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REVIEW

Vitrification: the pioneering past to current trends and perspectives of cryopreserving human embryos, gametes and reproductive tissue

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Abstract: After more than 2 decades of development in mammalian models and the clinical focus of a few pioneering laboratories, vitrification of human oocytes and embryos has transformed today's assisted reproductive technology (ART) industry. Our ability to cryopreserve gametes and embryos without fear of the damaging effects of ice formation has instilled great confidence in post-warming specimen viability. In turn, clinical treatment options are progressively eliminating fresh embryo transfer (ET; ie, freeze-all cycles), integrating preimplantation genetic screening and contemplating the use of cryopreserved oocytes as a viable resource. Vitrification's impact on clinical treatments is akin to the advent of sperm injection technology in the 1990s on male factor infertility. An appreciable and quantifiable difference was made, with procedural efficacy and global reliability essentially being guaranteed. Yet, there are challenges in the current trends and perspectives in how this technology is and will be optimized in the future. User variation in vitrification products and procedures warrants stricter adherence to quality control measures to enhance specimen biosafety and patient satisfaction, while reducing potential liability concerns. Furthermore, future progress in our understanding of the chemical and cryophysical processes of vitrification will insure the effective cryostorage of reproductive tissues and gametes at a level attained for embryo cryopreservation today.

Keywords: vitrification, embryos, oocytes, reproductive tissue, sperm, human

Insights into the pioneering past of vitrification

The concept and development of kinetic vitrification is credited to Father Basile J Luyet, a Professor of Biology at Saint Louis University. Luyet[1] reviewed historic research in cryobiology and published summaries of experiments which mostly involved freezing under natural conditions, without the presence of cryoprotective agents (CPAs).[2] Luyet[1] showed that supercooled solutions could become so viscous that they solidified without crystallization, forming a transparent glass state, and that this transparent frozen state was equivalent to "vitrification." Furthermore, he determined that although you may vitrify something successfully in the cooling phase, it did not mean you could sustain life, because when you warm it back up it may return to the crystalline state and cause cellular damage.[2] Later in the 1950s, Gonzales and Luyet[3] experienced limited success vitrifying chick hearts and neural tissue explants.[4] Insights into this pioneering history of cryobiology and the early efforts of other investigators have been reviewed.[5] As for Luyet, he went on to become the founding president of the Society for Cryobiology in 1964.

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In the late 1970s and early 1980s, renewed interests in the underlying concept of cryopreserving living tissue under the metastable solidification of water without ice growth (ie, vitrification) became a fascination of two independent, brilliant-minded postdoctoral fellows in the field of cryobiology. Neither Drs Greg Fahy nor Bill Rall (pre-1985) could have foreseen the tremendous effectiveness that vitrification would have on maintaining the cryoviability of oocytes and embryos. Fahy's applied aspirations focused on the challenges of whole tissue/organ preservation, believing that if the CPA concentration was sufficiently high, the crystallization of water molecules in the extracellular medium could be inhibited completely and become vitrified.[6,7] Meanwhile, Rall et al[8] were intrigued with innocuous ice formation inside cells during conventional freeze procedures that accommodated cellular survival. Using cryomicroscopy, it was observed that CPAs substantially increase viscosity of intracellular regions, causing water diffusion to cease and the liquid cytoplasm to form a metastable glass upon rapid cooling. Rall and coworkers[10] were confident that conventional embryo cryopreservation procedures could be simplified for on-farm use, and possible in-field conservation efforts, without a need for electronic equipment. After years of mutual respect and idea sharing, the scientific union of Fahy and Rall was formalized through the support of Harold Meryman, Scientific Director at the American Red Cross Blood Research Laboratory (Bethesda, MD, USA) in 1983. Under cold room experimental conditions, they successfully vitrified mouse embryos in 1985,[11] as previously reviewed.[12] In Rall's[13] efforts to develop an effective procedure working under room temperature conditions, it was determined that propylene glycol was highly toxic at higher concentrations. However, a third-generation vitrification solution (VS3a, a 6.5 M glycerol solution) produced high survival levels and positive pregnancy outcomes in closed one-step 0.25 mL straws, being comparable to conventional slow freezing of mouse embryos[14] and sheep blastocysts.[15,16]

By 1990, a new focus on vitrification began, one which emphasized minimizing the potential toxicity of vitrification solutions.[17] Our understanding of vitrification solutions and their potential toxicity to embryos was enhanced by the efforts of a few insightful pioneers in the field of reproductive cryobiology.[18–22] Their efforts led to the combined use of mixed permeating CPAs (eg, ethylene glycol [EG], dimethyl sulfoxide [DMSO] and glycerol), as well as non-permeating solutes (eg, sucrose and ficoll) that factored into reducing toxicity potential of individual vitrification solutions.

The integration of vitrification into clinical in vitro fertilization (IVF)

Nearly a decade passed before vitrification was proposed as a serious technology for improving our ability to cryopreserve human oocytes, cleaved embryos and blastocysts.[23–26] This interest in clinical vitrification was linked to the formation of unique cryodevices such as the Open Pulled Straw,[27,28] Cryoloops[29–31] Cryotops[32,33] and others,[23,24] whose thin surrounding film of vitrification solution and direct contact with liquid nitrogen (LN_2) achieved ultrarapid cooling rates. By the mid-2000s, it was the commercial industry, developing new devices (eg, HSV, Cryotip, Rapid-i and Cryolock) and solutions that propelled vitrification's use into clinical IVF laboratories. By 2010, the benefits of vitrified embryos and oocytes, having virtually no change from their fresh state, were gaining worldwide acceptance.[34,35] The diversity of issues surrounding the pros and cons of both open and closed device systems has been recently discussed by Vajta et al,[36] and is briefly mentioned in the following paragraphs. Historically, most clinical users of vitrification systems were misled that the ultrarapid cooling rates attained by the direct exposure of open devices to LN_2 were necessary to achieve high survival rates with embryos and oocytes. However, time and experience have proven that slower cooling closed systems vitrify equally well in contrast to other open systems, especially with regard to embryo cryopreservation.[26] Although the majority of oocyte cryopreservation experience and clinical data support the preferred use of open device systems, there is a need for more published reports and experimentation with closed devices before definitive conclusions are made, as mixed results have been reported.[37–43]

During this time period, the relative importance of warming rates to insuring successful vitrification was proven in a murine experimental model system by Seki and Mazur (the father of modern cryobiology).[44–47] Using a nonequilibrium, unstable vitrification solution model, they clearly proved that rapid to ultrarapid warming is the key determinant overriding conditions created at any cooling rate. Vitrification warming is a complex process, in that a closed device (eg, standard sealed straw) that achieves intermediate cooling rates (100–2000°C/min) performs well with an intermediate warming rate of ≥2950°C/min, whereas an open system utilizing high cooling rates (>10,000°C/min) experiences decreased survival at lower warming rates. Thus, open system devices achieving exceptionally high cooling rates, due to the low thermal mass of their microvolumes, are dependent on equally high warming rates for optimal success.[43] Using a nonequilibrium

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vitrification system, one should keep in mind that the faster one cools, the smaller the size of the invisible extracellular crystals in solution (ie, heterogeneous nuclei); the smaller the nucleated crystals are the greater their driving force to increase in size upon recrystallization during warming (ie, devitrification). In turn, one must warm more rapidly to combat the injurious effect of recrystallization.[43] Indeed, vitrification is a highly complex process,[36] whereby a reciprocal interaction exists between the cooling rate required to achieve vitrification of a solution and the concentration of CPA(s)/solutes.[48,49] Overall, these findings have lent support to the effective development and use of aseptic, closed vitrification device systems (HSV,[38,50] Vitrisafe,[39,43] microSecure,[40,51] SafeSpeed[52]), proving that open device systems were not a requirement for successful oocyte and embryo vitrification outcomes. In contrast, only higher volume, closed systems have proven effective to date for the vitrification of reproductive tissue.[53,54] The key component to optimizing post-warming survival, independent of device used, is to insure that the warming rate is greater than the cooling rate; of which the need for speed is inversely correlated with the concentration of the CPA used. The thermodynamics of cryophysical and chemical relationships has been reviewed[55,56] and eloquently discussed by Wowk.[57]

Although it took more than 20 years of development, vitrification has transformed the IVF industry, with regard to oocyte cryobanking[34,58,59] and the justified adoption of freeze-all IVF cycles[35] in conjunction with blastocyst culture and micromanipulation. As the story continues to unfold, in terms of devices and vitrification solutions, today blastocysts are vitrified with great confidence that their fresh-state viability will be completely sustained. This is particularly true in conjunction with blastocyst biopsy/preimplantation genetic screening (PGS)–single ET applications,[60,61] where over 99% survival can be typically achieved,[62] along with efficient pregnancy success across all age groups following single euploid ET. With embryo and oocyte vitrification being the most significant procedure applied to the assisted reproductive technology (ART) industry since the development of intracytoplasmic sperm injection (ICSI),[12] it is inconceivable why any IVF program would still be applying conventional slow-freeze (SF) procedures.[63] In this era of ART where many different vitrification devices and commercial solutions exist, as well as programs transitioning their cryoinventories from slow frozen to vitrified oocytes and embryos, it is important to realize that 1.0 M sucrose is an effective "universal" warming solution. It is not financially feasible, nor practical, to maintain various thawing solutions for slow frozen and vitrified samples. Parmegiani et al's[64] proposed use of 1.0 M sucrose solution for the step-down dilution of SF embryos is equally effective with vitrified embryos.[65] By simply halving the sucrose concentration into a three-step, or possibly four-step (+0.125 M), dilution (under ambient temperatures) in decreasing concentrations of sucrose at 2–3 min intervals, prior to final equilibration in isotonic medium, optimum post-thaw survival and viability can be achieved. In fact, in one pilot study in our laboratory, the CPAs in vitrified blastocysts were effectively eluted in 1.0 M sucrose over 5 min before direct isotonic equilibration.[66] Granted such an action creates unnecessary osmotic stress on an embryo, it does demonstrate the physiologic functionality of the phospholipid bilayer of blastomere membranes cryopreserved by vitrification. It is not known, however, whether the plasticity of the oolemma would be equally forgiving to sustain the viability of the largest single cell in the human body. Indeed, post-warming dilution protocols do vary considerably (ie, time interval and steps), dependent on cell permeability and the CPAs used, an area that could benefit from more controlled comparative experimentation.

Overcoming variables impeding clinical progress and legal liability

The commercial development of numerous types of vitrification devices and solutions has facilitated the application of clinical vitrification, but has also created technical inconsistencies between laboratories. Depending on the device and its learning curve, technical variation must be accounted for, as recently discussed.[67] What can be performed to minimize intra- and inter-laboratory procedural variation beyond the essential need for training? Numerous risk factors and safety issues associated with different vitrification methods should be considered (eg, LN_2 device handling; device design flaws; shipment concerns and viral cross-contamination of semen, embryos or ova),[25,36,40,67–69] but are not reviewed in this article. In essence, there are basic quality control (QC) factors warranting implementation to make vitrification a consistent, efficient, reliable and highly effective ART procedure that minimize liability and maximize success. The processes in need of QC considerations include the following: pre-vitrification organization, labeling, cryodilution, aseptic technique and possible aseptic storage, container loading and sealing/protection, reliability and repeatability, warming temperatures and CPA elution and LN_2 storage, handling and shipment. An ideal vitrification device and method should allow for a repeatable

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volume of vitrification solution, containing embryo/ova, to be loaded simply in a time-sensitive, reliable, controlled manner, devoid of air bubbles. The goal is to eliminate technical variation, while optimizing 100% recovery and high survival rates. Recovery rates should not be minimized, as they represent a potential design flaw to a device and create a serious liability risk to the group who ultimately discovers a problem with this issue.[67] Obviously, training and experience are critical to reducing technical variation and insuring reliable consistent outcomes.

Serious liability issues can arise regarding the reliability of given device systems and poor recovery potential (ie, >1% loss rate). When an embryo or egg(s) fails to be recovered, this situation can present serious problems, especially if the sample had been vitrified by another laboratory and/or the receiving laboratory is unfamiliar with a given device? This is especially so if the lost specimen represents the patient's last option. Was the device tapped or jostled pre- or post-vitrification, the specimen accidentally aspirated pre-vitrification or simply not identified post-warming? These are the questions which are ultimately asked and typically remain unresolved. In short, was it technical incompetence pre- or post-vitrification, or was it simply device failure in-between? The latter situation is not helpful for laboratories facing potential legal litigation, and unfortunately case precedence does exist in the US court system. In turn, the emphasis on universal reliability and repeatability must be seriously regarded. The reality is that highly qualified embryologists and experienced laboratories warming vitrified specimens are vulnerable to the procedural QC habits (good and bad) and device choices (open, closed or hybrid systems) used by other ART laboratories and their staff.[67] Failure to identify eggs can be particularly problematic in devices that do not allow direct visualization of the eggs on or in the device, because they can become highly translucent in 1.0 M sucrose solutions (ie, T1) immediately post-warming/elution. Unfortunately, most published reports simply hide their recovery failures in their otherwise good overall survival rates. This form of data manipulation, however, does not allow the industry to accurately assess the potential QC flaws inherent to certain device systems, nor anticipate possible liability risks. Conversely, vitrified oocyte recovery rates were recently published in a reasonable and informative manner in a randomized controlled trial (RCT) device study, revealing a 2.6% loss rate using semi-closed CryoTopSC devices compared to the aseptic closed CBS-HSV system (0%).[37]

Current trends and challenges with embryos, gametes and reproductive tissue

Blastocyst vitrification

The development and clinical application of blastocyst vitrification has experienced the greatest success in terms of maintaining the viability of fresh embryos,[70] being superior to conventional slow freezing.[26,71] Post-warming, blastocysts tend to appear completely intact, with the occasional appearance of a few necrotic outer trophectodermal cells. In contrast, approximately 25% of cleaved embryos may have one or two blastomeres degenerate, while the remaining embryo is highly viable.[72,73] Non-incorporated cells and fragments outside the trophectoderm of blastocysts commonly appear degenerate post-warming. It is very uncommon to observe a completely lysed blastocyst, and care should be taken to culture these embryos as they may have experienced an initial granular transitional phase, but sometimes appear quite normal after a couple hours in culture. Blastocyst reexpansion before ET is desired, but should not be used as a key determinant for viability as some embryos simply require more time to equilibrate. Osmotic responsiveness to sucrose dilutions and the maintenance of membrane integrity/cellular clarity are reliable indicators of post-warming embryo survival. Today, complete blastocyst survival rates routinely exceed 95%, and vitrified ET (VFET) live birth outcomes are routinely equal to or higher than fresh ET success,[74] likely due to impaired endometrial receptivity in fresh, hyperstimulation cycles.[75]

To promote and insure high blastocyst survival, pre-vitrification blastocoel collapsing (ie, fluid volume reduction) has been adopted, as reviewed by Liebermann and Conaghan.[76] The artificial collapsing of blastocysts prior to vitrification has been proven to be effective using a variety of methods,[77,78] with trophectoderm laser ablation being the simplest approach, sacrificing a single cell for the greater good of the whole embryo. Hatched blastocyst and fully expanded, zona intact blastocysts certainly have the greatest need for volume reduction before vitrification to enhance post-warming viability. However, it appears that this need to manually collapse a blastocyst is dependent on the type and concentration of the CPAs used. Most commercial vitrification solutions contain a mixture of DMSO and EG which possess high permeation coefficients, in contrast to glycerol, thus achieving osmotic equilibration and possible blastocoel reexpansion in a shorter interval. Therefore, it is not surprising that the use of an alternative glycerol–EG-based solution (Innovative Cryo Enterprises (ICE), Linden, NJ, USA) in our

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laboratory,[40,62] and others,[79] does not require artificial collapsing to achieve high post-warming survival, as the slower permeating glycerol effectively reduces water influx before cooling. Using this higher concentrated glycerol-based solution, devoid of DMSO, in a highly effective noncommercial aseptic, closed system,[51] has revealed that high pregnancy outcomes can routinely occur across all age groups without the pre-vitrification application of blastocoel collapsing.[40,62]

It has become increasingly evident, since first proposed by Grifo et al,[80] that the transfer of a single euploid vitrified/warmed blastocyst, independent of age, is the most efficient way to achieve a high implantation rate and live birth rate (IR/LBR), as clearly confirmed in other recent reports.[35,62] Furthermore, although there is no difference in IR/LBR using either day 5 or day 6 euploid blastocysts, the miscarriage rate tends to be higher using a day 6 early to full blastocysts (grade ≤3) or embryos with B-quality trophectoderm.[81] Whether PGS is applied, or not, it is routinely accepted that blastocyst viability is not compromised by vitrification, nor micromanipulation, and that delayed ET cycles using the warmed blastocyst can be advantageous.[62,80,82] To date, the possible epigenetic effects of embryonic vitrification solution exposures have not been elucidated; however, it has been shown that aneuploidy rates do not increase in blastocysts vitrified as oocytes.[83,84] Furthermore, it is doubtful whether there is a genetic consequence to vitrifying blastocysts considering their high post-warming IRs and live birth success. Based on the exceptional reliability of embryo vitrification and normal neonatal outcomes,[85] there is a growing trend in the IVF industry replacing fresh ET with freeze-all cycles, especially in older patient populations (>37 years old) and PGS-tested patients, as the prior fear of the best quality embryo may not survive post thaw no longer exists. Today, "freeze-all" VFET can efficiently achieve IRs in excess of 65% (up to 84%), independent of age, after single euploid ET (Table 1). While fresh ET success rates have remained relatively dormant between 2010 and 2015 (eg, donor egg LBR: 55.8–56.8% LBR/ET), frozen ET success has steadily increased from 38.4% to 46.6% total LBRs transferring fewer embryos/ET.[86] In turn, blastocyst vitrification in conjunction with PGS has facilitated improved embryo utilization rates aimed at more efficiently creating healthy singleton births (<31.9–36.5% from 2010 to 2015).

Oocyte vitrification

Breakthrough efforts in early oocyte slow freezing success integrated ICSI to reliably achieve fertilization and blastocyst development,[87] and subsequently healthy live births.[88–91] However, a couple of years later, early success in vitrifying and warming human oocytes[92–94] transformed this reproductive practice into a highly efficient freeze preservation method over the past decade.[34,58,59] Upon verifying the normal health and well-being of over 900 babies produced from embryos derived from slow-frozen and vitrified oocytes,[95,96] coupled with consistently high survival (>85%) and good fertilization rates (>70%) attained with vitrification, a Practice Committee of the American Society for Reproductive Medicine (ASRM) reclassified oocyte cryopreservation technology as "nonex-

Table 1 Select information from the national summary of ART statistics by the CDC reviewing the frozen ET results of non-donor egg cycles

Frozen embryos: autologous cycles	Age groups (years)				
	<35	35–37	38–40	41–42	43–44
SCCRM: 2014/2015					
No. of cycles	82/93	75/70	72/68	27/29	9/11
Mean #ET	1.1/1.0	1.2/1.1	1.2/1.1	1.1/1.1	1.4/1.0
%IR	77.9/71.3	61.0/69.4	60.0/71.4	84.0/64.3	6/13/6/11
%LBR/ET	73.1/68.8	66.2/65.7	55.0/69.1	69.6/58.6	5/9/6/11
%Healthy single/ET	56.4/61.3*	56.3/55.7	48.3/54.4	69.6*/48.3	4/9/6/11
Mean CDC 2014					
No. of cycles	26,182	13,539	10,078	3,792	1,811
Mean #ET	1.6	1.5	1.6	1.7	1.8
%IR	43.7	40.8	35.2	28.4	19.9
%LBR/ET	46.6	44.0	38.3	32.1	23.1
%Healthy single/ET	30.7	30.6	27.1	23.3	16.8

Notes: The data contrast the SCCRM clinic in 2014 and 2015 to the national average in 2014. SCCRM and its affiliated Ovation Fertility Laboratory have strived to optimize the quality of patient care by implementing a clinical practice of predominantly PGS/vitrification-all cycles. In turn, the use of single euploid ET maximizes implantation and healthy singleton live birth successes. *The SCCRM clinic achieved some of the highest healthy singleton LBR in the USA in 2015 (ie, healthy single: normal birthweight term singleton). Data from Centers for Disease Control and Prevention. Assisted Reproductive Technology Surveillance, National Summary Reports 2010–2015.[86]

Abbreviations: ART, assisted reproductive technology; CDC, Center for Disease Control; ET, embryo transfer; IR, implantation rate; LBR, live birth rate; PGS, preimplantation genetic screening; SCCRM, Southern California Center for Reproductive Medicine.

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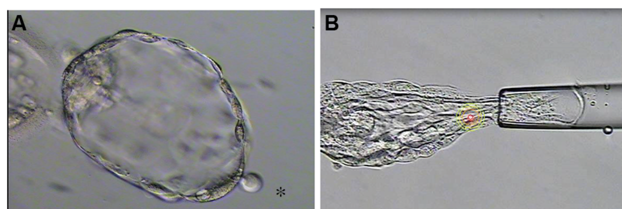

Figure 1 Laser manipulation of human embryos.
Notes: (**A**) The hatched blastocyst possessing a single nonviable, unincorporated trophectodermal cell (*) following pre-vitrification laser-mediated blastocoel collapse and post-warming in vitro culture. (**B**) Routine biopsying of trophectodermal cells via laser-assisted, micro-aspiration has confirmed the biosafety of infrared diode lasers and further proven the resiliency of embryos to micromanipulation and subsequent cryopreservation.

perimental" in 2013.[97] It is important to realize that the intent of this decision was to facilitate the application of this freeze preservation practice for female cancer patients, without the obstacles of informed research consent. A major determinant in lifting the "experimental" classification was the consistent evidence revealing that genetic anomalies occur at a rate of <2% in babies derived from vitrified oocytes, being no different than fresh oocytes.[98]

There is no doubt that the most important application for oocyte cryopreservation is to provide a "fertility preservation" option for women undergoing potential sterilizing medical treatments, or medical uncertainty during their remaining fertile years. As medical care continues to improve for women faced with life-threatening diagnoses, oocyte vitrification is indeed the best option to preserve a woman's future fertility if unmarried or unwilling to use donor sperm, knowing that blastocyst vitrification is a more well proven and reliable cryopreservation option.[99] Another important application is in emergency IVF cycle situations where upon on the day of oocyte retrieval there is no sperm present in a testicular biopsy or due to ejaculatory failure. Despite advanced planning, clinical laboratories worldwide are repetitively faced with this latter issue, but now have an effective solution to rescue an IVF cycle with vitrification.

The commercial banking and marketing of donor oocytes is probably the fastest growing and largest application of oocyte cryopreservation technology. Details on this growing industry have been reviewed by others.[100,101] Suffice it to say, the majority of publications boosting oocyte cryopreservation technologies as being comparable to the use of fresh oocytes are typically associated with egg-banking affiliated professionals. Granted, there have been RCTs supporting the efficacy of vitrified donor and autologous eggs.[34,102] Certainly, oocyte cryopreservation has proven to be a viable and effective clinical option.[59] For example, in a recent webinar presentation, Nagy[103] reported his experience at My Egg Bank (MEB) in the USA with 21,462 warmed eggs having an 88%/78% survival and fertilization rate, respectively, with an equally impressive 52% clinical pregnancy rate using vitrified donor eggs. Although fresh donor egg rates in the USA typically attain an average 55% live births, many top ART programs are able to routinely attain 65–85% LBRs. That level of success has yet to be reported using vitrified oocytes. In contrast, commercial egg banks repeatedly report sub-50% LBRs, as do published IVF programs.[34,58,104,105] Excluding the occasional outlier publishing an impressive data set,[98,106] the average clinical practice is not publishing their variable experience with oocyte cryopreservation that fails to attain the levels reported by commercial propaganda. There is, however, general agreement that blastocysts derived from vitrified oocytes are the same as fresh,[99,107,108] or from vitrified blastocysts for that matter. To optimize pregnancy and live birth success, it is generally accepted that if >8 oocytes are available, it is more favorable to culture and transfer at the blastocyst stage.[109] Yet, warming more oocytes per cycle does not necessarily insure that more blastocysts may be produced from a given batch source. Overall, the disparity observed in everyday laboratory practice, especially in terms of developmental competence, indicates that there is more to learn about oocyte cryopreservation, specifically how egg quality and development are influenced by factors such as hormonal stimulation, cytoplasmic maturation, organelle functionality and membrane permeability.[110] Furthermore, more RCTs are needed to compare open and closed vitrification devices, cooling rates and warming rates, as well as unstable and metastable non-equilibration solutions, if we are to truly optimize the reliability and repeatability of oocyte vitrification in the future.[37,39]

The difficulties associated with improving oocyte cryopreservation are related to understanding the special structure and sensitivity of this large single cell,[111] and considering the overall physiology of oocytes.[112] We may still need to evaluate the varying exposure time and concentration of the CPA(s) and the equilibrium/maturation interval pre and post-warming, while still inhibiting extra- and intracellular ice formation in conjunction with minimizing potential cytotoxicity, to optimize the viability and developmental capacity of embryos. Several studies evaluating the effect of vitrification and slow freezing on the meiotic spindle integrity and chromosome alignment revealed that less damage occurred in vitrified oocytes,[113,114] and that spindle recovery was more rapid following vitrification (1–2 h post-warming) compared to slow freezing.[115] However, other studies evaluating vitrified–warmed oocytes suggested that chromosomal alignment may be partly compromised.[115,116] Furthermore,

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other biological characteristics of human oocytes that might confer susceptibility to damage during the cryopreservation procedure include: 1) the low permeability coefficient of the oolemma, which makes the penetration of cryoprotectant substances more difficult, and their intracytoplasmic lipids which make them more sensitive to freezing than embryos;[117] 2) precocious oocyte activation induced by exposure to cryoprotectants which may disturb future development;[112,114] 3) loss of high mitochondrial polarity associated with a significantly reduced capacity to upregulate the levels of intracellular free calcium after thawing[118] and 4) microvacuolization in the ooplasm and ultrastructural alterations in specific oocyte microdomains have been linked to a reduced developmental potential of mature cryopreserved oocytes.[119] Furthermore, there remains a need to understand more about the level of gene regulation and energetics that could be responsible for decreasing the developmental potential of the vitrified cytoplasm.

The commercialization of oocyte cryopreservation was an unanticipated and somewhat disturbing trend as a direct by-product of ASRM's decision to qualify this technology as nonexperimental. This was not so much from the donor egg bank marketing perspective, but more in terms of the clinical promotion of elective fertility preservation of women (of all age groups) through egg banking. Clearly, there are age limits that should be considered, which influence the effectiveness of future fertility efforts.[108,120] Yet, the biggest problem has lied in every IVF clinic in the world suddenly considering themselves as qualified authorities on oocyte cryopreservation, capable of applying a textbook recipe without experience or any proven pregnancy success of their own, just because the technology was deemed "nonexperimental." More than 5 years later, oocyte vitrification practices have yet to be optimized nor their developmental competence issues fully understood. Granted, when it works well and yields good quality blastocysts, one can anticipate excellent pregnancy outcomes, similar to fresh eggs.[59,99,108] More often, however, the average laboratory frequently observes slower and lower blastocyst development. More importantly, more than 10% of the vitrified egg batches will likely fail to yield a transferrable embryo. This phenomenon was recently documented in a 137 and 368 autologous egg banking cycles in Spain and the USA, respectively, with 12–21.2% of the fertilized oocyte warming cycle failing to yield an embryo for transfer.[104,121] It is to be kept in mind that survival rates and normal fertilization events tend to be consistent (75–95% and 65–85%, respectively), with survival failure being a rare event (<2%).[105] Perhaps the greatest concern then is that naive, innocent female consumers (ie, perspective patients) are being misinformed about their risks, by financially motivated programs, that the freeze preservation of their potential fertility based on today's technology does not guarantee that a transferrable embryo will even result, let alone potentially create a healthy baby. As there is no stopping the elective fertility preservation movement, the latter unanticipated result begs us to redirect the question, does this technology truly not require continued experimentation to improve its overall efficacy, reliability and long-term effectiveness? A recent series of reciprocal nuclear transplantation experiments involving fresh and vitrified sheep oocytes has clearly confirmed that the vitrified cytoplasm of oocytes is the possible source of a zygotes' developmental incompetence, as opposed to chromatin defects in the meiotic spindle.[122] Due to the high costs, resource availability and ethical considerations of generating human oocytes for experimentation, research progress will undoubtedly be slow, but necessary.

Human ovarian tissue vitrification

Gosden et al[123] were the first to explore the realm of ovarian tissue cryopreservation using slow freezing methods with sheep ovaries. Similar freeze preservation success has subsequently been achieved in the human beings using both SF and vitrification methods.[53,124–127] Comparative vitrification solution trials have been initiated to identify optimal solutions for ovarian tissue freeze preservation.[54,128] Promising vitrification results have also been attained in a Macaque monkey model using a metastable solution composed of 25% EG, 25% glycerol and polymers in a closed system device.[129] Applying a two-step warming process, the latter investigators efficiently preserved the follicular morphology and stromal tissue of the ovarian cortex. In addition, there have been promising developments in the cryopreservation of whole organs (eg, kidneys) by perfusion with metastable vitrification solutions[130] that could have direct application with ovaries, as the effective external permeation of CPAs is concentration and rate dependent relative to tissue type and mass. Perfusion offers a mechanism to uniformly distribute CPAs throughout the tissue bed. Sheep ovaries have been successfully perfused for 10 min with a 10% DMSO solution followed by SF and thawed at 68°C for 20 s and then 37°C for 4 min.[131] Upon successful transplantation, these slow frozen perfused ovaries maintained physiological functionality after 6 years in vivo.[132] Successful perfusion and slow freezing of human ovaries has also been reported.[133] Ovarian tissue in humans, however, has generally been slowly cryopreserved as cortical biopsies or strips with proven in vivo transplantation success,[134] yet whole ovary perfusion and

vitrification are promising technologies for future clinical application.[135,136] Today, ovarian tissue cryopreservation and transplantation are still regarded as experimental.[137] Regardless, it is a viable option for patients who require immediate gonadotoxic medical treatment and is the only option available for prepubertal girls.[124,137,138] Overall, there is agreement in the fertility community that this technology should not be offered to patients with benign conditions or for the purpose of delaying childbearing, as embryo and oocyte vitrification are more efficient and effective approaches.

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Human sperm vitrification

In lieu of Luyet's original assertions, it is reasonable to believe that small cells with little intracellular water, such as sperm, can survive kinetic vitrification in the absence of permeating CPAs under rapid cooling conditions. Indeed, that has been the experience of Evgenia and Vladimir Isachenko and their colleagues in Cologne, Germany, whom first reported the successful cryopreservation of human sperm without cryoprotectants in 2002.[139] Using a 0.5 M sucrose vitrification solution, these investigators developed an effective kinetic vitrification system.[140–144] These efforts were particularly well suited for the cryopreservation of low numbers of sperm isolated in microvolume suspensions contained on or in different devices (eg, Cryoloop, grids/mesh, Cryotops, cut-straw microdrops and capillary tubes).[140,143,145,146] It was proven that sperm functionality, as measured by acrosomal integrity, mitochondrial activity, plasma membrane function, DNA fragmentation, motility and fertilizability, was similar to or better than conventional slow freezing.[141,142,144,147–149] Clinical efforts have further shown that vitrification of sperm in higher volumes (up to 0.5 mL) can be a practical and effective treatment achieving pregnancies and live births by IVF and intrauterine insemination (IUI) procedures.[141,142,145,147,150–154] Although kinetic sperm vitrification has been proven effective for over 15 years,[5,147] it has not been met with the same large-scale acceptance and industry application afforded to eggs and embryos. However, as experimentation continues and more users become familiar with this technology, a trend away from traditional sperm freezing in the presence of toxic CPAs could well occur overtime. It is worth adding that although testicular tissue vitrification has been successfully applied,[155] the current technology of rapid freezing of whole biopsy tissue (ie, seminiferous tubular masses) has proven to be effective in clinical application.[156] In turn, it is unlikely that the vitrification of sperm and testicular tissue will ever gain the industry-wide acceptance and popularity granted to embryos, oocytes and perhaps ovarian tissue in the future.

The future is guaranteed

Today, the advantages of vitrification appear to significantly outweigh any potential pitfalls, if one can clearly assess any. Under well-controlled vitrification conditions, there should be no damaging ice crystal formation to cause osmotic, physical or physiological disruption of cellular function. Procedures are performed simply, reliably and rapidly with relatively brief exposures to concentrated, biosafe CPAs, without the cost of electronic equipment and maintenance of a programmable freezer.[24]

Vitrification has made a lasting impact in the IVF industry over the past decade, literally changing the way reproductive endocrinologists and biologists approach infertility treatment. It has been successfully applied to oocytes and embryos by laboratories using both open and closed systems, as well as unstable or metastable nonequilibrium vitrification solutions. The relationship between device type and solution choice is complicated. Although some very effective DMSO/glycol and non-DMSO solutions have been developed, there is significant variation in the efficacy of their application depending on the vitrification method applied and the experience of the operator, commonly referred to as "technical signature."[79]

As with all ART procedures, there is always room for improvement in their application and outcomes. Steady advancements in reproductive tissue and oocyte vitrification will likely require continued experimentation to further understand membrane functionality, the role of extracellular stabilizing additives (eg, hyaluronate, hydrocellulose and butylated hydroxytoluene) and ice blocking agents (eg, polyvinyl alcohol polymer), organelle functionality and gene expression, cryoprotectant interactions and possible toxicities. Furthermore, quality management improvements aimed to reduce technical variation will all prove critical to optimizing vitrification in the future. Ideally, vitrification systems will be mindful of QC considerations to enhance procedural consistency and repeatability, with a common goal to eliminate technical signature by reducing intra- and interlaboratory variation. Indeed, the future of cellular viability is infinite in the wondrous world of metastable glass formation and the controlled elimination of recrystallization events, while maintaining normal physiological processes.

Disclosure

The authors report no conflicts of interest in this work.

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