparable to currently employed open systems. Another approach that deserves attention is the "Sarah" device (FertileSafe, Israel), which employs specially adapted 0.25 mL cryostraws to expose embryos to different preparation solutions, quenching in liquid nitrogen and warming in a sucrose solution. However, details regarding the time to complete the procedure and the degree of automation of the process have not been publicly disseminated, even if it shows promising results with other mammalian embryos (Ledda et al., 2018).
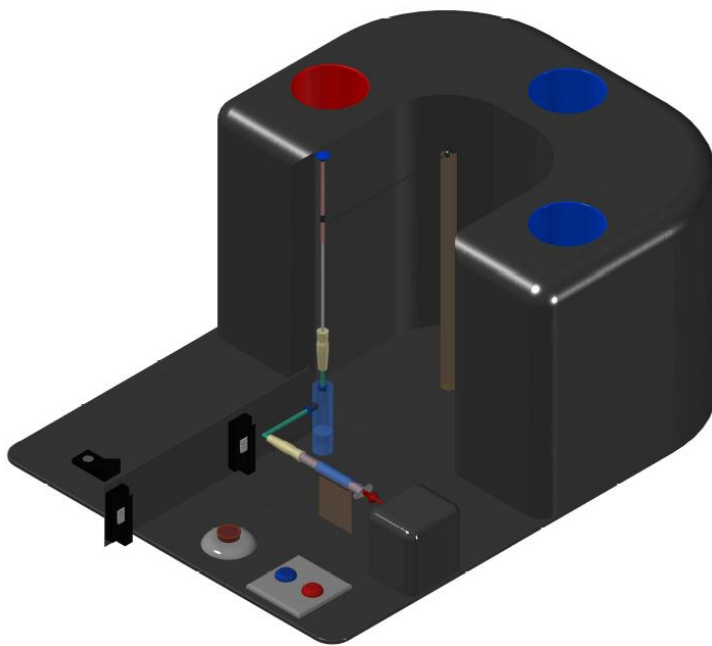

Figure 16. Functional 3D render scheme of a possible automation set-up for the SafeSpeed straw. A rotating arm holds it vertically and can transport the device to and from different wells. The straw is connected to a microfluidic solenoid valve for media exchange. This image is a simplification, for industrial protection reasons.

The SafeSpeed straw is a design that is susceptible of being adapted and incorporated in an automated vitrification device, a goal that is currently being pursued. The objective is to achieve a high degree of automation; the cells to be cryopreserved will be manually placed into a well from where they will be prepared for vitrification and loaded by controlled aspiration in a previously identified carrier, then sealed and safely quenched in liquid nitrogen for manual storage (Figure 16). The automation of the reverse process of automatic warming, recovery and CPA dilution of sealed carriers is also desirable.

6.2 X-Ray Computed Tomography

Current protocols to prepare oocytes and embryos for vitrification are based as much on rigorous theoretical formulations of the cells' osmotic behaviour as on empirical trial-and-error evidence accumulated over the years for the many factors that influence the vitrification outcome (Leibo et al., 2008). Considering that the degree of permeation of a certain CPA inside the cell over time will depend on temperature, time of exposure, presence of other solutes in the solution, etc., the variables soon grow exponentially. Mathematical tools for permeability modelling are alternatively a very useful approach to predict and optimize CPA exposure, yet they will only represent the biophysical reality to a certain extent, excluding operator-related variability and also are often misused (Katkov, 2011). The total intracellular concentration of solutes in an oocyte or embryo blastomer can also be indirectly deducted judging by their osmotic behaviour when exposed to a solution of known molarity (Vanderzwalmen et al., 2013).

X-ray computed tomography can be used to measure the concentration of Me_2SO inside solutions and tissues; the attenuation of the CT signal is proportional to the CPA concentration (Corral et al., 2015; ES2529265A1). With a spatial resolution of 50 μm, this technology could be used to assess the solute concentration within an oocyte at cryogenic temperatures and detect ice formation. Additionally, it could be used to determine the human Metaphase-II

oocyte's permeability parameters with greater accuracy. All this can lead to further improvements and optimisation in preparation protocols.

6.3 Introducing modifications of vitrification protocols in the clinical IVF practice

Currently, developments of the techniques employed to vitrify reproductive cells are governed by the premise that a compromise between safety and efficacy shall be maintained (De Munck & Vajta, 2017). The technique ought to be as aseptic and effective as possible. One could argue that SafeSpeed, as a closed carrier, meets this criterion of improving efficiency —thermal efficiency, at least— without compromising safety, and as such could be regarded as an advancement in the right direction. The use of the synthetic polymer hydroxypropyl cellulose also represents a safer and more stable alternative to human or animal derived proteins. The same could be said about our attempts at shortening the vitrification protocol: if the oocytes and embryos prove to be equally competent as with a longer protocol, reducing the duration of exposure to potentially cytotoxic cryoprotectants and suboptimal temperatures ought to be safer.

Ultimately, for every relevant modification of the procedure, a well designed validation route should be carried out before its use in the clinical context, from the preclinical stage in mammal models, to donated human material, through to clinical controlled prospective studies with follow-up (Harper et al., 2011).

References

- Agca, Y., Liu, J., Peter, A. T., Critser, E. S., & Critser, J. K. (1998). Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. *Mol Reprod Dev, 49*(4), 408-415. doi:10.1002/(SICI)1098-2795(199804)49:4<408::AID-MRD8>3.0.CO;2-R

- Agca, Y., Liu, J., Rutledge, J. J., Critser, E. S., & Critser, J. K. (2000). Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocyte complexes and its relevance to cryopreservation. *Mol Reprod Dev, 55*(2), 212-219. doi:10.1002/(SICI)1098-2795(200002)55:2<212::AID-MRD11>3.0.CO;2-M

- Al-Hasani, S., Ozmen, B., Koutlaki, N., Schoepper, B., Diedrich, K., & Schultze-Mosgau, A. (2007). Three years of routine vitrification of human zygotes: is it still fair to advocate slow-rate freezing? *Reprod Biomed Online, 14*(3), 288-293.

- Ali, J., & Shelton, J. N. (1993). Design of vitrification solutions for the cryopreservation of embryos. *J Reprod Fertil, 99*(2), 471-477.

- Ambrosone, L., Sartorio, R., Vescio, A., & Vitagliano, V. (1996). Volumetric properties of aqueous solutions of ethylene glycol oligomers at 25° C. Journal of the Chemical Society, Faraday Transactions, 92(7), 1163-1166.

- Amorim, C. A., Curaba, M., Van Langendonckt, A., Dolmans, M. M., & Donnez, J. (2011). Vitrification as an alternative means of cryopreserving ovarian tissue. *Reprod Biomed Online, 23*(2), 160-186. doi:10.1016/j.rbmo.2011.04.005

- Arav, A. (1992) Vitrification of oocytes and embryos. In New Trends in Embryo Transfer, edn 1, pp 255–264. Eds A Lauria & F Gandolfi. Cambridge: Portland Press.

- Bielanski, A., Nadin-Davis, S., Sapp, T., & Lutze-Wallace, C. (2000). Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology, 40*(2), 110-116. doi:10.1006/cryo.1999.2227

- Bielanski, A., & Vajta, G. (2009). Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units. *Hum Reprod, 24*(10), 2457-2467. doi:10.1093/humrep/dep117

- Borini, A., Lagalla, C., Bonu, M. A., Bianchi, V., Flamigni, C., & Coticchio, G. (2006). Cumulative pregnancy rates resulting from the use of fresh and frozen oocytes: 7 years' experience. *Reprod Biomed Online, 12*(4), 481-486.

- Boutron, P., & Kaufmann, A. (1978). Stability of the amorphous state in the system water--glycerol--dimethylsulfoxide. *Cryobiology, 15*(1), 93-108.

- Boutron, P., & Kaufmann, A. (1979). Stability of the amorphous state in the system water-glycerol-ethylene glycol. *Cryobiology, 16*(1), 83-89.

- Castelló, D., Cobo, A., Mestres, E., Garcia, M., Vanrell, I., Alejandro Remohí, J., . . . Costa-Borges, N. (2018). Pre-clinical validation of a closed surface system (Cryotop SC) for the vitrification of oocytes and embryos in the mouse model. *Cryobiology*. doi:10.1016/j.cryobiol.2018.02.002

- Ciotti, P. M., Porcu, E., Notarangelo, L., Magrini, O., Bazzocchi, A., & Venturoli, S. (2009). Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. *Fertil Steril, 91*(6), 2399-2407. doi:10.1016/j.fertnstert.2008.03.013

- Cobo, A., Remohí, J., Chang, C. C., & Nagy, Z. P. (2011). Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online, 23*(3), 341-346. doi:10.1016/j.rbmo.2011.05.014

- Coello, A., Campos, P., Remohí, J., Meseguer, M., & Cobo, A. (2016). A combination of hydroxypropyl cellulose and trehalose as supplementation for vitrification of human oocytes: a retrospective cohort study. *J Assist Reprod Genet, 33*(3), 413-421. doi:10.1007/s10815-015-0633-9

- Commission Directive 2006/86/EC of 24 October 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing, preservation, storage and dis- tribution of hu- man tissues and cells (Text with EEA relevance).

- Corral, A., Balcerzyk, M., Parrado-Gallego, Á., Fernández-Gómez, I., Lamprea, D. R., Olmo, A., & Risco, R. (2015). Assessment of the cryoprotectant concentration inside a bulky organ for cryopreservation using X-ray computed tomography. *Cryobiology*, *71*(3), 419-431.

- De Munck, N., Verheyen, G., Van Landuyt, L., Stoop, D., & Van de Velde, H. (2013). Survival and post-warming in vitro competence of human oocytes after high security closed system vitrification. *J Assist Reprod Genet, 30*(3), 361-369. doi:10.1007/s10815-013-9930-3

- De Munck, N., Petrussa, L., Verheyen, G., Staessen, C., Vandeskelde, Y., Sterckx, J., . . . Van de Velde, H. (2015). Chromosomal meiotic segregation, embryonic developmental kinetics and DNA (hydroxy)methylation analysis consolidate the safety of human oocyte vitrification. *Mol Hum Reprod, 21*(6), 535-544. doi:10.1093/molehr/gav013

- De Munck, N., Belva, F., Van de Velde, H., Verheyen, G., & Stoop, D. (2016). Closed oocyte vitrification and storage in an oocyte donation programme: obstetric and neonatal outcome. *Hum Reprod, 31*(5), 1024-1033. doi:10.1093/humrep/dew029

- De Munck, N., & Vajta, G. (2017). Safety and efficiency of oocyte vitrification. *Cryobiology, 78*, 119-127 doi:10.1016/j.cryobiol.2017.07.009

- Devroey, P., Polyzos, N. P., & Blockeel, C. (2011). An OHSS-Free Clinic by segmentation of IVF treatment. *Hum Reprod, 26*(10), 2593-2597. doi:10.1093/humrep/der251

- Edgar, D. H., & Gook, D. A. (2012). A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update, 18*(5), 536-554. doi:10.1093/humupd/dms016

- Edwards, R. G., Bavister, B. D., & Steptoe, P. C. (1969). Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature*, 221, 632-35.

- ESHRE Special Interest Group of Embryology, & Alpha Scientists in Reproductive Medicine. (2017). The Vienna consensus: report of an expert meeting on the development of art laboratory performance indicators. *Human Reproduction Open*, *2017*(2), hox011.

- Fabbri, R., Porcu, E., Marsella, T., Rocchetta, G., Venturoli, S., & Flamigni, C. (2001). Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod, 16*(3), 411-416.

- Fahy, G. M., MacFarlane, D. R., Angell, C. A., & Meryman, H. T. (1984). Vitrification as an approach to cryopreservation. *Cryobiology, 21*(4), 407-426.

- Fahy, G. M., Levy, D. I., & Ali, S. E. (1987). Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. *Cryobiology, 24*(3), 196-213.

- Fahy, G. M., Wowk, B., Wu, J., Phan, J., Rasch, C., Chang, A., & Zendejas, E. (2004). Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology, 48*(2), 157-178. doi:10.1016/j.cryobiol.2004.02.002

- Fahy, G. M. & Rall, W. (2007) Vitrification, an overview. In: Vitrification in Assisted Reproduction, A User's Manual and Troubleshooting Guide Ed: Tucker, M. J. & Liebermann, J. Informa Healthcare. 1-20.

- Fahy, G. M. (2010). Cryoprotectant toxicity neutralization. *Cryobiology, 60*(3 Suppl), S45-53. doi:10.1016/j.cryobiol.2009.05.005

- Fuller, B., & Paynter, S. (2004). Fundamentals of cryobiology in reproductive medicine. *Reprod Biomed Online, 9*(6), 680-691.

- Fuller, B. J., & Lee, C. Y. (2007). Hypothermic perfusion preservation: the future of organ preservation revisited?. *Cryobiology*, *54*(2), 129-145.

- Gallardo, M. (2015). Recalentamiento ultra-rápido: altas tasas de recuperación de ovocitos vitrificados en el sistema cerrado safespeed. Un nuevo paradigma en criopreservacion. Rev Asoc Est Biol Rep Noviembre 20(2);109.

- Gallardo, M. (2016). Hydroxypropyl cellulose is an effective surfactant agent for vitrification solutions: a prospective study on donor oocytes. European Society of Human Reproduction and Embryology. *Human Reproduction* 31(suppl1); 100-1.

- Gallardo, M., Hebles, M., Migueles, B., Dorado, M., Aguilera, L., González, M., . . . Risco, R. (2016). Thermal and clinical performance of a closed device designed for human oocyte vitrification based on the optimization of the warming rate. *Cryobiology, 73*(1), 40-46.

doi:10.1016/j.cryobiol.2016.06.001

- Gallardo, M., Hebles, M., Migueles, B., Dorado, M., Aguilera, L., González, M., . . . Risco, R. (2017). Hydroxypropyl cellulose supplementation in vitrification solutions: a prospective study with donor oocytes. *J Assist Reprod Genet, 34*(3), 417-422. doi:10.1007/s10815-016-0841-y

- Gook, D. A., Schiewe, M. C., Osborn, S. M., Asch, R. H., Jansen, R. P., & Johnston, W. I. (1995). Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. *Hum Reprod, 10*(10), 2637-2641.

- Grout, B. W., & Morris, G. J. (2009). Contaminated liquid nitrogen vapour as a risk factor in pathogen transfer. *Theriogenology, 71*(7), 1079-1082. doi:10.1016/j.theriogenology.2008.12.011

- Guerrero, J., Gallardo, M., Rodríguez-Arnedo, A., Ten, J., & Bernabeu, R. (2018). Comparison of two closed carriers for vitrification of human blastocysts in a donor program. *Cryobiology*. doi:10.1016/j.cryobiol.2018.03.002

- Hamawaki, A., Kuwayama, M., Hamano, S., (1999). Minimum volume cooling method for bovine blastocyst vitrification. Theriogenology 51, 165. Ref Type: Abstract.

- Harper, J., Cristina Magli, M., Lundin, K., Barratt, C. L., & Brison, D. (2011). When and how should new technology be introduced into the IVF laboratory?. *Human reproduction*, *27*(2), 303-313.

- He, X., Park, E. Y., Fowler, A., Yarmush, M. L., & Toner, M. (2008). Vitrification by ultra-fast cooling at a low concentration of cryoprotectants in a quartz micro-capillary: a study using murine embryonic stem cells. *Cryobiology, 56*(3), 223-232. doi:10.1016/j.cryobiol.2008.03.005

- Hotamisligil, S., Toner, M., & Powers, R. D. (1996). Changes in membrane integrity, cytoskeletal structure, and developmental

potential of murine oocytes after vitrification in ethylene glycol. *Biol Reprod, 55*(1), 161-168.

- Hunter, J., Bernard, A., Fuller, B., McGrath, J., & Shaw, R. W. (1992). Plasma membrane water permeabilities of human oocytes: the temperature dependence of water movement in individual cells. *J Cell Physiol, 150*(1), 175-179. doi:10.1002/jcp.1041500123

- Isachenko, V., Isachenko, E., Montag, M., Zaeva, V., Krivokharchenko, I., Nawroth, F., . . . van der Ven, H. (2005). Clean technique for cryoprotectant-free vitrification of human spermatozoa. *Reprod Biomed Online, 10*(3), 350-354.

- Isachenko, V., Montag, M., Isachenko, E., Dessole, S., Nawroth, F., & van der Ven, H. (2006). Aseptic vitrification of human germinal vesicle oocytes using dimethyl sulfoxide as a cryoprotectant. *Fertil Steril, 85*(3), 741-747. doi:10.1016/j.fertnstert.2005.08.047

- Isachenko, V., Todorov, P., Seisenbayeva, A., Toishibekov, Y., Isachenko, E., Rahimi, G., . . . Merzenich, M. (2018). Vitrification of human pronuclear oocytes by direct plunging into cooling agent: Non sterile liquid nitrogen *vs.* sterile liquid air. *Cryobiology, 80*, 84-88. doi:10.1016/j.cryobiol.2017.11.009

- Jelinkova, L., Selman, H. A., Arav, A., Strehler, E., Reeka, N., & Sterzik, K. (2002). Twin pregnancy after vitrification of 2-pronuclei human embryos. *Fertil Steril, 77*(2), 412-414.

- Jenniskens, P., & Blake, D. F. (1994). Structural transitions in amorphous water ice and astrophysical implications. *Science, 265*, 753-756.

- Jin, B., & Mazur, P. (2015). High survival of mouse oocytes/embryos after vitrification without permeating cryoprotectants followed by

ultra-rapid warming with an IR laser pulse. *Sci Rep, 5*, 9271. doi:10.1038/srep09271

- Kasai, M., Niwa, K., & Iritani, A. (1981). Effects of various cryoprotective agents on the survival of unfrozen and frozen mouse embryos. Journal of reproduction and fertility, 63(1), 175-180.

- Kasai, M., Hamaguchi, Y., Zhu, S. E., Miyake, T., Sakurai, T., & Machida, T. (1992). High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. Biology of reproduction, 46(6), 1042-1046.

- Karlsson, J. O., Cravalho, E. G., Borel Rinkes, I. H., Tompkins, R. G., Yarmush, M. L., & Toner, M. (1993). Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. *Biophys J, 65*(6), 2524-2536. doi:10.1016/S0006-3495(93)81319-5

- Karlsson, J. O., Szurek, E. A., Higgins, A. Z., Lee, S. R., & Eroglu, A. (2014). Optimization of cryoprotectant loading into murine and human oocytes. *Cryobiology, 68*(1), 18-28. doi:10.1016/j.cryobiol.2013.11.002

- Katayama, K. P., Stehlik, J., Kuwayama, M., Kato, O., & Stehlik, E. (2003). High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril, 80*(1), 223-224.

- Katkov, I. I. (2011). Amicus Plato, sed magis amica veritas∗: Plots must obey the laws they refer to and models shall describe biophysical reality!. *Cryobiology,* 62(3), 242-244.

- Kauzmann, W. (1948). The nature of the glassy state and the behavior of liquids at low temperatures. *Chemical reviews*, *43*(2), 219-256.

- Kleinhans, F. W. (1998). Membrane permeability modeling: Kedem-Katchalsky vs a two-parameter formalism. *Cryobiology, 37*(4), 271-289. doi:10.1006/cryo.1998.2135

- Kuleshova, L., Gianaroli, L., Magli, C., Ferraretti, A., & Trounson, A. (1999). Birth following vitrification of a small number of human oocytes: case report. *Hum Reprod, 14*(12), 3077-3079.

- Kuleshova, L. L., & Shaw, J. M. (2000). A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen. *Hum Reprod, 15*(12), 2604-2609.

- Kuleshova, L. L., Shaw, J. M., & Trounson, A. O. (2001). Studies on replacing most of the penetrating cryoprotectant by polymers for embryo cryopreservation. *Cryobiology, 43*(1), 21-31. doi:10.1006/cryo.2001.2335

- Kuwayama, M., Vajta, G., Ieda, S., & Kato, O. (2005). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online, 11*(5), 608-614

- Kuwayama, M. (2007). Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology, 67*(1), 73-80. doi:10.1016/j.theriogenology.2006.09.014

- Landa, V., & Teplá, O. (1990). Cryopreservation of mouse 8-cell embryos in microdrops. *Folia Biol (Praha), 36*(3-4), 153-158

- Lane, M., Schoolcraft, W. B., & Gardner, D. K. (1999). Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril, 72*(6), 1073-1078.

- Larman, M. G., & Gardner, D. K. (2011). Vitrification of mouse embryos with super-cooled air. *Fertil Steril, 95*(4), 1462-1466. doi:10.1016/j.fertnstert.2010.12.003

- Lassalle, B., Testart, J., & Renard, J. P. (1985). Human embryo features that influence the success of cryopreservation with the use of 1, 2 propanediol. *Fertility and sterility*, *44*(5), 645-651.

- Ledda, S., Kelly, J. M., Walker, S. K., Natan, Y., & Arav, A. (2018). 47 A New Device and Method for Successful Vitrification of In Vitro-Produced Ovine Embryos. *Reproduction, Fertility and Development*, *30*(1), 163-163.

- Leibo, S. P., McGrath, J. J., & Cravalho, E. G. (1978). Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. *Cryobiology, 15*(3), 257-271.

- Leibo, S. P. (2008). Cryopreservation of oocytes and embryos: optimization by theoretical versus empirical analysis. *Theriogenology*, *69*(1), 37-47.

- Liebermann, J., Tucker, M. J., Graham, J. R., Han, T., Davis, A., & Levy, M. J. (2002). Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. *Reprod Biomed Online, 4*(2), 146-150.

- Lovelock, J. E., & Bishop, M. W. H. (1959). Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature*, *183*(4672), 1394.

- Luyet, B. J., & Gehenio, P. M. (1947). Thermo-electric recording of vitrification and crystallization in ultra-rapid cooling of protoplasm. *Biodynamica, 6*(109-112), 93-100.

- Markarian, S. A., Asatryan, A. M., & Zatikyan, A. L. (2005). Volumetric properties of aqueous solutions of diethylsulfoxide at temperatures from 298.15 K to 343.15 K. The Journal of Chemical

- Mazur, P. (1963). Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol, 47*, 347-369.

- Mazur, P., & Paredes, E. (2016). Roles of intracellular ice formation, vitrification of cell water, and recrystallisation of intracellular ice on the survival of mouse embryos and oocytes. *Reprod Fertil Dev*. doi:10.1071/RD16021

- McWilliams, R. B., Gibbons, W. E., & Leibo, S. P. (1995). Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and disaccharides. *Hum Reprod, 10*(5), 1163-1171.

- Medicine, A. S. I. R. (2012). The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting. *Reprod Biomed Online, 25*(2), 146-167. doi:10.1016/j.rbmo.2012.05.006

- Medicine, P. C. o. A. S. f. R., & Technology, S. f. A. R. (2013). Mature oocyte cryopreservation: a guideline. *Fertil Steril, 99*(1), 37-43. doi:10.1016/j.fertnstert.2012.09.028

- Meryman, H. T. (2007). Cryopreservation of living cells: principles and practice. *Transfusion, 47*(5), 935-945. doi:10.1111/j.1537-2995.2007.01212.x

- Molina, I., Mari, M., Martínez, J. V., Novella-Maestre, E., Pellicer, N., & Pemán, J. (2016). Bacterial and fungal contamination risks in human oocyte and embryo cryopreservation: open versus closed vitrification systems. *Fertil Steril, 106*(1), 127-132. doi:10.1016/j.fertnstert.2016.03.024

- Mori, C., Yabuuchi, A., Ezoe, K., Murata, N., Takayama, Y., Okimura, T., ... & Okuno, T. (2015). Hydroxypropyl cellulose as an option for supplementation of cryoprotectant solutions for embryo vitrification in

human assisted reproductive technologies. *Reproductive biomedicine online*, *30*(6), 613-621.

- Mukaida, T., Wada, S., Takahashi, K., Pedro, P. B., An, T. Z., & Kasai, M. (1998). Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Hum Reprod, 13*(10), 2874-2879.

- Mullen, S. F., Agca, Y., Broermann, D. C., Jenkins, C. L., Johnson, C. A., & Critser, J. K. (2004). The effect of osmotic stress on the metaphase II spindle of human oocytes, and the relevance to cryopreservation. *Hum Reprod, 19*(5), 1148-1154. doi:10.1093/humrep/deh201

- Mullen, S. F., Li, M., Li, Y., Chen, Z. J., & Critser, J. K. (2008). Human oocyte vitrification: the permeability of metaphase II oocytes to water and ethylene glycol and the appliance toward vitrification. *Fertil Steril, 89*(6), 1812-1825. doi:10.1016/j.fertnstert.2007.06.013

- Nei, T. (1976). Freezing injury to erythrocytes. I. Freezing patterns and post-thaw hemolysis. *Cryobiology, 13*(3), 278-286.

- Newton, H., Pegg, D. E., Barrass, R., & Gosden, R. G. (1999). Osmotically inactive volume, hydraulic conductivity, and permeability to dimethyl sulphoxide of human mature oocytes. *J Reprod Fertil, 117*(1), 27-33.

- Panagiotidis, Y., Vanderzwalmen, P., Prapas, Y., Kasapi, E., Goudakou, M., Papatheodorou, A., . . . Maroulis, G. (2013). Open versus closed vitrification of blastocysts from an oocyte-donation programme: a prospective randomized study. *Reprod Biomed Online, 26*(5), 470-476. doi:10.1016/j.rbmo.2013.01.016

- Papatheodorou, A., Vanderzwalmen, P., Panagiotidis, Y., Petousis, S., Gullo, G., Kasapi, E., . . . Prapas, Y. (2016). How does closed system vitrification of human oocytes affect the clinical outcome? A prospective, observational, cohort, noninferiority trial in an oocyte

donation program. *Fertil Steril, 106*(6), 1348-1355. doi:10.1016/j.fertnstert.2016.07.1066

- Paynter, S. J., Cooper, A., Gregory, L., Fuller, B. J., & Shaw, R. W. (1999). Permeability characteristics of human oocytes in the presence of the cryoprotectant dimethylsulphoxide. *Hum Reprod, 14*(9), 2338-2342.

- Paynter, S. J., Fuller, B. J., & Shaw, R. W. (1999). Temperature dependence of Kedem-Katchalsky membrane transport coefficients for mature mouse oocytes in the presence of ethylene glycol. *Cryobiology, 39*(2), 169-176. doi:10.1006/cryo.1999.2199

- Pegg, D. E., Wang, L., & Vaughan, D. (2006). Cryopreservation of articular cartilage. Part 3: the liquidus-tracking method. *Cryobiology, 52*(3), 360-368. doi:10.1016/j.cryobiol.2006.01.004

- Pitzer, K. S., Peiper, J. C., & Busey, R. H. (1984). Thermodynamic properties of aqueous sodium chloride solutions. Journal of Physical and Chemical Reference Data, 13(1), 1-102.

- Polge, C., Smith, A. U., & Parkes, A. S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature, 164*(4172), 666.

- Pradzynski, C. C., Forck, R. M., Zeuch, T., Slavíček, P., & Buck, U. (2012). A fully size-resolved perspective on the crystallization of water clusters. *Science, 337*(6101), 1529-1532. doi:10.1126/science.1225468

- Rall, W. F., Reid, D. S., & Polge, C. (1984). Analysis of slow-warming injury of mouse embryos by cryomicroscopical and physiochemical methods. *Cryobiology, 21*(1), 106-121.

- Rall, W. F., & Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature, 313*(6003), 573-575.

- Rall, W. F. (1987). Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology, 24*(5), 387-402.

- Registo, S.E.F (2015). Registro Nacional de Actividad, Informe estadístico de técnicas de reproducción asistida. Sociedad Española de Fertilidad.
http://www.cnrha.msssi.gob.es/registros/pdf/Informe_Global_Registro_actividad_2015.pdf

- Rienzi, L., Gracia, C., Maggiulli, R., LaBarbera, A. R., Kaser, D. J., Ubaldi, F. M., . . . Racowsky, C. (2017). Oocyte, embryo and blastocyst cryopreservation in ART: systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Hum Reprod Update, 23*(2), 139-155. doi:10.1093/humupd/dmw038

- Risco, R., Elmoazzen, H., Doughty, M., He, X., & Toner, M. (2007). Thermal performance of quartz capillaries for vitrification. *Cryobiology, 55*(3), 222-229. doi:10.1016/j.cryobiol.2007.08.006

- Saenz, J., Toner, M., & Risco, R. (2009). Comparison between ideal and nonideal solution models for single-cell cryopreservation protocols. *J Phys Chem B, 113*(14), 4853-4864. doi:10.1021/jp807274z

- Sathananthan, A. H., Trounson, A., & Freeman, L. (1987). Morphology and fertilizability of frozen human oocytes. *Gamete Res, 16*(4), 343-354. doi:10.1002/mrd.1120160408

- Schiewe, M. C., Zozula, S., Anderson, R. E., & Fahy, G. M. (2015). Validation of microSecure vitrification (μS-VTF) for the effective cryopreservation of human embryos and oocytes. *Cryobiology, 71*(2), 264-272. doi:10.1016/j.cryobiol.2015.07.009

- Schiewe, M. C., Zozula, S., Nugent, N., Waggoner, K., Borba, J., Gamboa, L., & Whitney, J. B. (2017). Modified MicroSecure Vitrification: A Safe, Simple and Highly Effective Cryopreservation Procedure for Human Blastocysts. *J Vis Exp*(121). doi:10.3791/54871

- Schoolcraft, W. B., Treff, N. R., Stevens, J. M., Ferry, K., Katz-Jaffe, M., & Scott, R. T. (2011). Live birth outcome with trophectoderm biopsy, blastocyst vitrification, and single-nucleotide polymorphism microarray-based comprehensive chromosome screening in infertile patients. *Fertil Steril, 96*(3), 638-640. doi:10.1016/j.fertnstert.2011.06.049

- Seki, S., & Mazur, P. (2009). The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology, 59*(1), 75-82. doi:10.1016/j.cryobiol.2009.04.012

- Seki, S., & Mazur, P. (2012). Ultra-rapid warming yields high survival of mouse oocytes cooled to -196°c in dilutions of a standard vitrification solution. *PLoS One, 7*(4), e36058. doi:10.1371/journal.pone.0036058

- Seki, S., Jin, B., & Mazur, P. (2014). Extreme rapid warming yields high functional survivals of vitrified 8-cell mouse embryos even when suspended in a half-strength vitrification solution and cooled at moderate rates to -196°C. *Cryobiology, 68*(1), 71-78. doi:10.1016/j.cryobiol.2013.12.001

- Shaw, P. W., Bernard, A. G., Fuller, B. J., Hunter, J. H., & Shaw, R. W. (1992). Vitrification of mouse oocytes using short cryoprotectant exposure: effects of varying exposure times on survival. *Mol Reprod Dev, 33*(2), 210-214. doi:10.1002/mrd.1080330214

- Shaw JM, Kuleshova LL, Macfarlane DR, Trounson AO. (1997). Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran. *Cryobiology,* 35,219–29.

- Shaw, J. M., & Jones, G. M. (2003). Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum Reprod Update, 9*(6), 583-605.

- Smith, A.U. Effects of low temperatures on living cells and tissues. In Harris RJC, editor. Biological Applications of Freezing and Drying. New York: Academic Press. (1954). 1-53

- Stiles W. On the cause of cold death of plants. Protoplasma 1930; 9: 459–68.

- Stoop, D., De Munck, N., Jansen, E., Platteau, P., Van den Abbeel, E., Verheyen, G., & Devroey, P. (2012). Clinical validation of a closed vitrification system in an oocyte-donation programme. *Reprod Biomed Online, 24*(2), 180-185. doi:10.1016/j.rbmo.2011.10.015

- Toner, M., Cravalho, E. G., Karel, M., & Armant, D. R. (1991). Cryomicroscopic analysis of intracellular ice formation during freezing of mouse oocytes without cryoadditives. *Cryobiology, 28*(1), 55-71.

- Trounson, A., & Mohr, L. (1983). Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature, 305*(5936), 707.

- Tsujimoto, M., Imura, S., & Kanda, H. (2016). Recovery and reproduction of an Antarctic tardigrade retrieved from a moss sample frozen for over 30 years. *Cryobiology, 72*(1), 78-81. doi:10.1016/j.cryobiol.2015.12.003

- Vajta, G., & Kuwayama, M. (2006). Improving cryopreservation systems. Theriogenology, 65(1), 236-244.

- Vajta, G., Nagy, Z. P., Cobo, A., Conceicao, J., & Yovich, J. (2009). Vitrification in assisted reproduction: myths, mistakes, disbeliefs and confusion. Reproductive biomedicine online, 19, 1-7.

- Vajta, G., Rienzi, L., & Ubaldi, F. M. (2015). Open versus closed systems for vitrification of human oocytes and embryos. *Reprod Biomed Online, 30*(4), 325-333. doi:10.1016/j.rbmo.2014.12.012

- Vanderzwalmen, P., Ectors, F., Grobet, L., Prapas, Y., Panagiotidis, Y., Vanderzwalmen, S., . . . Zech, N. H. (2009). Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM. *Reprod Biomed Online, 19*(5), 700-707.

- Vanderzwalmen, P., Zech, N., Lejeune, B., Wirtleitner, B., Zech, M., & Ectors, F. (2010). Vitrification and the use of high concentrations of cryoprotectants: is it a justified argument to prefer slow freezing?. *Gynecol Obstet Fertil, 38*(9), 536-540. doi:10.1016/j.gyobfe.2010.07.008

- Vanderzwalmen, P., Zech, N., Prapas, Y., Panagiotidis, Y., Papatheodorou, A., Lejeune, B., . . . Ectors, F. (2010). Closed carrier device: a reality to vitrify oocytes and embryos in aseptic conditions. *Gynecol Obstet Fertil, 38*(9), 541-546. doi:10.1016/j.gyobfe.2010.07.011

- Vanderzwalmen, P., Connan, D., Grobet, L., Wirleitner, B., Remy, B., Vanderzwalmen, S., . . . Ectors, F. J. (2013). Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions. *Hum Reprod, 28*(8), 2101-2110. doi:10.1093/humrep/det107

- Vincent, C., & Johnson, M. H. (1992). Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. *Oxf Rev Reprod Biol, 14*, 73-100.

- Whittingham, D. G., Leibo, S. P., & Mazur, P. (1972). Survival of mouse embryos frozen to -196 degrees and -269 degrees C. *Science, 178*(4059), 411-414.

- Wowk, B. (2010). Thermodynamic aspects of vitrification. *Cryobiology, 60*(1), 11-22. doi:10.1016/j.cryobiol.2009.05.007

- Yavin, S., Aroyo, A., Roth, Z., & Arav, A. (2009). Embryo cryopreservation in the presence of low concentration of vitrification

solution with sealed pulled straws in liquid nitrogen slush. *Hum Reprod, 24*(4), 797-804. doi:10.1093/humrep/den397

- Youm, H. S., Choi, J. R., Oh, D., & Rho, Y. H. (2017). Survival Rates in Closed and Open Vitrification for Human Mature Oocyte Cryopreservation: A Meta-Analysis. *Gynecol Obstet Invest*. doi:10.1159/000484243

- Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., ... & Van der Poel, S. (2009). The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology, 2009. *Human reproduction*, *24*(11), 2683-2687.

Supplementary Material

Original Research Article 1

Hydroxypropyl cellulose supplementation in vitrification solutions: a prospective study with donor oocytes

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<u>**Abstract**</u>

Purpose Hydroxypropyl cellulose (HPC), a polysaccharide that forms a viscous gel under low temperatures, is a promising substitute of the blood-derived macromolecules traditionally used in cryopreservation solutions. The performance of a protein-free, fully synthetic set of vitrification and warming solutions was assessed in a matched pair analysis with donor oocytes.

Methods A prospective study including 219 donor MII oocytes was carried out, comparing the laboratory outcomes of oocytes vitrified with HPC-based solutions and their fresh counterparts. The primary performance endpoint was the fertilization rate. Secondary parameters assessed were embryo quality on days 2 and 3.

Results 70/73 (95.9%) vitrified MII oocytes exhibited morphologic survival 2 h post-warming, with 49 (70.0%) presented normal fertilization, compared to 105 of 146 (71.9%) MII fresh oocytes. Similar embryo quality was observed in both groups. A total of 18 embryos implanted, out of 38 embryos transferred (47.3%), resulting in 13 newborns.

Introduction

The ability to efficiently cryopreserve oocytes and embryos by vitrification [1] has become essential in the field of human assisted reproduction. There are two main factors which influence the efficacy of vitrification: (i) the cooling/warming rates (which are influenced by the type of vitrification device used) and (ii) the glass-forming tendency (which is determined by the composition of the solution) [2–8]. Of course, each of these two factors depends on multiple parameters [9, 10]. With current techniques, the rates of survival of embryos and oocytes to the vitrification process have reached very high levels [11–13].

Human oocyte vitrification is a routine procedure today [14]. Historically, it has presented a greater challenge than embryo vitrification for many reasons. Oocytes' low surface to volume ratio, and the low permeability coefficient of their plasma membrane, hinders the exchange between cryoprotectant agents (CPAs) and water [15, 16]. However, this issue has been overcome by the synergic combination of permeating CPAs, mainly ethylene glycol and dimethyl sulfoxide, and non-permeating CPAs, such as sucrose and trehalose, and adjusting the concentration and the exposure time to CPAs needed to achieve a successful vitrification with currently attainable cooling and warming rates [17–22].

Another component of the solutions used for vitrification and warming is the macromolecular supplementation. It increases the viscosity of the solution to enhance the glass-forming tendency, enables the in vitro manipulation required for the vitrification procedure, and prevents the attachment of the gametes and embryos to the surfaces of pipettes, dishes, and devices used in the process [23]. Protein supplementation with human serum albumin has traditionally been employed for this task, alone or in the form of synthetic serum substitute (SSS), or dextran se- rum supplement (DSS), and it is still used in most commercial formulations. However, as a human-source material, it presents

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risks of contamination and production variability associated with it [24], so a replacement synthetic component has been sought. The international regulatory policies are also encouraging the use of solutions free of human or animal components.

The fully synthetic macromolecule hydroxypropyl cellulose (HPC) has been a successful candidate: it is a variable length polysaccharide that, with certain molecular weights, when added to a solution, can achieve very similar physical properties to the albumin-based formulations, forming a viscous gel under low temperatures. It is listed as pharmacopeia and is a common food additive and drug excipient [25]. Preliminary studies have shown promising results of HPC supplementation of vitrification solutions on murine oocytes and blastocysts in terms of survival and embryo development [26, 27]. Recently, direct comparative studies revealed the similarity of HPC and SSS-based formulations for human oocytes vitrification [25, 28].

In the present study, we use a formulation of protein- free vitrification and warming solutions for the vitrification of oocytes from an egg donation program. Laboratory outcomes of vitrified-warmed oocytes are compared against fresh oocytes from the same donor from a different stimulation cycle. This design allows us to evaluate the impact of vitrification on the developmental abilities of donor oocytes and assess the performance of an HPC-based vitrification formulation for its use in daily laboratory practice.

Materials and methods

Study design and outcome measures

A matched pair analysis within donor was performed, involving results from 219 MII oocytes from 19 oocyte donors between June 2014 and January 2015. Each recipient was assigned with two cohorts of oocytes from the same donor; one composed by six to nine fresh oocytes from a synchronized donation cycle (control group), and the other cohort composed two to five oocytes were

vitrified/rewarmed oocytes from a previous cycle of the same donor (experimental group). Both groups of oocytes underwent ICSI in parallel. The primary end-point was the fertilization rate of fresh and vitrified oocytes, with secondary assessments of embryo quality performed on day 2 and day 3.

Study population

All donors included on the study complied with the regula- tions on ART donors, described elsewhere [29], and the following inclusion criteria: over 18 and under 30 years old, good physical and psychological health, no personal nor familiar history for hereditary diseases, normal karyotype, negative tests for sexually transmitted diseases, and without any medical or gynecological disorders. Donors were subjected to a short agonist stimulation treatment and were excluded in case of a response to stimulation <12 oocytes. All recipients for the egg donation program were offered to participate in the study, until the quota was filled. Couples with a severe male factor surgically extracted spermatozoa and very severe oligoastenozoospermia (motile sperm count <500.000/ml after preparation) were not included.

Ethical considerations

All recipients were informed thoroughly about the protocol of the study and signed an informed consent form. The study was conducted as described in the protocol, which was developed in accordance to the principles of Helsinki and the national Policy of Good Clinical Practice (ISO 14155:2011), and was approved by the internal review board of the institutional research committee.

Donor and recipient stimulation

To synchronize the donation, donors took contraceptive pills during the previous month to the stimulation. Stimulation dose was decided according to donor's BMI (150–200 daily IU of rFSH, alpha folitropin, Gonal-F, Merck). On day 5 of stimulation, analysis of the estradiol levels and ultrasound scans was performed for dose adjustment. When a follicle reached 14 mm, they

started GnRH antagonist treatment with 0.25 mg Cetrotide (Cetrorelix, Merck) until at least three follicles reached 20 mm, when ovulation was triggered with 0.3 mg of triptoreline GnRH agonist (Decapeptyl; IpsenPharma).

Recipients were kept on contraceptive pills for the synchronized donation. Once discontinued, after menstruation, they started endometrial substitutive treatment with 6 mg daily of estradiol hemihydrate (Estradot, Novartis, Switzerland). On the day of the donor's ovarian puncture, they began taking progesterone vaginal or oral supplement, 600 mg daily (Utrogestan, Seid, Spain), continued until the results of the embryo transfer were known.

Ovarian puncture

A total of 19 oocyte donation cycles were included in the study. Oocyte recovery was performed by eco-guided ovarian puncture. An ultrasound transducer was placed into the vaginal fornix to visualize the ovary and follicles, and a single lumen ovum aspiration needle (Cook Medical) was inserted in the transducer and advanced into the ovarian follicles, where oocytes were recovered by aspiration with a syringe, until all the follicles had been punctured.

Embryology procedures

Two hours post-retrieval, the fresh control group of oocytes was denuded using hyaluronidase (HYASE-10X, Vitrolife), and the experimental group of oocytes was rewarmed as described below. Both control and experimental groups underwent ICSI simultaneously, 38–40 h post-hCG in the case of fresh oocytes and 2 h post-warming in the case of vitrified oocytes. Embryos were cultured on microdrops (G-IVF, G1Plus, G2Plus, Vitrolife) under mineral oil (tissue culture oil, Sage, CooperSurgical) until the third day of development, when they were either transferred, cryopreserved, or kept on culture up to day 6, according to their quality.

Vitrification protocol

Vitrification solutions were supplemented with 0.06– 0.125 mg/ml of hydroxypropyl cellulose (pharmaceutical standard, 80,000 Da average molecular weight, Sigma Aldrich) and did not contain albumin or any other proteins. Vitrification of the experimental group oocytes was performed at laboratory room temperature (RT), in our case 23–26 ± 0.5 °C, with SafeSpeed vitrification media (SafePreservation). The vitrification kit consists of three solutions: washing solution (WS, no CPAs), equilibration solution (ES 7.5% ethylene glycol and 7.5% dimethyl sulfoxide) and vitrification solution (VS 15% ethylene glycol and 15% dimethyl sulfoxide) [30].

All solutions were exposed at least for an hour to laboratory RT. Oocytes were gradually exposed to the cryoprotective agents by placing them in a first droplet of 50 μl of WS, which was joined with the tip of the pipette to another droplet of 50 μl of ES. After 2 min, both droplets were joined with a third ES drop and allowed an additional 2 min of CPA diffusion to the primary droplet where the oocytes were located. Afterwards, oocytes were transferred to 100 μl of ES, where they remained up to 10 min, until the oocytes appeared to be fully re-expanded. Once the equilibration was complete, oocytes were transferred to a 200-μl droplet of VS and washed at least three times to eliminate any leftover ES. Then the oocytes were placed on the thin plastic strip of a Cryotop cryodevice, as described by Kuwayama et al. [31], and plunged vertically in liquid nitrogen. The amount of time the oocytes remained in VS for washing and loading purposes before plunging in liquid nitrogen was approximately 60 s.

Warming protocol

SafeSpeed warming media consists in three solutions: thawing solution (TS) (1 M sucrose), dilution solution (DS) (0.5 M sucrose), and WS (no CPAs) [30]. For at least 1 h, closed vials of DS and WS were exposed to laboratory RT, and TS vial was placed in the incubator at 37 °C. The vitrification straw containing

the oocytes was transferred from a styrofoam box with liquid nitrogen to a double-well dish (Becton-Dickinson, 60 × 15 mm Falcon Center-Well Organ Culture Dish) containing 1 ml of TS at 37 °C. The liquid nitrogen container and the double-well dish used for warming were placed as close as possible (10–20 cm) to allow for a fast transfer (less than a second), to facilitate a high warming rate. After 1 min, oocytes were carefully moved to a 200-μl droplet of DS, where they remained 3 min. Two washing steps of 5 and 1 min, respectively, were performed in 200 μl droplets of WS. Warmed oocytes remained in culture for 2 h prior to ICSI.

Primary outcomes

Morphological survival

Morphological survival was dictated 2 h post-warming. Oocytes evaluation was based on the integrity of oocyte features, such as intact polar corpuscle, normal oolemma, and absence of vacuoles [32]. Oocytes clearly degenerated or considered not suitable for ICSI were deemed non-viable.

Fertilization rate

Fertilization rate was defined as the proportion of oocytes with two pronuclei at the time of fertilization check (17 ± 1 h post- insemination) [32]. The number of oocytes showing no signal of fertilization, abnormal fertilization (1 or >2 pronuclei), and degenerated oocytes was recorded.

Secondary outcomes

Embryo development was checked at 44 ± 2 h (day 2) and 68 ± 2 h (day 3) post-ICSI. For comparative purposes, the embryo assessment algorithm proposed by Cobo et al. [33] was used, considering day 2 good quality embryos as those pre- senting 2–4 blastomeres, ≤15% type I–II fragmentation, and no multinucleation. Day 3 good quality embryos were those with 6–8 blastomeres and ≤20% type I–II fragmentation.

Clinical outcomes

Clinical pregnancy was confirmed by ultrasound visualization of a gestational sac with fetal heartbeat. The implantation rate was calculated as the ratio of gestational sacs with fetal heartbeat by the number of embryos transferred. The live birth rate was calculated as live birth events per embryo transfer, with twin deliveries considered as a single event. Any perinatal complications were recorded.

Statistical analysis

The study was designed to detect a difference of 15% in the primary endpoint, fertilization rate, and between vitrified and fresh donor oocytes (N = 216 ICSI'ed), with a power of 80% and a significance level (α) of 0.05. The correlation between qualitative ordinal variables was analyzed using chi-square and Fisher's exact test. All statistical analysis was performed using the IBM SPSS statistics 17.0 package.

Results

The number of subjects included in the study, their age, and the number of oocytes assigned to the recipients is shown on Table 1.

Primary endpoint: fertilization rates

Out of 73 vitrified MII oocytes, 70 (95.9%) presented morphologic survival 2 h post-warming, and 49 of them (70.0%) presented normal fertilization, compared to 105 (71.9%) of 146 MII fresh oocytes ($p > 0.05$) (Table 2).

Secondary endpoints: embryo quality and development

Table 2 reveals no differences ($p > 0.05$) in cleavage rates or the embryo quality of vitrified or fresh oocytes on day 2 or day 3.

Clinical results

Clinical results of the cycles were collected without comparative purposes and are shown on Table 3. They were not subjected to statistical analysis due to the low sample size. Thirty-eight embryos were transferred in total, with a total implantation rate of 44.7% and a clinical pregnancy rate of 52.6%, resulting in 12 live birth events.

	Donors	Recipients
Number	13	19
Age (y.o.)	25.6 (2.7)	41.5 (4.2)
# fresh oocytes (control)	—	7.7 (1.2)
# vitrified oocytes (experimental)	—	3.8 (1.1)

Table 1 Characteristics of the donors and recipients included in the study and the ratio of control/experimental oocytes assigned. Oocytes from some donors were assigned to more than one recipient. In the row labeled as number, the values represent the number of donors or recipients. In the rest of the rows, data is presented as mean (SD).

Discussion

It is well established that human or animal-derived protein supplementation of the solutions used for gamete and embryo vitrification has a positive effect on the survival rates obtained [24]. Solutions are typically supplemented with 10–20% of SSS or DSS—albumin and glycoprotein solutions—that, among other things, provide viscosity to the solution and a surfactant property that enables the handling and pipetting of the gametes/embryos [23]. These formulations have been used for years and are currently in use with very satisfactory results. However, there is an interest in replacing blood-derived components from the vitrification solutions with synthetic substitutes, such as HPC.

		Fresh	Vitrified	p value
# of oocytes		146	73	–
Survival		–	70/73 (95.9)	–
Normal fertilization		105/146 (71.9)	50/70 (71.4)	0.768
Day 2	Cleavage rate	99/105 (94.3)	45/50 (90.0)	0.346
	Good quality embryos	71/99 (71.7)	37/45 (82.2)	0.071
Day 3	Cleavage rate	90/99 (90.9)	39/45 (86.6)	0.451
	Good quality embryos	63/90 (70.0)	27/39 (69.2)	0.171

Table 2 Oocyte distribution and laboratory outcomes. Results expressed as percentages (%). Morphological survival was dictated 2 h post-warming. Oocytes evaluation was based on the integrity of oocyte features, such as intact polar corpuscle, normal oolemma, and absence of vacuoles. Fertilization rate was defined as the proportion of oocytes with two pronuclei at the time of fertilization check (17 ± 1 h post- insemination). Day 2 good quality embryos were those presenting 2–4 blastomeres, ≤15% type I–II fragmentation, and no multinucleation. Day 3 good quality embryos were those with 6–8 blastomeres and ≤20% type I–II fragmentation

Desirable advantages that are sought in HPC-supplemented, protein-free solutions are reduced production cost and variability, and eliminating the hypothetical risk of viral contamination associated with protein purified from blood [24]. The addition of hydroxypropyl cellulose instead of serum to vitrification solutions can provide the same viscosity that enables in vitro manipulation and enhances the glass-forming tendency of the solution, without the aforementioned drawbacks [25, 28].

The present study is a direct comparison of the laboratory outcomes of two groups of oocytes: a control group of fresh oocytes and an experimental group of vitrified oocytes using an HPC-supplemented set of vitrification and warming solutions. Oocytes from both groups derived from the same donor were microinjected and cultured in parallel by the same team of embryologists in the same environment, minimizing the number of variables influencing the outcomes of fertilization and embryo development.

	Total	Fresh	Mixed	Vitrified
Transfers	19	10	8	1
Embryos transferred	38	21	15	2
Embryos transferred (mean per transfer)	2.0	2.1	1.9	2
Clinical pregnancies (%)	10 (52.6)	5 (50.0)	4 (50.0)	1 (100)
Implanted embryos (%)	18 (47.3)	8 (38.1)	8 (53.3)	2 (100)
Live births (%)	8 (42.1)	4 (40.0)	4 (50.0)	1 (100)

Table 3 Report of the total clinical outcomes of the study patients, and comparison between transfers performed with embryos from the control group of fresh oocytes, embryos from the experimental group of vitrified oocytes, and mixed transfers in which an embryo of each group was replaced

As mentioned, the two cohorts, even if from the same donor, were recovered from two different stimulation cycles: a source of variability in terms of oocyte quality. Nonetheless, the fact that no differences were observed in between both groups reveals that the process of vitrification and warming did not impair the capacity of the oocytes to fertilize and develop. This outcome is in agreement with the literature [25, 28] and supports the efficacy of HPC-based vitrification solutions.

The primary outcome, the fertilization rate of fresh and vitrified oocytes, was compared without finding any significant differences. The biological process of fertilization, represented by the apparition of the two pronuclei, requires the intervention of complex oocyte machinery that could be dam- aged during vitrification. A successful fertilization event was used as primary endpoint to assess the survival post- vitrification and warming [12, 13]. A successful fertilization event was used as primary endpoint to assess the survival post-vitrification and warming [12, 13]. In addition, a similar rate of good quality embryo formation on day 2 and 3 occurred between groups. For completeness, a complete follow-up to delivery is also included.

The results obtained in our experiments for survival, fertilization, and cleavage outcomes obtained match the vitrification key performance indicators (KPIs)

recently set [32, 34]. Our results are also comparable with previously reported data demonstrating comparable viability and development of vitrified oocytes to fresh controls [33, 35].

Up to now, other authors had presented data showing the effectiveness of protein-free, HPC-supplemented solutions combined with ethylene glycol, dimethyl sulfoxide, and trehalose as the non-permeant cryoprotectant agent. They have compared its results with protein-based solutions: Inoue [26] and Kuwayama [27] presented results from bovine embryos and oocytes and human oocytes, showing comparable survival rates using HPC and SSS supplemented solutions. Mori et al. [25] compared media supplemented at 1 and 5% (v/v) with a stock solution of 60 mg HPC/ml Milli-Q water against 5–20% SSS supplementation, describing similar physical properties and survival rates in mouse and human oocytes and blastocysts with both solutions. Most recently, a retrospective study by Coello et al. [28] compared the use of solutions supplemented with 0.06 mg HPC/ml and trehalose against 20% SSS + sucrose, showing similar laboratory and clinical outcomes. Our results are the first report of the combination of HPC as a surfactant agent and sucrose as the osmotic agent; as up to now, it only had been tested in combination with trehalose.

Larger prospective randomized studies comparing the outcomes of oocyte vitrification with protein vs. HPC supplementation, in which embryos are cultured to blastocyst stage and the live birth rate is used as a primary endpoint, are the ultimate comparative test and would be necessary. Yet the results reported in this article add to the growing body of evidence documenting efficacy of the use of fully synthetic, protein-free vitrification solutions.

Compliance with ethical standards The study was conducted as de- scribed in the protocol, which was developed in accordance to the principles of

Helsinki and the national Policy of Good Clinical Practice (ISO 14155:2011), and was approved by the internal review board of the institutional research committee. All recipients were informed thoroughly about the protocol of the study and signed an informed consent form.

References

1. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at −196°C by vitrification. Nature. 1985;313:573–5.

2. Bakhach J. The cryopreservation of composite tissues: principles and recent advancement on cryopreservation of different type of tissues. Organogenesis. 2009;5(3):19–126.

3. Baudot A, Alger L, Boutron P. Glass-forming tendency in the sys- tem water-dimethyl sulfoxide. Cryobiology. 2000;40(2):151–8. 4. Benson EE. Cryopreservation theory; plant cryopreservation: a practical guide. Springer 2008 5. Boutron P, Mehl P, Kaufmann A, Angibaud P. Glass-forming tendency and stability of the amorphous state in the aqueous solutions of linear polyalcohols with four carbons. I. Binary systems water- polyalcohol. Cryobiology. 1986;23(5):453–69.

4. Risco R, Elmoazzen H, Doughty M, He X, Toner M. Thermal performance of quartz capillaries for vitrification. Cryobiology. 2007;55(3):222–9.

5. Seki S, Mazur P. Ultra-rapid warming yields high survival of mouse oocytes cooled to −196 °C in dilutions of a standard vitrification solution. PLoS One. 2012;7:e36058.

6. Toner M, Carvalho EG, Karel M. Thermodynamics and kinetics of intracellular ice formation during freezing of biological cells. J App Phys. 1990;67:1582–93.

7. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. Theriogenology. 2007;67:81–9.

8. Wowk B. Thermodynamic aspects of vitrification. Cryobiology. 2010;60(1):11–22.

9. Cobo A, Serra V, Garrido N, Olmo I, Pellicer A, Remohí J. Obstetric and perinatal outcome of babies born from vitrified oo- cytes. Fertil Steril.

2014;102(4):1006–15.

10. Rienzi L, Ubaldi F, Iacobelli M, Ferrero S, Minasi MG, Martinez F, et al. Day 3 embryo transfer with combined evaluation at the pro- nuclear and cleavage stages compares favorably with day 5 blastocyst transfer. Hum Reprod. 2002;17:1852–5.

11. Solé M, Santaló J, Boada M, Clua E, Rodríguez I, Martínez F, et al. How does vitrification affect oocyte viability in oocyte donation cycles? A prospective study to compare outcomes achieved with fresh versus vitrified sibling oocytes. Hum Reprod. 2013;28:2087– 92.

12. Practice Committee of the American Society for Reproductive Medicine, & Practice Committee of the Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: a guideline. Fertil Steril. 2013;99(1):37–43.

13. Ruffing NA, Steponkus PL, Pitt RE, Parks JE. Osmometric behavior, hydraulic conductivity, and incidence of intracellular ice forma- tion in bovine oocytes at different developmental stages. Cryobiology. 1993;30:562–80.

14. Van den Abbeel E, Schneider U, Liu J, Agca Y, Critser JK, Van Steirteghem A. Osmotic responses and tolerance limits to changes in external osmolalities, and oolemma permeability characteristics, of human in vitro matured MII oocytes. Hum Reprod. 2007;22: 1959–72.

15. Fahy GM. The relevance of cryoprotectant Btoxicity^ to cryobiol- ogy. Cryobiology. 1986;23:1–13.

16. Széll A, Shelton JN. Osmotic and cryoprotective effects of glycerolsucrose solutions on day-3 mouse embryos. J Reprod Fertil. 1987;80:309–16.

17. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. Theriogenology. 2007;67:73–80.

18. Stachecki JJ, Garrisi J, Sabino S, Caetano JP, Wiemer K, Cohen J. A new

safe, simple, and successful vitrification method for bovine and human blastocysts. Reprod Biomed Online. 2008;17:360–7.

19. Vanderzwalmen P, Connan D, Grobet L, Wirleitner B, Remy B, Vanderzwalmen S, et al. Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions. Hum Reprod. 2013;28(8):2101–10.

20. Arav A. Cryopreservation of oocytes and embryos. 40th Anniversary Special Issue. Theriogenology. 2014;81:96–102.

21. Vajta G, Rienzi L, Cobo A, Yovich J. Embryo culture: can we perform better than nature? Reprod Biomed Online. 2010;20(4): 453–69.

22. Shaw JM, Kuleshova LL, Macfarlane DR, Trounson AO. Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran. Cryobiology. 1997;35:219–29.

23. Mori C, Yabuuchi A, Ezoe K, Murata N, Takayama Y, Okimura T, et al. Hydroxypropyl cellulose as an option for supplementation of cryoprotectant solutions for embryo vitrification in human assisted reproductive technologies. Reprod Biomed Online. 2015;30(6): 613–21.

24. Inoue F. Hydroxypropyl cellulose as a macromolecular supplement for cryopreservation by vitrification of bovine ooytes and blasto- cysts and human oocytes. Fertil Steril. 2011;96(3):S212–3.

25. Kuwayama M. Efficiency of non-protein solutions using hydroxy-propyl cellulose on survival of bovine and human oocytes and embryos after vitrification. Fertil Steril. 2013;100(3):S174.

26. Coello A, Campos P, Remohí J, Meseguer M, Cobo A. A combi- nation of hydroxypropyl cellulose and trehalose as supplementation for vitrification of human oocytes: a retrospective cohort study. J Assist Reprod Genet. 2015;33(3):413–21.

27. Clua E, Tur R, Coroleu B, Boada M, Barri PN, Veiga A. Analysis of factors

associated with multiple pregnancy in an oocyte donation programme. Reprod Biomed Online. 2010;21:694–9.

28. Risco R, Hebles M, Saa AM, Vilches-Ferron AM, Sanchez-Martin P, Lucena E, et al. Safespeed technology—the myth of ultrahigh cooling rates: a close device and a serum-free media for the vitrifi- cation human oocytes/embryos with the highest recovery rates. Hum Reprod. 2013;28(S1):i180.

29. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrifi- cation method for cryopreservation of human oocytes. Reprod Biomed Online. 2005;11:300–8.

30. Alpha SIRM. The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting. Reprod Biomed Online. 2012;25(2):146.

31. Cobo A, Kuwayama M, Pérez S, Ruiz A, Pellicer A, Remohí J. Comparison of concomitant outcome achieved with fresh and cryo- preserved donor oocytes vitrified by the Cryotop method. Fertil Steril. 2008;89:1657–64.

32. Maggiulli R, Scarica C, Dovere L, Ievoli E, Ubaldi FM, Rienzi L. Efficiency of human oocyte and embryo vitrification. Curr Trends Clin Embriol. 2015;1:2–11.

33. Rienzi L, Romano S, Albricci L, Maggiulli R, Capalbo A, Baroni E, et al. Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. Hum Reprod. 2010;25(1):66–73.

Original Research Article 2:

Thermal and clinical performance of a closed device designed for human oocyte vitrification based on the optimization of the warming rate

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Abstract

Although it was qualitatively pointed out by Fahy et al. (1984), the key role of the warming rates in non-equillibrium vitrification has only recently been quantitatively established for murine oocytes by Mazur and Seki (2011). In this work we study the performance of a closed vitrification device designed under the new paradigm, for the vitrification of human oocytes.

The vitrification carrier consists of a main straw in which a specifically designed capillary is mounted and where the oocytes are loaded by aspiration. It can be hermetically sealed before immersion in liquid nitrogen for vitrification, and it is warmed in a sterile water bath at 37 C. Measured warming rates achieved with this design were of 600.000 oC/min for a standard DMEM solution and 200.000 oC/min with the vitrification solution for human oocytes.

A cohort of 143 donor MII sibling human oocytes was split into two groups: control (fresh) and vitrified with SafeSpeed device. Similar results were found in both groups: survival (97.1%), fertilization after ICSI (74.7% in control vs. 77.3% in vitrified) and good quality embryos at day three (54.3% in control vs. 58.1% in vitrified) were settled as performance indicators. The pregnancy rate was 3/6 (50%) for the control, 2/3 (66%) for vitrified and 4/5 (80%) for mixed transfers.

1. Introduction

Human oocyte cryopreservation by vitrification is very efficient, and is no longer considered as experimental [23]. As stated by the American Society of Reproductive Medicine guidelines on MII oocyte cryopreservation, there is enough evidence on its safety, with a high similarity between laboratory and clinical outcomes from fresh and vitrified oocytes. However, hitherto most reported data comes from studies using open, plastic-strip vitrification systems [3,25,33]; therefore it is still in doubt whether such efficiency levels can be achieved with a closed vitrification device [6,7,14,19,20,34,35], in which the biological sample does not have direct contact with liquid nitrogen [35].

According to the paradigm of vitrification under which open systems were designed [2,35], the efficacy of a vitrification carrier depends mostly on the cooling rate achieved [1,15]. Therefore, open carriers rely on the direct exposure of the oocytes with liquid nitrogen to maximize such cooling rates [31]. A drawback of this approach is the hypothetical possibility of contamination by infectious microorganisms present in the liquid nitrogen, and the risk of cross-contamination between patients' samples. That said, there is plenty of literature reporting on the efficacy of plastic-strip open systems on both oocytes and embryos and no cases of contamination have been reported [35]. Also, numerous strategies to improve the biosafety of these systems have been developed [9,13,16,21,22], and it is even discussed whether there is a real probability of cross-contamination in the clinical practice [5,35].

To overcome biosafety issues, closed devices have been designed to guarantee asepsis, providing isolation to the liquid nitrogen. However this feature compromises their cooling/warm- ing rates and whether they are as efficient as open systems is a subject of debate [14,19,20,35]. Regardless of this, the most recent literature suggests that the vitrification paradigm has changed: studies in murine model have established that the

cooling rate is "of less consequence" for a successful vitrification than that of the warming rate, which now appears to be the dominant variable over the cooling rate [17,30,31], and even over the CPA concentration of the vitrification solution [12,32]. Whether this translates to human oocytes is not fully established, but it is being acknowledged in recent publications [6].

The introduction of this new degree of freedom —the warming rate— makes possible, at least in principle, the specific design of a closed system in which the central idea is not the cooling rate (as is the case in the open devices) but the warming rate. SafeSpeed is a closed vitrification carrier (Safepreservation, Spain) that has been recently designed under the new vitrification paradigm for a maximized warming rate: the biological samples are loaded on a thin, flexible, thermally efficient capillary that yields not only high cooling rates, but most importantly, very high warming rates [27].

In this manuscript a description of the closed carrier for vitri- fication and the experiments to measure the thermal rates achieved with it are provided. Afterwards, the protocol and the outcomes when used for vitrification of donor oocytes in an IVF programme are presented.

2. Materials and methods

2.1. SafeSpeed closed vitrification carrier

The SafeSpeed is a carrier for the vitrification of biological samples (Fig. 1). The device is composed by a thin capillary, which is assembled into a main straw with a diameter of 24 mm and 135 mm of length. The rear of the straw can be connected to an aspiration system, necessary to load the samples inside the capillary, using two placement marks as reference for its final position (Fig. 1). Once the biological sample is loaded by aspiration the device is sealed at both ends —the capillary and the rear end— to make it hermetically closed. Then, it is ready to be subjected to cooling by

plunging the capillary into liquid nitrogen. For subsequent storage, a slideable transparent plastic cover protects the capillary. For re-warming, the device is quickly transferred from the liquid nitrogen to a 37 °C water bath. Safepreservation, Spain, commercially produces this device, and it holds the CE marking as a product for the cryopreservation of human oocytes and embryos.

2.2. Measurement of thermal performance of the vitrification carrier

i) Set-up: For determining the warming rate we used a tem- perature measuring system consisting of an ultra-fine thermo- couple probe (COCO-001; OMEGA Engineering Inc., USA). After the corresponding amplification, the signal was introduced in one of the analogic input channels of a DAC converter (USB.1208LS; Measurement Computing Inc., USA). A program written in Labview 6.0 (reading rate 600 Hz) gives us the whole thermal history of the thermocouple junction.
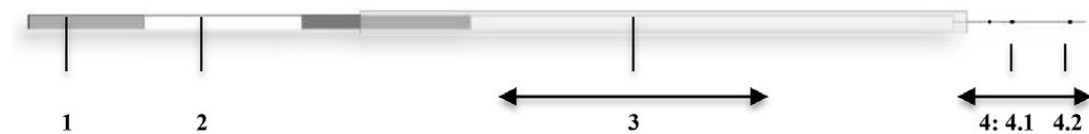

Fig. 1. SafeSpeed Carrier. Detailed outline of the SafeSpeed carrier and its components. The capillary where the oocytes are introduced is assembled into a main straw with a sealable rear-end and a slide cover for protection of the capillary during storage. 1: Sealable rear-end. 2: Labelling area. 3: Slide protector cover. 4: Ultra-thin capillary. 4.1: Sample placement marks, 4.2: Sealing mark.

The thermocouple was introduced inside the SafeSpeed device, and the copper-constantan junction was carefully placed in the position where the oocyte is allocated. Then, the SafeSpeed device was filled with the solution and sealed at the tip of the capillary. Next, the device was quenched in liquid nitrogen, held 10 s (a time that is more than 2 orders of magnitude higher than that necessary to reach the thermal equilibrium with the

cryogenic media), and subsequently re-warmed in a 37 °C water bath, exactly as in the standard vitrification/warming protocol for oocytes.

ii) Warming rates in relation to the time of flight: To determine the correlation between the time of flight (the motion from the exit of the liquid nitrogen to the entrance into the water bath) and the warming rates achieved, the motion of the capillary was recorded at 480 frames per second (Fig. 2). The ultra-fast video camera, CASIO EX-ZR100, allowed us to visualize, with a temporal resolution of milliseconds, the position of the carrier device, and in particular, the position of the thermocouple junction, placed, as explained above, in the position where the oocyte/embryo would remain. Its optical properties are: 12.1 megapixels, Objective/focal distance (f): 3.0 to 5.9/f 1/4 4.24e53.0 mm and 12.5X optical zoom. The number frames per second (fps) can be chosen between 120, 240, 480 y 1000. In our case we used 480 fps as the best compromise between the spatial and temporal resolution of the camera.

With Sony Vegas (Sony, USA) we determined, frame by frame (Fig. 3), the time of flight: the lapse of time for the thermocouple junction to be taken from liquid nitrogen to the water bath (Fig. 4). With a second algorithm in Matlab we determined the warming rate. Afterwards, by making the matching of these two sets of data (warming rate and time of flight) we established its correlation. The average warming rate was calculated in the temperature interval from -150 °C to -20 °C.
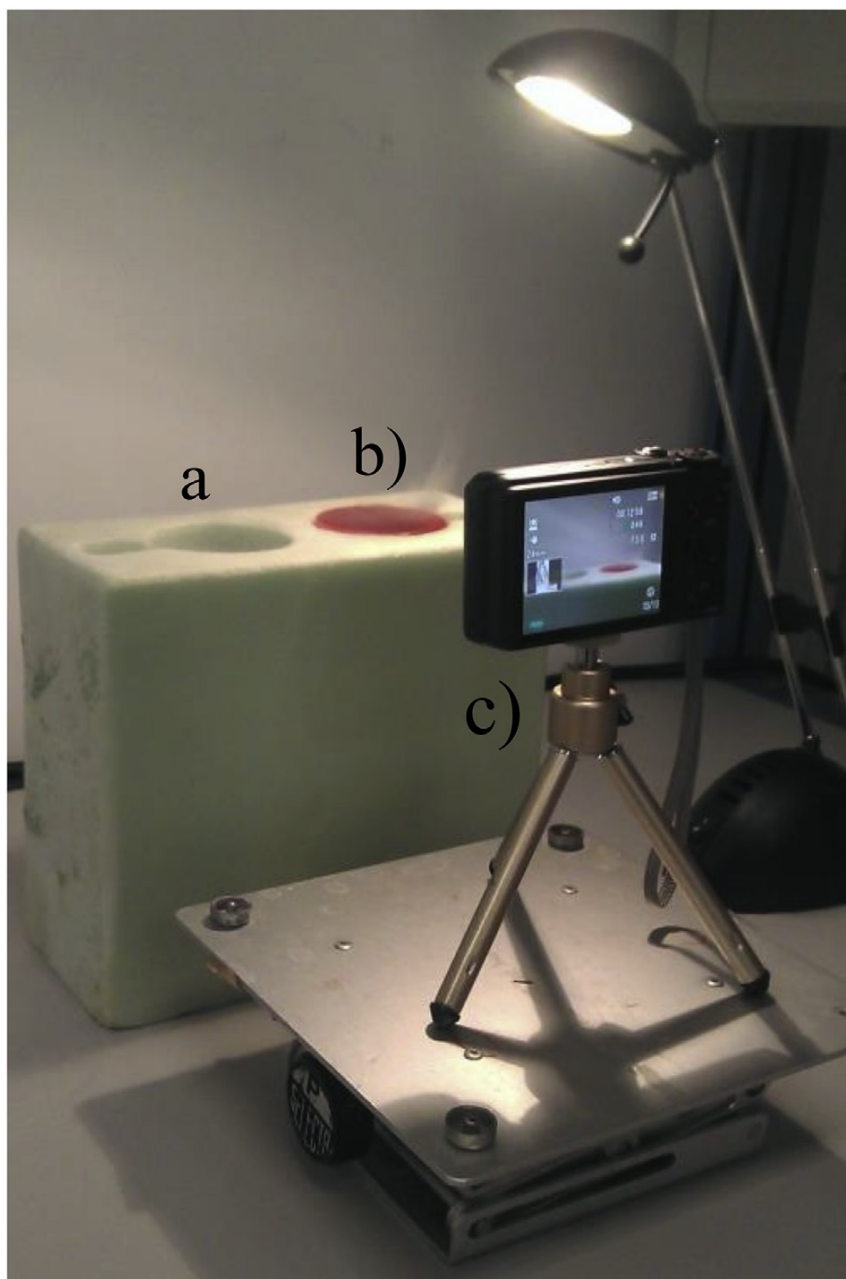
Fig. 2. Set up for image acquisition of the re-warming motion. The following elements are shown: a) Liquid nitrogen container filled to the rim. b) Removable sterilized water bath at 37 C. c) High speed (1000 fps) video camera (CASIO EX-ZR100). The full motion of the transfer of the capillary containing the thermocouple from the liquid nitrogen to the warm water bath is recorded by the camera.

iii) Warming rates in relation to the vitrification solution: In order to compare the warming rates of different cryoprotectants a set of experiments were carried out loading the capillary with the following solutions: a) Standard DMEM culture media, (Dulbecco's Modified Eagle Medium, GIBCO) b) DMEM with 1. 5M 1,2- propanediol and 0.3 M sucrose, c) VS vitrification solution (Safe- Speed vitrification solutions, Safepreservation, Spain) with 15% ethylene glycol and 15% dimethyl sulfoxide (v/v).

2.3. Clinical study design

143 MII sibling oocytes from donation cycles between January and November 2014 were included in this prospective randomized study. Participants of the study were 14 recipients of the egg donation program in Ginemed Clinicas, Seville, Spain. Each recipient was assigned with one cohort of sibling oocytes from the same donor. Half of the cohort was composed of fresh oocytes from a synchronic donation cycle (control group) and the other half were vitrified/warmed oocytes from a previous cycle of the same donor (experimental group). Both groups of oocytes were fertilized and underwent culture in parallel. The laboratory outcomes of both groups were compared: the primary performance end-point of the study was the fertilization rate of fresh and vitrified oocytes. Secondary parameters assessed were: cleavage rates, embryo quality in day 2 and day 3, and percentage of discarded embryos. Pregnancy and implantation rate were also included in the follow up of the study.

2.4. Study population

All donors included in the study fulfilled the Spanish regulations on ART donors (RD 1301/2006), described elsewhere [33] and fulfilled Ginemed Clínicas inclusion criteria: over 18 and under 30 years old, good physical and psychological health, no personal nor family history of hereditary diseases, normal karyotype, negative tests for sexually transmitted

diseases and without any medical or gynecological disorders. Donors were subjected to a short agonist stimulation treatment and were excluded in case of a response to stimulation <12 oocytes. All recipients who attended Ginemed Clínicas for an egg donation program were consecutively offered to participate in the study, until the quota was filled. Cases of couples with a severe male factor, such as surgically extracted spermatozoa and very severe oligoastenozoospermia (motile sperm count <500.000/ml after preparation) were not included.

Fig. 3. Determination of the time of flight. Frame by frame visualization of the video enables (Sony Vegas software), with a temporal resolution of milliseconds, the determination of the exact lapse of time between the exit from liquid nitrogen and the entrance in the warm water bath.
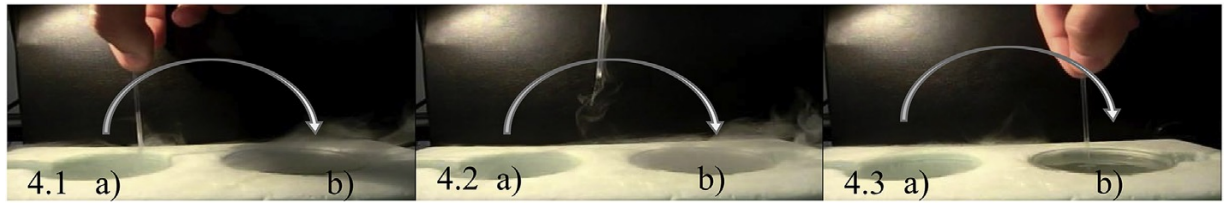
Fig. 4. The motion performed for the warming process. a) Liquid nitrogen container filled to the rim. b) Removable sterilized water bath at 37 C. The carrier device with the capillary submerged in liquid nitrogen is quickly transferred from a) to b) (white arrow). Note that the warm water bath is situated at a close distance (2 cm) to the liquid nitrogen container and at the same height to enable a fast motion and a reduced time of flight.

2.5. Ethical considerations

All recipients were informed thoroughly about the protocol of the study and signed an informed consent form. The study was conducted as described in the protocol, which was developed in accordance to the principles of Helsinki and the Spanish Policy of Good Clinical Practice (ISO 14155:2011), and was approved by the Internal Review Board of the Research Committee of Ginemed Clínicas.

2.6. Donor stimulation

For the donation, donors took contraceptive pills during the previous month to the stimulation. Five days after finishing con- traceptives, stimulation began with 150e200 IU daily of r-FSH (alpha follitropin, Gonal-F, Merck, Spain), depending on BMI. After day five, determination of estradiol levels and ultrasound scans were performed for dose adjustment. When a follicle reached 14 mm, they started GnRH antagonist treatment with 0.25 mg Cetrotide (Cetrorelix, Merck, Spain) until at least three follicles reached 20 mm. At this point, ovulation was triggered with 0.3 mg of triptoreline GnRH agonist (Decapeptyl; IpsenPharma, Spain).

2.7. Recipient stimulation

Recipients were kept on contraceptive pills for the synchronized donation. Once suspended, when menstruation happened, endo- metrial substitutive treatment started with 6 mg daily of estradiol hemihydrate (Estradot, Novartis, Switzerland). After 14 days of treatment, it was assessed that an optimal endometrium had been achieved. On the day of the retrieval from the donor, the partner of the receiving patient delivered a sperm sample for IVF. At that time, they began taking progesterone vaginal or oral supplement, 600 mg daily (Utrogestan, Seid, Spain), continued until the results of the embryo transfer were known.

2.8. Ovarian puncture

Oocyte recovery was performed by eco-guided ovarian punc- ture. An ultrasound transducer was placed into the vaginal fornix to visualize the ovary and follicles, and a single lumen ovum aspira- tion needle (Cook Medical, IN, USA) was inserted in the transducer and advanced into the ovarian follicles, where oocytes were recovered by aspiration with a syringe, until all the follicles had been punctured.

2.9. Embryology procedures

Two hours post-retrieval, oocytes were denuded using hyal- uronidase (HYASE-10X, Vitrolife, Sweden). Embryos from both groups were cultured on micro drops (G-IVF, G1Plus, G2Plus, Vitrolife, Sweden) under mineral oil (Tissue Culture Oil, Sage, Cooper Surgical, USA), under controlled temperature, humidity and CO_2 concentration (37 C, 95% RH, 6% CO_2). Both control and experimental groups underwent ICSI simultaneously, 38e40 h post-hCG. Immediately after denudation, oocytes from the experi- mental group were vitrified.

2.10. Vitrification Vitrification was performed at laboratory room temperature

(RT), in our case 23 ± 0.5 C. SafeSpeed vitrification media kit (Safepreservation, Spain) was used. It consists in three solutions: WS (no

CPAs), ES (7.5% ethylene glycol and 7.5% dimethyl sulfoxide) and VS (described above; 15% ethylene glycol and 15% dimethyl sulfoxide). All solutions were exposed at least for an hour to labo- ratory RT. Oocytes were gradually exposed to the CPAs: they were placed in a first droplet of 50 ml of WS (Washing Solution), which was then joined using the tip of the pipette to another droplet of 50 ml of ES (Equilibration Solution). After two minutes, both drop- lets were joined with a third of the same ES, waiting another 2 min, allowing the diffusion of the CPAs.

Afterwards, oocytes were transferred to a 100 ml droplet of ES, where they remained from 6 to 10 min, until they appeared to be fully re-expanded. Once the equilibration was complete, oocytes were transferred to a 200 ml droplet of VS and washed by pipetting, to eliminate any leftover ES.

Immediately after, they were loaded by aspiration inside the capillary of the SafeSpeed device (Safepreservation, Spain): Oocytes were placed in between the two black security marks of the capillary (Fig. 1). Then, both ends of the device, the capillary and the rear part, were sealed with a dedicated sealer (SafeSealer, Spain). The SafeSpeed was then quenched into a liquid nitrogen bath, deep enough (20 cm of depth), to allow a deep plunging without risks of hitting its bottom. The capillary was then protected by pulling down the slide cover, before releasing it in the liquid nitrogen bath.

2.11. Warming

SafeSpeed warming media consists of three solutions: TS (Thawing solution, 1 M sucrose), DS (Diluent solution; 0.5 M su- crose) and WS (Washing solution, CPA-free). For at least one hour, closed vials of DS and WS were exposed to laboratory room tem- perature, and TS vial was placed in the incubator at 37 C.

For warming, a recipient containing sterilized water at 37 C is placed on the side of the liquid nitrogen recipient containing the straw, at a distance of 2 cm and with its rim at the same height. All these details are critical parts of the strategy to maximize the warming rate, as explained in the "Results" section. The slide cover must be pulled up to expose the capillary containing the oocytes, and the rear end of the SafeSpeed device is cut open. The straw is transferred, minimizing the flight time (see Fig. 5), from the liquid nitrogen bath to the warm water bath, where it is stirred for one second in the water and then removed, drying the capillary carefully in a sterile tissue afterwards.

The carrier was then connected to the aspiration system, the capillary cut over the sealed area, and the oocytes expelled in a 200 ml droplet of TS. In the TS solution oocytes were washed to eliminate any leftover VS media with a stripper tip of the appropriate diameter (170 mm). Within 60 s they were transferred very gently to a 200 ml droplet of DS, where they remained 3 min, followed by two washing steps of 5 and 1 min(s), performed in 200 ml droplets of WS. Afterwards they were cultured (37 C, 6% CO_2) for 2 h to allow meiotic spindle reformation before undergoing ICSI.

2.12. Primary performance indicators

Morphological survival

Morphological survival was assessed 2 h post-thawing. Oocytes evaluation was based on the integrity of oocyte features, such as intact polar body, normal oolemma, absence of vacuoles, etc. [18]. Oocytes clearly degenerated or considered not suitable for ICSI were considered negative for survival.

Fertilization Rate

Fertilization rate was defined as the proportion of oocytes with 2 pronuclei at the time of fertilization check (17 ± 1 h post insemi- nation)

[18]. The number of oocytes showing no signal of fecundation, abnormal fecundation (1 or >2 pronuclei) and degenerated oocytes was registered.

2.13. Secondary performance indicators

Embryo development was checked at 44 ± 2 h (Day 2) and 68 ± 2 h (Day 3) post-ICSI. The embryo assessment algorithm proposed by Cobo et al. [3] was used, considering that the embryos were of good quality on the second day of development if they presented 2-4 blastomeres, 15% type I-II fragmentation and no multinucleation. On day 3, good quality embryos were those with 6-8 blastomeres and 20% type I-II fragmentation.

2.14. Clinical results

To confirm clinical pregnancy, the serum level of ß-hCG was measured 12-14 days post-transfer. Implantation was confirmed by ultrasound visualization of a gestational sac.

2.15. Statistical analysis

The baseline quantitative variables are presented as mean ± standard deviation, and the qualitative variables are presented as their absolute value and percentage. To test the association between the exposure to vitrification/warming and each laboratory endpoint, Pearson's Chi-Square test and Fisher's exact

3. Results

3.1. Warming rates with SafeSpeed closed vitrification carrier

i) Warming rates in relation to the time of flight: Fig. 5 represents the typical behavior of the warming rate (for VS) as a function of the time it takes the capillary to complete the motion from liquid nitrogen to water: there is an exponential in- crease in the warming rate as the time of flight is reduced, with a critical point around 300 ms. Longer time of flights translate in lower warming rates that could compromise the outcome of the recovery of the oocyte [17].

ii) Warming rates in relation to the CPA cocktail

The average (mean ± SEM) value obtained was 200.000 ± 10.000 oC/min for VS, 450.000 ± 10.000 oC/min for 1.5 M 1,2-propanediol and 0.3 M sucrose and 600.000 ± 20.000 oC/min for CPA-free culture media. All times of flight used for calculations were below 300 ms.
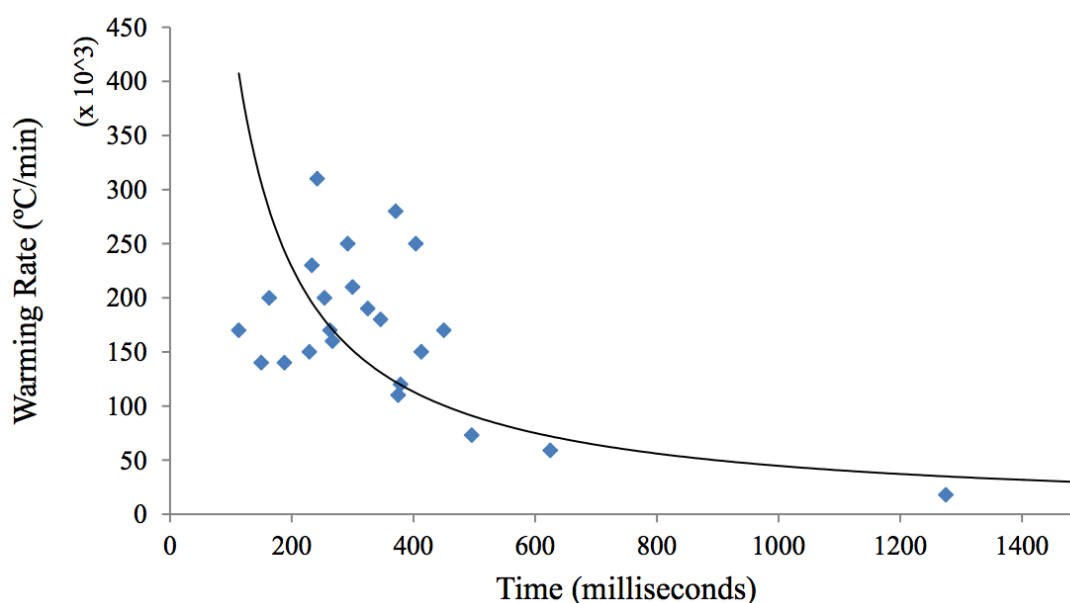

Fig. 5. Relevance of the re-warming step. The graph shows the typical behavior of the warming rate (oC/min) vs. the time of flight (the time to complete the warming motion from liquid nitrogen to the 37 C water bath) for VS vitrification solution. There is an exponential increase of the warming rate as the time of flight is reduced. A plateau seems to be reached for time of flights below 300 ms; in this area, the average warming rate for VS vitrification solution is 200.000 oC/min.

3.2. Clinical study results

3.2.1. Study subjects

The number of subjects included in the study, their age, and the number of oocytes assigned to the recipients are shown on Table 1.

Table 1. Baseline characteristics data

Patients	14
Age (years)	42.3 (5.7)
Number of oocytes assigned	9,1 (1.4)

Table 1: Baseline characteristics of the recipients of the egg donation programme participating in the study. Data as mean (SD).

3.2.2. Primary endpoint: survival and fertilization rates

Out of 68 vitrified MII oocytes, 66 (97.1%) presented morpho- logical survival 2 h post warming, and 51 of them (77.3%) presented normal fertilization, compared to 56 of 75 (74.7%) fresh oocytes ($p > 0.05$) (Table 2).

3.2.3. Secondary endpoints: embryo quality and development

Table 2 also summarizes the embryo development at day 3: our results showed similar cleavage rates and similar number of good quality embryos (58.1% from vitrified oocytes against a 54.3% from fresh oocytes) on both groups ($p > 0.05$). The total number of embryos that were appropriate for transfer or cryopreservation was also similar whether they originated from fresh or vitrified/warmed oocytes.

3.2.4. Clinical results

As shown in Table 3, there were 6 cases where only embryos from the fresh group were transferred. In 3 transfers only embryos from the vitrified/warmed group were transferred. And there were 5 mixed transfers with embryos from both groups. Similar implantation rates were obtained within groups, with 9 out of 14 recipients achieving clinical pregnancy.

Table 2. Laboratory outcomes

	Fresh	Vitrified/ Warmed	OR (95% CI)	P
Survival	-	66/68 (97.1)	-	-
Normal Fertilization	56/75 (74.7)	51/66 (77.3)	1.15 (0.53 to 2.51)	0.72
Non-Fertilized	11/75 (14.7)	7/66 (10.6)	0.69 (0.25 to 1.90)	0.39
Abnormal Fertilization	5/75 (6.6)	4/66 (6.05)	0.90 (0.23 to 3.51)	0.88
Degenerated after ICSI	3/75 (4.0)	4/66 (6.05)	1.55 (0.33 to 7.18)	0.58
Day 3 Cleavage rate	46/56 (80.3)	43/51 (84.3)	1.16 (0.42 to 3.23)	0.76
Day 3 Good Quality Embryo Rate	25/46 (54.3)	25/43 (58.1)	1.17 (0.50 to 2.70)	0.72
Transferred or Cryopreserved	40/46 (86.9)	38/43 (88.4)	1.14 (0.32 to 4.04)	0.84

Table 2: Comparison of the primary (fertilization rate) and secondary endpoints of fresh and vitrified sibling oocytes. Data expressed as absolute values and percentage (%), OR (95% CI) and p-value.

Table 3. Clinical outcomes

	Embryo Transfers		
	Fresh Group	Mixed Transfers	Vitrified /Warmed Group
Nº of transfers	6	5	3
Mean nº of embryos transferred	2.17 (0.41)	2.33 (0.51)	2.33 (0.58)
Implantation Rate (per embryo transferred)	3/13 (23.1)	4/14 (28.6)	2/7 (28.6)
Pregnancy rate (per transfer)	3/6 (50.0)	4/5 (80.0)	2/3 (66.0)
Total Pregnancy rate	9 /14 (64.3)		

Table 3: Clinical results of the embryo transfers performed. Data as mean (SD) or absolute values and percentages (%).

4. Discussion

The average warming rate obtained with the closed carrier was 600.000 °C/min when using a CPA-free DMEM culture media, 450.000 °C/min for a 1.5 M 1,2-propanediol, 0.3 M sucrose, and 200.000 °C/min using the VS vitrification solution for human oocytes, when the warming motion was completed in under 300 ms. The correlation between warming rates and time of flight between the liquid nitrogen and the warm water bath fits in our experiments to a potential curve of the form: $B(ºC/min) \approx 10^8\ t^{-1}$

where B is the warming rate in (ºC/min) and t is the time of flight, in milliseconds (ms). The exponential increase of the warming rate (B) with

the reduction of the duration of the warming motion (t), shows the relevancy of performing a fast rewarming of the vitrification carrier, as described in the section warming of this work.

The warming rate measurement experiments with DMEM and DMEM supplemented with 1.5 M 1,2-propanediol and 0.3 M sucrose were done, even if this was not the cryoprotectant solution used in this manuscript, because there is reported evidence of the capability of vitrification of this solution in similar devices [11, 26]. Other authors have also explored the use of low CPA formulations combined with an increase in the warming rates [12]. The use of SafeSpeed for oocyte vitrification with this and other low concentration cryoprotectant cocktails is, however, something to be explored.

The vitrification and subsequent warming of donor oocytes in this closed carrier did not impair their competence: the primary outcome, fertilization rate, was similar to fresh sibling oocytes, as well as their cleavage, development and reproductive outcomes. The vitrification carrier used in this study was recently designed under the new paradigm of vitrification that Seki and Mazur have developed in their latest publications in a murine model [12,17,30e32]. According to it, the warming rate is the dominant variable affecting survival in the vitrification process. This is because in the warming step there is risk of recrystallization of intracellular ice, which can grow to a size that will ultimately compromise the viability of the cryopreserved cell [17,31].

It must be acknowledged that most open vitrification systems yield excellent results for the cryopreservation of human oocytes [36]. The ability to cryopreserve oocytes with such success has resulted in a wide and ever growing array of applications [4], which have had a tremendous positive impact on the assisted reproduction technologies. The reason for the success of plastic-strip open systems is that, through their design, they inherently achieve a high warming rate too [32], although their design was

not necessarily optimized for such an end. However, there are published strategies for achieving very high warming rates also in open devices consisting in using an infrared laser pulse against the oocyte [12]. The laser approach has already been successfully used for avoiding devitrification of the sample during a cryopreservation protocol [10].

In the SafeSpeed carrier the warming rate is maximized by the following factors: the warming is performed in a significant volume of warm water, in a very fast motion, and the capillary yields excellent thermal performance [27]. The fact that the oocytes are encapsulated inside a capillary is not a drawback, but on the contrary, it enables this improvement in the warming rate by allowing the possibility of warming in bigger volumes of warm media/water without the risk of losing the oocytes/embryos. It also minimizes the risk of contamination associated with direct contact with liquid nitrogen [14], even though capillary-based vitrification systems require careful manipulation to be completely aseptic [35].

Other authors have reported improvements in the vitrification outcomes of human oocytes with protocol modifications directed to increase the warming rate in human [6]. Nevertheless, it is yet to be determined to which extent each variable (CPA concentration and toxicity, cooling rate and warming rate) plays a role in the survival of a human oocyte to vitrification and how they interact to find a perfect combination amongst them [8,24,28]. Overall, the application of ultra-fast vitrification on human assisted reproduction is very successful, and it has become a key tool in daily clinical practice [4]. Current results are satisfactory [36] but as we gain a better understanding of the principles that make vitrification work, the techniques must keep improving too and with these experiments we aimed to take a step in such direction; towards a safer, more effective, less-time consuming, less technically demanding, and ultimately, a universal protocol for the vitrification of human gamete and embryos.

Conflicts of interest

One of the authors (R.R.) is the Co-Director and Chief Scientific Officer of SafePreservation S.L., which sells material for cryopreservation.

The rest of the authors (M.G., M.H., B.M., M.D., L.A, M.G., P.P., L.M., P.S-M. & F.S-M.) certify that they do not have any conflict of interest with any financial organization regarding the material discussed in the manuscript.

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References

[1] A. Arav, Y. Zeron, A. Ocheretny, A new device and method for vitrification increases the cooling rate and allows successful cryopreservation of bovine oocytes, Theriogenology 53 (2000) 248e249.

[2] A. Arav, Cryopreservation of oocytes and embryos, Theriogenology 81 (1) (2014) 96e102.

[3] A. Cobo, M. Kuwayama, S. Pe rez, A. Ruiz, A. Pellicer, J. Remohí, Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method, Fertil. Steril. 89 (6) (2008) 1657e1664.

[4] A. Cobo, C. Diaz, Clinical application of oocyte vitrification: a systematic re- view and meta-analysis of randomized controlled trials, Fertil. Steril. 96 (2) (2011) 277e285.

[5] A.Cobo,J.Bellver,M.J.delosSantos,J.Remohí,Viralscreeningofspentculture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles, Fertil. Steril. 97 (1) (2012) 74e78.

[6] N. De Munck, G. Verheyen, L. Van Landuyt, D. Stoop, H. Van de Velde, Survival and post-warming in vitro competence of human oocytes after high

security closed system vitrification, J. Assist. Reprod. Genet. 30 (3) (2013) 361e369.

[7] N.Desai,J.Goldberg,C.Austin,ThenewRapid-iTMclosedvitrificationsystemis technically easy to use and gives excellent outcomes with both blastocyst and cleavage stage embryos, Fertil. Steril. 98 (3) (2012) S129eS130 (P-60).

[8] G. Fahy, D.R. MacFarlane, C.A. Angell, H.T. Meryman, Vitrification as an approach to cryopreservation, Cryobiology 21 (1984) 407e426.

[9] A.M.Filicori,Efficiencyofasepticopenvitrificationandhermeticalcryostorage of human oocytes, Reprod. Biomed. 23 (2011) 505e512. Online.

[10] A.J. Fowler, M. Toner, Prevention of hemolysis in rapidly frozen erythrocytes by using a laser pulse, Ann. New Acad. Sci. 858 (1) (1998) 245e252.

[11] X. He, E.Y. Park, A. Fowler, M.L. Yarmush, M. Toner, Vitrification by ultra-fast cooling at a low concentration of cryoprotectants in a quartz micro-capillary: a study using murine embryonic stem cells, Cryobiology 56 (3) (2008) 223e232.

[12] B. Jin, F.W. Kleinhans, P. Mazur, Survivals of mouse oocytes approach 100% after vitrification in 3-fold diluted media and ultra-rapid warming by an IR laser pulse, Cryobiology 68 (3) (2014) 419e430.

[13] L.L. Kuleshova, J.M. Shaw, A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen, Hum. Reprod. 15 (2000) 2604e2609.

[14] M. Kuwayama, G. Vajta, S. Ieda, O. Kato, Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination, Reprod. Biomed. 11 (2005) 608e614. Online.

[15] M. Kuwayama, G. Vajta, O. Kato, S.P. Leibo, Highly efficient vitrification method for cryopreservation of human oocytes, Reprod. Biomed. 11 (2005)

300e308. Online.

[16] M.G. Larman, D.K. Gardner, Vitrification of mouse embryos with super-
cooled

air, Fertil. Steril. 95 (2011) 1462e1466.

[17] P.Mazur,S.Seki,Survivalofmouseoocytesafterbeingcooledinavitrification

solution to -196 C at 95 C to 70,000 C/min and warmed at 610 C to
118,000 C/min: a new paradigm for cryopreservation by vitrification,
Cryobiology 62 (2011) 1e7.

[18] A.S.I.R. Medicine, The Alpha consensus meeting on cryopreservation key
performance indicators and benchmarks: proceedings of an expert meeting,
Reprod. Biomed. 25 (2) (2012) 146e167. Online.

[19] Y. Panagiotidis, P. Vanderzwalmen, Y. Prapas, E. Kasapi, M. Goudakou,
A. Papatheodorou, T. Passadaki, S. Petousis, Veletza S. NikolettosN, N.
Prapas, G. Maroulis, Open versus closed vitrification of blastocysts from an
oocyte- donation programme: a prospective randomized study, Reprod.
Biomed. 26 (2013) 470e476. Online.

[20] A. Papatheodorou, P. Vanderzwalmen, Y. Panagiotidis, N. Prapas, K.
Zikopoulos, I. Georgiou, Y. Prapas, Open versus closed oocyte vitrification
system: a prospective randomized sibling oocyte study, Reprod. Biomed. 26
(2013) 595e602. Online.

[21] L. Parmegiani, A. Accorsi, G.E. Cognigni, S. Bernardi, E. Troilo, M.
Filicori, Sterilization of liquid nitrogen with ultraviolet irradiation for safe
vitrification of human oocytes or embryos, Fertil. Steril. 94 (4) (2010)
1525e1528.

[22] L. Parmegiani, A. Accorsi, S. Bernardi, A. Arnone, M. Cognigni
GE,Filicori, A reliable procedure for decontamination before thawing of human
speci- mens cryostored in liquid nitrogen: three washes with sterile liquid
nitrogen (SLN2), Fertil. Steril. 98 (2012) 870e875.

[23] Practice Committee of the American Society for Reproductive Medicine, Practice committee of the society for assisted reproductive technology. Mature oocyte cryopreservation: a guideline, Fertil. Steril. 99 (1) (2013) 37e43.

[24] W. Rall, Factors affecting the survival of mouse embryos cryopreserved by vitrification, Cryobiology 24 (1987) 387e402.

[25] L. Rienzi, S. Romano, L. Albricci, R. Maggiulli, A. Capalbo, E. Baroni, S. Colamaria, F. Sapienza, F. Ubaldi, Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study, Hum. Reprod. 25 (1) (2010) 66e73.

[26] R. Risco, H. Elmoazzen, M. Doughty, X. He, M. Toner, Thermal performance of quartz capillaries for vitrification, Cryobiology 55 (3) (2007) 222e229.

[27] R. Risco, M. Hebles, A.M. Saa, A.M. Vilches-Ferron, P. Sanchez-Martin,

E. Lucena, M. Lucena, SafeSpeed technology - the myth of ultrahigh cooling rates: a close device and a serum-free media for the vitrification human oocytes/embryos with the highest recovery rates, Hum. Reprod. 28 (2013) 180.

[28] N.A. Ruffing, P.L. Steponkus, R.E. Pitt, J.E. Parks, Osmometric behavior, hy- draulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages, Cryobiology 30 (1993) 562e580.

[29] Sealed Envelope Ltd, Power Calculator for Binary Outcome Non-inferiority Trial, 2012 [Online] Available from: https://www.sealedenvelope.com/ power/binary-noninferior/.

[30] S. Seki, P. Mazur, Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice, Biol. Reprod. 79 (2008) 727e737.

[31] S. Seki, P. Mazur, The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure, Cryobiology 59 (2009) 75e82.

[32] S. Seki, P. Mazur, Ultra-rapid warming yields high survival of mouse oocytes cooled to 196 C in dilutions of a standard vitrification solution, PLoS One 7 (4) (2012) e36058.

[33] M. Sole , J. Santalo , M. Boada, E. Clua, I. Rodríguez, F. Martínez, B. Coroleu, P.N. Barri, A. Veiga, How does vitrification affect oocyte viability in oocyte donation cycles? A prospective study to compare outcomes achieved with fresh versus vitrified sibling oocytes, Hum. Reprod. 28 (8) (2013) 2087e2092.

[34] D. Stoop, M.N. De, E. Jansen, P. Platteau, E. Van den Abbeel, G. Verheyen, P. Devroey, Clinical validation of a closed vitrification system in an oocyte- donation programme, Reprod. Biomed. 24 (2012) 180e185. Online.

[35] G. Vajta, L. Rienzi, F.M. Ubaldi, Open versus closed systems for vitrification of human oocytes and embryos, Reprod. Biomed. 30 (4) (2015) 325e333. Online. [36] G. Vajta, A. Reichart, F. Ubaldi, L. Rienzi, From a backup technology to a strategy-outlining approach: the success story of cryopreservation, Expert Rev. Obstet. Gynecol. 8 (2) (2013) 181e190**.**

Glossary of ART terminology

- **Assisted reproductive technology (ART):** all treatments or procedures that include the in vitro handling of both human oocytes and sperm or of embryos for the purpose of establishing a pregnancy. This includes, but is not limited to, in vitro fertilization and embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or a sperm donor.

- **Blastocyst:** an embryo, 5 or 6 days after fertilization, with an inner cell mass, outer layer of trophectoderm, and a fluid-filled blasto- cele cavity.

- **Clinical pregnancy:** a pregnancy diagnosed by ultrasonographic visualization of one or more gestational sacs or definitive clinical signs of pregnancy. It includes ectopic pregnancy. Note: Multiple gestational sacs are counted as one clinical pregnancy.

- **Controlled ovarian stimulation (COS) for ART:** pharmaco- logic treatment in which women are stimulated to induce the devel- opment of multiple ovarian follicles to obtain multiple oocytes at follicular aspiration.

- **Cryopreservation:** the freezing or vitrification and storage of gametes, zygotes, embryos, or gonadal tissue.

- **Embryo:** the product of the division of the zygote to the end of the embryonic stage, 8 weeks after fertilization. (This definition does not include either parthenotes—generated through partheno- genesis— nor products of somatic cell nuclear transfer.)

- **Embryo transfer (ET):** the procedure in which one or more embryos are placed in the uterus or fallopian tube.

- **Fertilization:** the penetration of the ovum by the spermatozoon and combination of their genetic material resulting in the formation of a zygote.

- **Frozen-thawed embryo transfer cycle (FET):** an ART proce- dure in which cycle monitoring is carried out with the intention of transfering frozen-thawed embryo(s). Note: An FET cycle is initi- ated when specific medication is provided or cycle monitoring is started with the intention to treat.

- **Gestational sac:** a fluid-filled structure associated with early pregnancy, which may be located inside or outside the uterus (in case of an ectopic pregnancy).

- **Implantation:** the attachment and subsequent penetration by the zona-free blastocyst (usually in the endometrium) that starts 5 to 7 days after fertilization.

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

- **In vitro fertilization (IVF):** an ART procedure that involves extracorporeal fertilization.

- **Infertility (clinical definition):** a disease of the reproductive sys- tem defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.

- **Intracytoplasmic sperm injection (ICSI):** a procedure in which a single spermatozoon is injected into the oocyte cytoplasm.

- **Live birth:** the complete expulsion or extraction from its mother of a product of fertilization, irrespective of the duration of the pregnancy, which, after such separation, breathes or shows any other evidence of life, such as heart beat, umbilical cord pulsation, or definite movement of voluntary muscles, irrespective of whether the umbilical cord has been cut or the placenta is attached.

- **Micromanipulation:** a technology that allows micro-operative procedures to be performed on the spermatozoon, oocyte, zygote, or embryo.
- **Oocyte donation cycle:** a cycle in which oocytes are collected from a donor for clinical application or research.
- **Oocyte recipient cycle:** an ART cycle in which a woman receives oocytes from a donor.
- **Preimplantation genetic diagnosis (PGD):** analysis of polar bodies, blastomeres, or trophectoderm from oocytes, zygotes, or embryos for the detection of specific genetic, structural, and/or chromo- somal alterations.
- **Vitrification:** an ultra-rapid cryopreservation method that pre- vents ice formation within the suspension which is converted to a glass-like solid.
- **Zygote:** a diploid cell resulting from the fertilization of an oocyte by a spermatozoon, which subsequently divides to form an embryo.

Glossary terms from *International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology* (Zegers-Hochschild et al. 2009).