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# Vitrification: Fundamental Principles and Its Application for Cryopreservation of Human Reproductive Cells

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## Abstract

The fundamental understanding of cryobiology through experimentation in the 1960s, 1970s, and 1980s has led to the development of today's vitrification technology. Although human embryo and oocyte vitrification was slow to evolve, it has become an invaluable technology in the field of reproductive medicine. The aim of this chapter is to discuss some of the underlying basic principles behind forming a metastable glass phase during rapid cooling in liquid nitrogen (LN<sub>2</sub>) and the prevention of recrystallization events upon warming. We then highlight how this understanding has led to its highly effective and reliable usage in clinical IVF. Furthermore, we describe how quality control factors (e.g., ease of use, repeatability, reliability, labeling security, and cryostorage safety) can vary between vitrification device systems, potentially influencing clinical outcomes and creating possible liability issues. An open-minded approach to continued experimentation is a necessity, especially pertaining to oocyte freeze preservation, if we are to optimize the vitrification of reproductive cells and tissue in the future.

**Keywords:** cryopreservation, embryo, oocyte, quality control, vitrification

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## 1. Introduction

Studies investigating the effect of cooling on biological cells have been conducted since at least the late 1700s [1]. For much of this history, relatively uncontrolled methods were utilized. The discovery [2], or rediscovery [3], of the protective effects of small-molecule solutes such as glycerol and sucrose, when incorporated into carrier solutions used during cooling and warming, has greatly expanded the efforts to develop robust freeze preservation methods.

An exponential number of reports successfully preserving biological material have been published in the scientific literature over the last six decades.

Because long-term preservation of biomaterial requires cessation of biochemical reactions, storing this material is typically done at very low subzero temperatures. As a result, ice usually forms in the sample, often with deleterious consequences. The avoidance of ice formation, particularly intracellular ice, is known to be one of the most important factors contributing to successful cryopreservation [4]. Preventing intracellular ice formation (IIF) during cooling and warming, and maintaining cellular viability during this process, is achieved by increasing the concentration of solutes in the cytoplasm. This is done by one of the two ways. The first way, referred to as either slow cooling or equilibrium freezing, allows the cell's sufficient time to dehydrate as a result of extracellular ice formation and subsequent exosmosis during the cooling process. This dehydration increases the concentration of the solutes in the cytoplasm, preventing lethal intracellular ice formation during subsequent cooling and warming if done appropriately. A thorough discussion of the principles of cryopreservation has been reviewed by Peter Mazur [5]. The second method intentionally loads high concentrations of solutes into cells prior to cooling below the freezing point of the solution. With this method, the solution containing the cells (and the cells themselves) maintains an amorphous state during subsequent cooling and warming. This later procedure, generally referred to as vitrification, is the focus of the current chapter.

While practical methods to successfully vitrify cells were not developed until the mid-1980s, the concept of vitrification as a means for cryopreservation has a much longer history. In the 1930s, Stiles suggested that, with the use of very rapid cooling, cytoplasm may not extensively crystallize. The result of which could be maintenance of the system [6]. In 1937, Father Basile J. Luyet, one of the founding members of the Society for Cryobiology and its first President, developed the concept of vitrification into a major research proposal [7]. For reasons described elsewhere [8], Father Luyet never succeeded in developing a method to vitrify cells successfully but laid the foundation for the development of vitrification methods by those that followed.

It was not until the 1980s that a method for reviving cells after vitrification was demonstrated unequivocally [9]. Gregory Fahy had worked on developing methods for vitrification for over a decade, and his contributions, coupled with those of William Rall, proved that mouse embryos could be vitrified using several methods. It was subsequently shown that a practical, ambient approach to vitrify and warm embryos could efficiently and effectively produce healthy live births in the mouse [10] and sheep model [11].

After the initial report in 1985, numerous experiments describing successful vitrification of embryos from other species were published. It is beyond the scope of the present work to describe these reports in detail, but such information can be obtained in recently published reviews [12–16].

## **2. Achieving a vitreous state when cooling an aqueous solution: physical aspects**

At temperatures above an aqueous solution's melting point, the water remains liquid as a result of the Gibbs free energy being lower in comparison to that in the solid phase [17]. As

a solution is cooled, it becomes progressively more favorable for ice to form. However, even when the temperature goes below the thermodynamic equilibrium point, ice formation is initially unfavorable as a result of an energy barrier to ice nucleation.

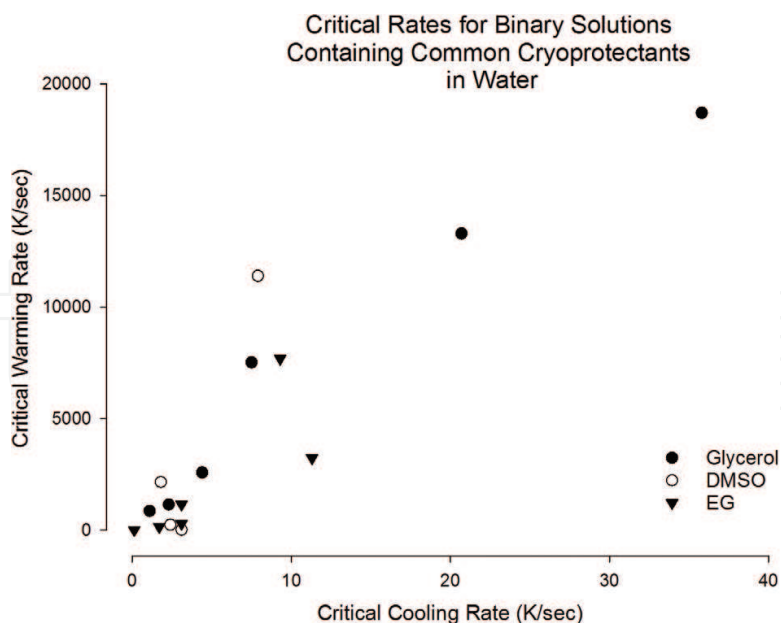
As cooling proceeds, it becomes more favorable for ice nuclei to form. This is often the result of water molecules becoming arranged in a favorable configuration on a foreign particle suspended in the solution. This process is called heterogeneous nucleation. Homogeneous nucleation (i.e., where an ice nuclei forms as a result of self-aggregation of water molecules) is not favorable until relatively low temperatures ( $\sim -39^\circ\text{C}$  in pure water). Therefore, preventing heterogeneous nucleation during cooling is important to attaining the vitreous state.

Avoiding homogeneous nucleation is difficult when cooling dilute solutions. However, increasing the concentration of solutes in a solution depresses the homogeneous nucleation temperature ( $T_{\text{hom}}$ ), and it is possible to depress  $T_{\text{hom}}$  below the glass transition temperature ( $T_g$ ) with a sufficient solute concentration [18]. Unfortunately, solutions with solute concentrations high enough to depress  $T_{\text{hom}}$  below  $T_g$  are usually too toxic to biological systems to be of practical use.

Fortunately, it is possible to depress  $T_{\text{hom}}$  low enough with relatively nontoxic solutions such that kinetics begins to exert an appreciable effect on the probability of ice nucleation and growth. It is the combination of thermodynamic and kinetic effects that allow ice crystal nucleation and growth to be avoided during cooling of these solutions and is the means by which vitrification of oocytes and embryos are achieved presently.

During warming, the likelihood that extensive ice crystal formation will occur is greater for a given solution having been cooled at a specific rate, resulting in markedly higher warming rates being necessary to maintain the vitreous state. The mechanism behind devitrification events has been previously discussed [19]. In brief, during cooling, the nucleation of ice in a solution can be prevented until very low temperatures. These ice nuclei are often very small (submicroscopic), and a solution cooled with only moderate concentrations of cryoprotectants is, for all intent and purposes, vitreous. However, these nuclei remain present during warming up to the melting point of the solution. Because crystal growth occurs more rapidly as the temperature increases, if warming is not extremely rapid (greater than the cooling rate), extensive ice crystal growth will occur from these previously formed nuclei. This phenomenon is referred to in the literature as devitrification and is believed to be just as damaging to biomaterial as ice crystal formation during cooling [19]. Hence, developing cryopreservation methods to avoid extensive crystallization rely upon rapid warming more so than rapid cooling, a point often overlooked in the literature (See **Figure 1**).

It should be pointed out that ice formation during warming is not necessarily damaging to biological systems, particularly if it occurs for only very brief periods of time and the crystals remain very small. It is believed that this may be due, in part, to the type of ice crystal structure initially formed at low temperatures, as ice has many crystalline forms. However, if the system is afforded sufficient time during warming, the molecules in the ice crystals may rearrange to form the more favorable (i.e., from a thermodynamic standpoint) hexagonal crystal structure as well as larger crystals. It is believed that this structure of ice is the most damaging to biological systems [19].



**Figure 1.** The relationship between the critical cooling rate (CCR) and the critical warming rate (CWR) of solutions containing a permeating cryoprotectant dissolved in water is shown. Note that the CWR is generally two to three orders of magnitude greater than the CCR (Data from Table 1 in Hopkins et al. 2012 [21]).

Historically, studies investigating the relationships between devitrification and rates of temperature change as well as solute concentrations have been limited to relatively slow cooling and warming rates due to technical limitations ( $<100$  K/min). As such, estimations of the critical concentrations for the realm of cooling and warming rates that are utilized in the embryology laboratory have often been extrapolated from those data [20] with uncertain accuracy. Fortunately, a recent study has shed light on the critical concentration of solutes in the ranges of cooling and warming rates encountered in oocyte and embryo vitrification [21]. Some of the results from that study were not surprising—revealing a greater critical warming rate compared to the cooling rate for a given solute concentration (**Figure 1**), for example. Also, the critical warming rate is strongly dependent upon cooling rate, even at the high rates of warming in the latter study. Other results, however, were enlightening. The experimental data suggests that the critical warming rate is in fact lower than previously estimated from theoretical models (c.f. [22], as one example) suggesting that the current systems may be more stable than previously estimated. On the contrary, experimental ice growth after nucleation was much faster than theoretically predicted [23], approaching  $25 \mu\text{m sec}^{-1}$  at  $-33^{\circ}\text{C}$  from approximately zero growth below  $-80^{\circ}\text{C}$ . From a practical standpoint, this means that ice, starting at a nucleus in the center of a mature human oocyte (radius =  $63 \mu\text{m}$ ; [20]), would proceed to the edge of the cell at  $-30^{\circ}\text{C}$  in roughly 2 seconds. This highlights the importance of warming as quickly as possible when vitrifying oocytes and embryos.

### 3. Cryoprotectants as components of vitrification solutions

An exhaustive review of this topic is beyond the scope of the current work, and, therefore, interested readers are referred to thoughtful reviews published previously, as well as the



primary literature cited within those papers [24–27]. As with freezing methods, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), and glycerol are common components of vitrification solutions used for reproductive cells and tissues. These solutes possess favorable properties such as high solubility and cellular permeability and relatively low toxicity. For systems where extremely rapid cooling and warming are not possible (i.e., pieces of tissue and the entire organs), these compounds must be used in fairly high concentrations to preclude ice formation. Unfortunately, at these concentrations, the toxicity of the solutions becomes a serious concern, and investigations to determine superior vitrification solutes have been conducted as a result [27, 28].

In some investigations the search for superior vitrificants has been conducted by assessing the effect on the concentration necessary to vitrify (CNV) of the molecular structure of the closely related compounds. As an example, the position of the hydroxyl side groups (and the associated presence or absence of methyl groups) on diols has a significant effect on the vitrifiability of solutions. For example, at low cooling rates, the concentration of 1,2-propanediol necessary for vitrification is 44% by mass, whereas with the isomeric form 1,3-propanediol, the CNV is increased substantially (to 57% by mass), making the more commonly used isomer a superior vitrificant. Similarly, for a fixed concentration of solute, the position of the hydroxyl pairs plays a significant role in attaining and maintaining an amorphous state. A solution of 30% 1,3-butanediol (by mass) dissolved in phosphate-buffered saline containing 4% sorbitol has a critical warming rate (i.e., rate needed to prevent adverse devitrification effects) of  $2.73 \times 10^9$ °C/min. However, this rate is reduced by nearly four orders of magnitude for its isomer 2,3-butanediol ( $2.9 \times 10^5$ °C/min) [29].

Similarly, adding methyl groups to commonly used diols greatly enhances their stability [30]. The critical warming rates for 50% (by mass) ethylene glycol, ethylene glycol monomethyl ether, and ethylene glycol dimethyl ether are 250, 80, and 5°C/min, respectively. About 30% propylene glycol monomethyl ether has a critical warming rate of  $7 \times 10^{30}$ °C/min, whereas 35% propylene glycol has a critical warming rate of  $2 \times 10^{70}$ °C/min [30]. These effects are believed to be strongly associated with the relative ability of the compounds to hydrogen bond with water [27].

Unfortunately, a significant correlation between vitrifiability and biological toxicity has been noted [31], making what might seem to be relatively simple modifications to currently used vitrification solutions too toxic for practical applications. One alternative has been the inclusion of nonpermeating solutes as a means to reduce the concentration of permeating (and presumably more toxic) compounds in vitrification solutions. There are two general classes of agents used, relatively small sugars (usually disaccharides like sucrose and trehalose) and larger molecular weight polymers (i.e., Ficoll and polyvinyl alcohol). The former can enhance the vitrifiability of solutions for a given concentration of permeating agent(s) and also enhance the vitrifiability of cytoplasm via dehydration. The latter generally have lower osmotic effects on cells but can enhance vitrifiability by one or more means [18].

Due to their large size, these macromolecules contribute markedly to the viscosity of vitrification solutions, thus suppressing the kinetics of molecular motion and consequent ice forming tendency as solutions are cooled. Additional developments include the discovery that certain polymers are able to interfere with ice nucleation and growth, presumably by directly

interacting with the surface of ice nuclei and small crystals [32]. These types of compounds (e.g., X-1000 and Z-1000 as marketed by 21st Medicine, Inc.) [32, 33] are becoming common additives to vitrification solutions as they are particularly effective in this regard. It should be pointed out that the ice blockers may not make a significant difference for vitrification under so-called ultrarapid cooling regimes. However, there are benefits to vitrifying by cooling and warming more slowly [34, 35], and such methods may eventually prove to be superior to some of those currently being utilized.

#### 4. Quality control considerations in vitrification systems

Since the first effective method for vitrification was demonstrated using mouse embryos [9], results from numerous experiments designed to vitrify embryos from other mammals have been reported. Investigations at that early time period utilized standard 0.25 ml cryostraws as sample carriers [10, 36]. Chilling injury was determined to be a challenge to cryopreserving bovine and porcine embryos, particularly cleavage-stage embryos. This was also discovered to be a serious concern for oocytes. Following the elucidation of the kinetics of chilling injury on cattle oocytes [37], more successful methods to cryopreserve these sensitive cells developed as a result of increasing the rate of cooling. This was achieved by using approaches that reduced the volume of solution being cooled and reducing or eliminating the effects of the sample carriers on heat transfer from the sample [37].

In the adoption of vitrification to the human ART industry, thinner straws and flat to semi-flat sample supports composed of various materials gained prominence for use to increase the cooling and warming rates of the samples [38]. While these devices tended to improve outcomes, many have relied upon directly exposing the samples to liquid nitrogen [39]. This results in an increased theoretical risk of sample cross-contamination from contaminated liquid nitrogen [40], yet no such disease transmission has ever occurred via an embryo or oocyte [41]. Furthermore, these systems have been reported to be very challenging to use, resulting in a significant “technical signature” of the outcomes [42–44]. Commercial influences have pushed vitrification devices into the marketplace, in fact more than 25 different device systems have been utilized. This commercial push to market devices has created serious potential quality control (QC) problems, such as inherent design flaws of some devices in secure labeling, open system storage, and suboptimal recovery and inconsistent survival rates; these factors could present unnecessary and undesirable industry variation with substandard outcomes. While a device system may be perfected, or not, within a laboratory, when samples are transferred for warming to another laboratory, reduced outcomes may occur. This interlaboratory variation poses potential liability issues to both programs [45, 46]. Even when both programs are competent in their procedures, the relationships between cooling and warming thermodynamics as discussed above can have applied consequences. For example, open device systems like Cryo-Locks, Cryo-Tops, or Cryo-Tech that have become a worldwide industry standard emphasize the use of micro-volumes of DMSO/EG solutions varying from 0.5 to 0.05  $\mu\text{l}$ . This 10-fold variation in volume, or greater, directly influences both the risk of dehydration and cooling/warming rate potentials of the sample. A program

emphasizing ultralow volumes utilizing visual dehydration (i.e., complete loss of solution seen around sample) prior to LN<sub>2</sub> direct exposure to maximize ultrarapid cooling rates is likely exposing oocytes/embryos to potentially damaging or unnecessary osmotic stress and an increased risk of suboptimal warming rates by an end user unfamiliar with their particular vitrification nuance in the technique. In turn, a poor outcome by the end user is not necessarily a reflection of their competence. Device systems using higher volumes, or more importantly set volumes, are less vulnerable to warming rate variation under standard conditions.

Alternative efforts have focused on developing aseptic vitrification devices/procedures that offer simplicity and reliability of use, high survival/viability rates, and biosecurity. The CBS™ 0.3 ml embryo/semen straw is an ideal storage container, offering biosecurity, and tamper-proof, dual-colored labeling for ease of identification. In conjunction with vitrifying in an open pulled straw (OPS; [47]), a cut standard straw (CSS; [48]), or sterile flexipette (microSecure-VTF; [44]), effective low-cost options are proving to be very practical and successful, in contrast to expensive commercial systems like the CBS™-HSV and VitriSafe™ devices [49, 50]. The success of implementing these double-container systems is predicated more on achieving rapid warming rates (in excess of 5000°C/min) than on their moderately rapid cooling rates (up to 1500°C/min). Thus, the simplicity of the system and the ability to effectively remove the device for rapid warming are critical factors to these aseptic methods. These factors were taken into consideration in the development and validation of the microSecure-VTF system [51], which has proven to be a user-friendly technique offering high inter-technician repeatability and reliability (100% recovery rates), high survival rates, and high live birth rates with human oocytes and blastocysts [44, 52, 53]. There are also hybrid vitrification device systems like Rapid-i [54] and the Cryotop SC [55] which ultrarapidly cool the device prior to sealing them into a straw container under LN<sub>2</sub> vapor conditions, placing the container at risk of incomplete seals (i.e., particularly the Cryotop SC and homemade cut straw-double container systems). The latter event could allow LN<sub>2</sub> seepage to occur and problematic warming events to transpire if not accounted for properly [56]. Variations in device systems that place the end user at risk of unexpected poor outcomes, like non-recovery and high degeneration rates, create serious liability issues to IVF programs, as recently discussed [46]. Liability concerns can also stem from poor manufacturer design or user compliance to quality management practices.

When contemplating which vitrification device to use, there are some critical factors to evaluate to accurately judge its potential usefulness [57]. We believe it is important to assess labeling potential, technical ease, simplicity and repeatability, LN<sub>2</sub> storage, recovery potential, and survivability. A device that offers secure (internalized), dual-colored labeled containers (e.g., CBS™ embryo straws) is considered optimum. In evaluating technical ease, one should assess handling simplicity/repeatability of cryo-loading and warming, as well as identification potential. Can the desired vitrified sample be promptly, clearly, and accurately identified without ambient exposure? In terms of LN<sub>2</sub> exposure, is the device closed with secure seals, easy to handle, well protected, and space efficient (e.g., greater than five devices/goblet)? Furthermore, technician's safety in the handling of devices in LN<sub>2</sub> should be seriously evaluated and not overlooked. Finally, one should determine the recovery and survival potential of using a particular device before implementing it into commercial practice. In terms of



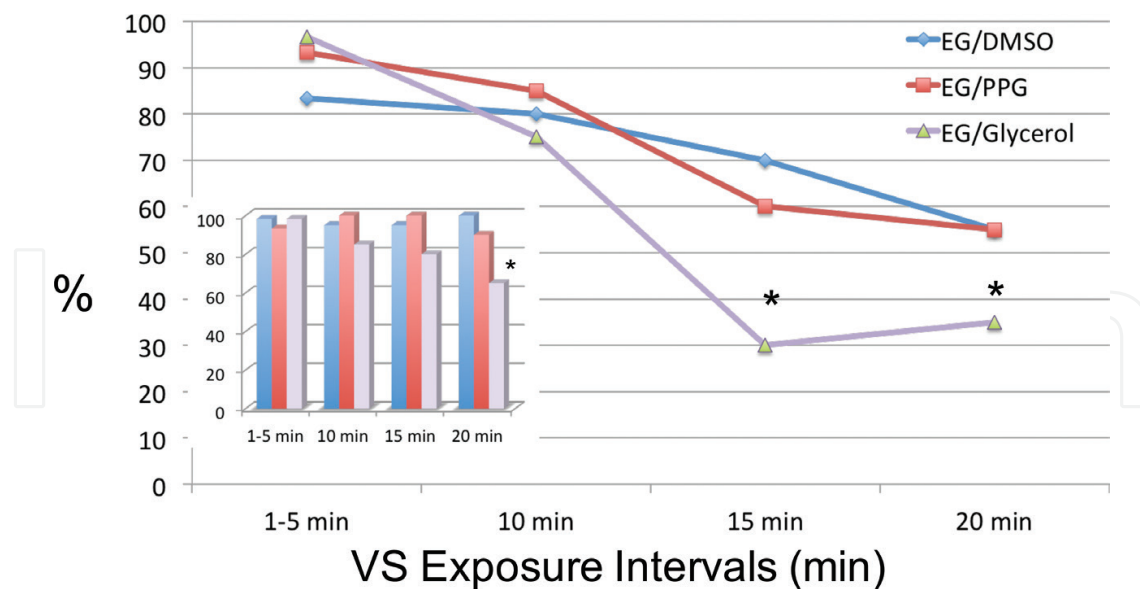
“technical signature,” remember that any good laboratory can master a device with sufficient practice and experience, but can an inexperienced lab using that same device have comparable results? Therein lies the rub, a potential QC nightmare awaits as so many different types of suboptimum vitrification devices have been mass marketed without full consideration to the factors listed above.

Ultimately, the successful application of all vitrification methods, independent of the device used, is dependent on three principle quality control components (i.e., the 3 “Cs” to successful vitrification): clarity of the mind (i.e., organization), concentration (i.e., focus on task at hand), and consistency (meticulous, technical repeatability; [44]). For example, complete organization (i.e., clarity) is imperative to avoid any variation in strictly timed dilution and loading/plunging/warming steps. Meanwhile, the cryo-dish setup and routine manner of warming and diluting oocytes and embryos (intra- and inter-solution steps, i.e., consistency) can be critical to the effective and timely use and reuse of vitrification solutions. Because of the rate-limiting nature of these dilution steps to avoid toxicity, user concentration and focus are important to insure that multiple straws and devices can be prepared in a short, precise time period. It is the latter issue that poses the greatest challenges to animal industry application of vitrification where large numbers of oocytes and blastocysts may need to be processed in a given day (e.g., in vitro production facility).

## 5. Experimental aspects of today’s clinical vitrification

After 20 years of development, vitrification has transformed the IVF industry, with regard to oocyte cryobanking [58–60] and the justified adoption of vitrification-all IVF cycles [61] in conjunction with blastocyst culture and micromanipulation. The combined use of nontoxic levels of permeating cryoprotective agents, coupled with supplemental macromolecules, as discussed above, has facilitated the safe vitrification of human oocytes and embryos. 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/PGS-single embryo transfer application ([62, 63] where over 99% survival can be typically achieved [64] along with efficient pregnancy success across all age groups following single euploid embryo transfer [65]. Embryo and oocyte vitrification has been the most significant procedure applied to the ART industry since the development of ICSI [44]. In our own experimental efforts to verify the safety and reliability of  $\mu$ S-VTF in a metastable solution ( $>7.9$  M glycerol/DMSO-free; Innovative Cryo Enterprises, NJ, USA), we determined that blastocyst viability was sustained after up to five times re-vitrified (rVTF) with and without equilibrating sucrose dilutions post-warming [66]. Although the commonly used 15% DMSO/15% EG commercial vitrification solution is less cytotoxic to human blastocysts following extended exposure ( $>10$  min; **Figure 2**), its inferior metastability is less resilient to repeated rVTF.

In the last decade, oocyte vitrification has proven to be a reliable option for the “fertility preservation” of women facing potential sterilization medical treatments. Based on the normal health and well-being of live-born babies, and consistently good survival, fertilization,



**Figure 2.** The effect of vitrification solution (VS) type by exposure interval is conveyed in terms of initial post-warming survival (0 hr; inner bar graph) and 24 h sustained development (line graph). Statistical differences ( $p < 0.05$ ) between groups within interval are indicated by an asterisk (\*). The more concentrated, metastable EG-glycerol solution tended ( $p > 0.10$ ) to show reduced survival and development of human blastocysts after 10 min of exposure, with overt differences in toxicity seen by 15 min.

and pregnancy rates in some randomized clinical trials, oocyte cryopreservation technology was deemed “nonexperimental” in 2013 by the American Society for Reproductive Medicine (ASRM; [67]). It should be noted that the determining factor to remove the “experimental” classification was dictated by medical insurance factors to aid the former female population. Although oocyte vitrification has numerous potential clinical benefits, including IVF cycle rescue and elective fertility preservation when a sperm provider fails to produce or is unavailable, respectively, its “nonexperimental” classification has been potentially misleading [46]. Blastocysts derived from vitrified oocytes are comparable to those derived from fresh oocytes, in terms of euploidy and live birth rate potential [68, 69]; however, the overall developmental competence of zygotes to the blastocyst stage continues to be delayed and reduced overall [69].

Little to no progress has been made over the past 5 years to correct or understand why overall blastocyst development may be reduced between cryopreserved batches. The inefficiencies of oocyte cryopreservation go beyond the device system used or technical variation and undoubtedly rest on improving our understanding of the membrane integrity and cytoplasmic sensitivity of this large single cell [45, 70, 71]. There remains a need to understand more about cytosolic factors at the level of gene regulation and energetics of vitrified-warmed oocytes that could be responsible for decreasing their developmental potential [72–74]. Due to the high costs, resource availability, and ethical considerations of generating human oocytes for experimentation, research progress is slow but necessary. There is a need to continue exploring the role of safer, metastable vitrification practices (e.g., solutions, equilibration intervals, dilution methods), as well as the cytoplasmic preparedness of oocytes to be cryopreserved.

## 6. Summary

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 biomechanics and the role of extracellular stabilizing additives (e.g., hyaluronate, hydrocellulose, and butylated hydroxytoluene) and ice blocking agents (e.g., 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 require mindfulness to quality control issues to enhance procedural consistency and repeatability. Our common goal should be to eliminate technical signature by reducing intra- and interlaboratory variation. Indeed, our future ability to sustain cellular viability and physiological processes is infinite in the wondrous world of glass formation and the controlled elimination of recrystallization events.

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