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Abstract

The need to find an efficient method to store gametes and embryos was driven both by medical and agricultural necessities. Gametes were the first cells used in early attempts of cryopreservation, yet these proved to be the most elusive. This chapter details the story of the development of techniques for gamete and embryo freezing, starting with hot air balloons and ending with cryotop open vitrification systems. Since gametes were the first cells to be frozen and the last to successfully thaw, their story provides an overview of the development of the science of cryopreservation.

Keywords: cryopreservation, oocytes, sperm, embryos, vitrification, slow freezing

1. Introduction

The advent of embryo cryopreservation 25 years ago [1, 2] was revolutionary as well as critical in reproductive medicine. Cryopreservation and storage of gametes and embryos provide cost and procurement efficiencies in treatment options, which otherwise would be inaccessible without substantial financial resources.

Cryopreservation maximizes fertility potential per retrieval cycle, providing a repository for individual's gametes/embryos that may not exist elsewhere. Thus reproductive potential is not limited to reproductive years, but available as one manifests the need. Use of this technology has paved the way toward single embryo transfer (SET), thereby decreasing the risk of multiple gestation pregnancy and associated health risks.
Additional application has unfolded to address needs associated with convenience and transport for the purposes of using gestational carriers, family planning, travel, and using donor oocytes/embryos in support of non-fertile couples in conception and familial continuity.

Advances in cryopreservation are paralleled and even rooted in key developments in assisted reproductive technologies (ARTs). Historically, advanced culture techniques, complex culture media, and supplements specific to the support of late stage embryonic development attempted to mimic in vivo conditions [3]. Together with carefully controlled culture environment, blastulation rates have increased exponentially from decades prior [4, 5].

In addition to these supportive measures, newer technologies including time lapse morphological assessment allow for the development of observation-based algorithms as prognostic indicators of embryonic competence. Collectively these factors form the paradigm in increased opportunities for cryopreservation and subsequent improved selection criteria for single embryo transfer. In fact, this is the current trend.

The goal of healthy ART outcome should not be clouded by commercial success rates, and while not a mandate, single embryo transfer (SET) is now widely accepted as the default position in good prognosis patients. According to a meta-analysis of randomized controlled trials, SET versus double embryo transfer (DET) in a fresh In Vitro Fertilization (IVF) treatment cycle resulted in a lower pregnancy rate, lower rate of multiple births and preterm birth, and better odds of delivering a term singleton live birth. The reported SET versus DET pregnancy rate disparity is virtually eliminated with an additional frozen SET cycle [6]. However, the immediate consequence is that in order to achieve similar results, the patient may require/need multiple cycles of embryo transfers.

Approximately 30–50% of embryos make it to a blastocyst stage. The average number of embryos frozen per IVF cycle is age dependent: women of age >35 have fewer than two embryos frozen, while younger women responding better to ovarian stimulation and producing more eggs, result in a higher likelihood of having excess embryos available for freezing [7]. The Department of Health and Human Services estimates that in 2015 more than 600K frozen embryos were stored nationwide in the USA [8]. Figures for the year 2012 released by the Human Fertilization and Embryo Authority (UK) report that of the >3.5 million embryos created since 1991, 840K (24%) were cryopreserved for clinical use. In Canada, it is estimated that >60K frozen embryos are in storage [9]. The current trend of freezing all the embryos with no fresh embryo transfer [10] in IVF treatment would suggest these numbers will likely grow much faster. Despite this uncertainty, these values underscore the importance of cryopreservation technologies.

2. Historical perspectives

Since the discovery of the tissue preserving effect of low temperatures, it has been an aspiration to maintain the vitality of human tissues by freezing. Soon after early attempts at tissue cryopreservation had failed, the main hurdle in achieving this goal became apparent; water
crystallizes upon freezing and the sharp edges of the crystals disrupt cell membranes and destroy the cells. From that point onward the history of the study of cryopreservation is a description of the relentless attempts to prevent intracellular crystallization at subzero temperatures. This journey has been even more elusive for those trying to cryopreserve oocytes.

It has been known for many centuries that subzero temperatures can preserve tissue, mostly following the accidental discoveries of intact ancient animals frozen in ice for many years. However, from studying patients inflicted by frostbite it was clear that freezing may also cause tissue destruction [11].

2.1. Early days

Interestingly, the cells that were chosen for the early studies on the effects of freezing and thawing on cell viability were gametes. Spermatozoa were chosen due to their availability, small size, and their motility, which was a simple marker of viability. Oocytes were chosen since their size is large enough to allow for simple morphological evaluation.

Spallanzani, in 1776, was the first to study the effect of subzero temperatures on stallion semen and silkworm eggs [12]. He discovered that when thawed the sperm regained its motility. This was the first report of a successful sperm freeze thaw. However, it was not until 1938 that Jahnel, while searching for a remedy for syphilis, found that sperm cooled to −79°C for 40 days regained some of its motility upon thaw and reinvigorated efforts to devise an efficient freezing method [13].

Very early in the study of cryopreservation two opposing schools of thought had been developed in parallel; slow freezing with gradual desiccation of the cell and ultra-rapid freezing of small volumes also known as vitrification.

2.2. Vitrification

The term vitrification originates from the Latin word “vitreum” (glass) that describes the transformation of a substance into a non-crystalline amorphous solid. The process commonly involves rapid cooling of a liquid so that it passes through the glass transition to form a vitrified solid.

The French, Joseph Luis Gay-Lusac, in 1804 ascended in a hot air balloon and noticed that the water drops (size around 8–10 μm) in the clouds are not frozen despite the sub-zero temperatures (−5°C) [14]. He later went on to find that water can be subcooled to −12°C when contained in small tubes [15]. In 1858, Albert J.R. Mousson, a Swiss physicist, had found that the smaller the sprayed water droplets (diameter < 0.5 mm), the longer they can stay subcooled [16]. The liquid state of the water droplets in subzero temperatures is attributed to rapid cooling forming a non-crystalized solid. Luyet in his book coined the term “crystallization zone”, which relates to the range of temperatures in which crystals form. He concluded that in order to avoid crystallization one must traverse this zone faster than the time it takes to form crystals [14]. It later became apparent that the small volumes are essential for achieving the high cooling
velocity since it is proportional to the ratio of surface area to volume. It was also noticed that different solutions of similar volumes cool at different rates. The concentration of the solutes was shown to affect the “thermal mass (heat capacity)”, which represents the ability of a substance to store thermal energy and is inversely proportional to the velocity of cooling. Pure water has a very high heat capacity and therefore is almost impossible to cool fast enough to exceed crystal growth unless very small volumes are used [17]. Walton and Judd measured the velocity of ice crystal growth in undercooled water and found it to be 65 mm/s, thereby providing the basis for calculation of the necessary speed of cooling to avoid crystallization [18]. Fahy and Rall found that in order to vitrify pure water a cooling rate of $100 \times 10^6\,^\circ\text{C/min}$ is necessary. Since such cooling velocities are not feasible, to achieve vitrification one needs to increase to solute concentration (cryoprotectants) and reduce the solutions volume. This is the current basis for clinically applied vitrification [19].

The work done by Luyet and Hodapp with colloid solutions (gelatin or agar) had led to the first successful vitrification of sperm [20]. They were able to show that the water content of the solutions was the determining factor on whether vitrification was achievable. With a 50% gelatin solution they were able to vitrify layers of 0.3 mm; however, when using a 10% gelatin solution, they could only vitrify a layer a few microns thick [15]. The drawback of these concentrated solutions was their cell toxicity. Therefore, there is a need to balance the solutions’ cooling velocity on one hand and the solutes’ cell toxicity on the other. It was not until 1985 that an ice-free cryoprotectant system was developed that could attain vitrification and achieve live birth for vitrified thawed mouse embryos [21–23]. Others were able to achieve high post-thaw survival rates with vitrified hamster oocytes, as well as with immature and mature murine oocytes [24–26].

Attempts to simplify the vitrification solution using a high concentration of a single cryoprotectant (dimethyl sulphoxide, DMSO) were initially successful for mouse and hamster oocytes, but later proven to be toxic causing aneuploidy, malformations and a high rate of miscarriage [27–30]. These publications halted further attempts to vitrify oocytes and focused the attention on the alternative, slow freezing.

2.3. Slow freezing

Parkes et al., in 1945 discovered, accidentally, that the rate of cooling is associated with post-thaw survival rate. They found that large containers used for freezing semen, in which, due to the large volume, the rate of cooling is slower, gave the best post-thaw motilities [31]. Hence, opposite to vitrification, slower cooling rates were associated with better cell vitality. The explanation for this observation was the physical principle of osmotic dehydration; as ice crystals formed in the suspending solution, the relative concentration of solutes in the unfrozen fraction of the solution increased and thereby increasing its osmolality. The cells suspended in the solution will respond to the higher osmolality by losing water. Therefore, slower cooling rates are associated with greater cellular dehydration and reduced risk of intracellular ice crystals formation, leading to a better post-thaw viability. Further work by Chang on rabbit ova recognized the importance of cooling rate on the maintenance of viabil-
ity, the artificial activation of oocytes by rapid cooling and the achievement of litters from embryos stored at 0°C [32, 33].

Mazur was the first to describe cell-specific optimal cooling rates [34]. He was able to formulate an equation that was based on the rate at which the cells responded to osmotic pressure (hydraulic conductivity) and the effect of temperature on the movement of water across the cell membrane (temperature coefficient of water permeability) and could therefore predict cell-specific optimal cooling rates. Leibo et al. constructed a graph describing cooling rate against survival rate [35]. He showed over a 1000-fold difference in the optimal rate of cooling between oocytes (0.3°C/min) and erythrocytes (1000°C/min) due to the oocytes low hydraulic conductivity and high temperature coefficient of water permeability.

In order to guarantee ice crystal formation in the cryo-solution that will ensure the increase in its osmolality and cell desiccation, a process of ice crystal seeding was developed [36].

Two groups worked in the early 1970s independently on slow freezing of embryos. Both groups had published in 1972 the first survival of murine embryos after slow freezing [1, 2] and live offspring [1]. Both groups used slow freezing and a cryosolution containing 1 mol/l of DMSO. Wilmut and Rowson published in 1973 on the first farm animal (a calf) to be born after a transfer of a frozen thawed embryo [37].

With the advent of clinical use of IVF at the beginning of the 1980s a significant effort was made to optimize human embryo freezing in order to increase the efficiency of IVF by storing excess oocytes and embryos. This came to fruition with the first pregnancies and birth from frozen thawed embryos that were frozen using slow freezing and DMSO [38, 39]. Soon after, these were followed by publications reporting on human live births subsequent to the use of other cryoprotectants such as propanediol and sucrose. These methods proved to be more reliable and more widely adopted [40–42]. The success of human embryo freezing ignited a public debate on the ethics of embryo freezing. These ethical dilemmas prompted research on the possibility of clinical application of oocyte freezing, which was deemed to be more ethically acceptable.

In 1986, Chen reported a twin pregnancy following slow freezing of human oocytes with DMSO [43]. Chen reported high post-thaw survival, fertilization and development rates of oocytes frozen with this technique, however, attempts to replicate his success by others failed [44–46]. Furthermore, in line with the observation in animals, a high proportion of thawed human oocytes resulted in polyploid embryos [44, 47]. The poor results of oocyte cryopreservation relative to the success with embryo freezing brought clinical oocyte freezing to a halt.

2.4. Cryoprotectants

A cryoprotectant is a substance used to protect biological tissue from freezing damage. Arctic and Antarctic insects, fish and amphibians create cryoprotectants (antifreeze compounds and antifreeze proteins) in their bodies to minimize freezing damage during cold winter periods. Their exact mechanism of action is yet not fully understood. 1949, Polge et al., once again by accident, discovered the cryoprotective effects of glycerol [48]. They found that the glycerol solution protects from crystal formation during freezing by cellular dehydration. This
discovery had led to successful semen storage of farm animals in 1953 and human sperm in 1964 [49]. Cryoprotectants are divided into two groups: intracellular (such as DMSO, glycerol and propylene glycol) and extracellular (such as sucrose, polyvinyl pyrrolidone, hydroxyethyl starch and dextran). One of their modes of action is lowering of the freezing point of the solution. Use of an intracellular cryoprotectant such as DMSO will prevent intracellular ice formation, while the seeding drives extracellular crystallization and the resulting increase in the osmolality of the cryosolution leading to cellular dehydration [50]. Cryoprotectants may also protect the cell membrane from the drastic changes occurring during the transition between fluid and solid states. Cryoprotectants may, however, be toxic to the cells, therefore over the years a relentless search for less toxic and efficient cryoprotectants ensued as well as for protocols combining several cryoprotectants in order to reduce individual solute concentration and the associated cell toxicity.

2.5. The return of vitrification

In 1985, Rall and Fahy were able to successfully vitrify a strew of a relatively large volume (0.25 ml) containing mouse embryos with a mixture of DMSO, acetamide and polyethylene glycol that was snap frozen in liquid nitrogen [22]. Shortly after the publication on the first births from slow-frozen oocytes, the first pregnancy and live birth from vitrified oocytes was published [51]. Developments that led to this breakthrough included the understanding that the length of exposure of the cells to the vitrification solution should be minimized to reduce toxicity [52], as well as replacing DMSO with ethylene glycol and mixtures of several cryoprotectants [53]. These changes brought about successful vitrification of bovine, murine as well as human oocytes with multiple live births [54–56]. These advancements were accompanied by the development of appropriate carriers to facilitate rapid cooling such as open-pulled straws [57], electron microscopy grids [55] and nylon loops [58]. By the end of the 1990s, vitrification was applied to human embryos achieving live births with both blastocyst and cleavage stage embryos [59, 60]. The vitrification of oocytes, despite these developments, was lagging until the introduction of appropriate carriers. The development of Cryotop in Japan was the breakthrough that allowed the adoption of oocyte vitrification into routine clinical practice. It allowed for an extremely rapid cooling rate that was facilitated by a minimal volume and resulted in a very high survival rate and live births [61–63]. A few methodological modifications that were made to the kit simplified its use and supported its wide spread distribution. Two large comparative studies established its lead role in oocyte cryopreservation [64, 65].

3. Cryopreservation protocols

Cryopreservation protocols are numerous and optimized for the cell type being frozen. These protocols fall into two major categories: equilibrium freezing and non-equilibrium freezing. Critical to either process is the partial elimination of water in the cell to avoid ice crystal damage. This chapter focuses on the two main methodologies employed in freezing reproductive cells.
Conventional slow freeze methodology is characterized as equilibrium freezing. Cells are pre-equilibrated in cryo-protecting agent (CPA) and gradual temperature depression in a controlled rate freezer optimized for the cell type being frozen is initiated. As super-cooling is achieved, a manual seeding process is required to initiate ice crystal formation outside of the cell. Continuous equilibrium of the cells is achieved by increasing the osmotic gradient initiated from the increasing proportion of ice in the surrounding medium. As a result, the cell dehydrates, thereby lowering the freezing point of the cell. At a point, with the cell being almost devoid of water, ice crystal formation is negligible and freezing occurs. This method is viewed as “forgiving” in practice, given increased pre-equilibration exposure times to relatively low concentrations of CPAs and as such promotes efficiencies by accommodating batch freezing of multiple samples.

As water excursion depends on the rate of cooling, risk can be mitigated. Rapid cooling can trap excess water inside the cell, leading to the formation of intracellular ice crystals, whereas slow cooling promotes high intracellular solute concentration by severe volume shrinkage. Both have deleterious effects on the cell.

In addition, cells that are cooled slowly are susceptible to cryo damage. Mechanisms of cryo damage include upregulation of heat and cold-shock proteins in response to cold temperatures [66, 67]. Induction of apoptosis [68], a mechanism of cryo damage, may not be immediately visible but delayed for several hours as cells try to recover from such cryopreservation stresses [69].

Largely contrasting this technology, non-equilibrium freezing was developed to overcome the many shortfalls of slow freeze methodology. Cells exposed to (usually 7.5–10%) lower strength cryoprotectant solution undergo dehydration and permeation with CPAs. Subsequent (30–60 s) rapid exposure to higher (40%) hyperosmotic solution results in complete dehydration of the cell. The sample is plunged directly into liquid nitrogen. This avoids deleterious ice crystal formation with high concentrations of CPAs and supremely rapid cooling rates (15,000–30,000°C/min). The extreme elevation in solution viscosity promotes solidification or a glass-like, suspended state as opposed to crystallization. This method requires high level manual dexterity, is labor-intensive, while offering decreased incubation times can consistently and reliably accommodate only one sample being frozen at a time. Highly skilled technicians may stagger multiple samples as per protocol, yet this leaves success rates subject to human variation. As a benefit, this method is easily introduced without the need of expensive equipment. Though unconventional, an added benefit is a comparable survival after repeat vitrification and warming of the same sample [70, 71].

Recent technological advancement into this freeze methodology is semi-automated vitrification. This platform allows simultaneous cryopreservation of up to four embryos in a closed system, addressing the long-term debate of cross-contamination in shared liquid nitrogen. Non-clinical preliminary data comparing GAVIT™ (Genea BIOMEDX) to commercial manual method in mouse and donated human blastocyst stage embryos is promising [72]. Further clinical evaluation and advancement to oocytes and all embryonic stages is under way. Given success of this platform, process standardization demonstrating improved ART efficiencies
may implore the few labs resistant to convert to vitrification technologies to reconsider; albeit cost considerations excluded.

4. Cryo-protecting agents

The biophysical changes that take place during temperature depression, extracellular ice formation and the creation of a potentially lethal hyperosmotic environment leads to further dehydration of the cell. Termed “freeze dehydration” this effect is implicated in organelle disruption and loss, as well as fusion or changes in cell membranes [73]. At more depressed temperatures, the viscosity of the highly concentrated solution inside and outside of the cells remains as a glassy matrix, which is relatively stable for long-term preservation.

Additional cell damage may be caused by intracellular ice formation, which is more prominent during inappropriate rapid cooling as time might be insufficient for water to move down the chemical potential gradient established by the difference in solution concentrations between the two sides of the membrane. If a cell can be cooled to a ‘glass region’, under conditions inhibiting ice crystal formation, successful preservation can be achieved. This is termed vitrification as previously described.

While the methodologies of slow freeze and vitrification technologies may vary within clinics, the underlying principles are fundamentally the same. Combinations of reagents provide a delicate balance between the protective and toxic effects of CPAs aiming to maintain the functional capacity of organelles, while avoiding the two main causes of cell death associated with cryopreservation: solute toxicity [74] and ice formation [34].

CPAs are generally small molecular weight solutes with high aqueous solubility, bearing polar groups that interact weakly with water [75]. CPAs act to (i) moderate the effects of the rising solute (electrolyte) concentrations in the intra and extracellular environment, (ii) stabilize intracellular protein structure and (iii) provide increasing viscosity during temperature depression that may kinetically slow or inhibit ice crystal formation. There has been a myriad of solutes that exhibit some CPA activity: amino acids (e.g. alanine, glycine, proline), amides (e.g. acetamide, formamide), diols (e.g. 1,2-propanediol, ethanediol), sugars (glucose, lactose, ribose raffinose, dextrans, hydroxyethyl starch), large polymers (polyethylene glycol, polyvinylpyrrolidone, polyvinyl alcohol) and alcohols (methanol) although some at low efficiencies [76, 77]. Modern cryopreservation protocols are largely based on few reported CPAs that are considered moderately or very effective in preserving nucleated cells [78].

CPAs can also be deleterious as osmotic effects resulting in too rapid excursion of water across a cell membrane can cause membrane rupture. Similarly, hydrogen bonding may disrupt the hydration shell around macromolecules. Chemical toxicity of high concentrations of CPAs is another cause for concern, largely in vitrification methods, the nature of which is not entirely understood [79, 80]. The suggested protein denaturation effect, even DNA conformational changes and fragmentation have been debated [80, 81]. Finally, CPAs have been shown to alter cytoskeletal components in mammalian oocytes, particularly filaments and the meiotic spindle.
The reversibility of this disruption is concentration and CPA dependent and varies amongst species [54, 83–85]. CPA reagents are classified as permeating, non-permeating and stabilizing.

Permeating cryoprotectants (e.g., glycerol, propane diol, dimethyl sulphoxide or ethylene glycol) cross the cell membrane through an osmotic gradient displacing water. These agents act to reduce ice crystalization and reduce cell dehydration but are toxic at higher concentrations. More specifically, in vitrification, the role is to completely inhibit ice formation. Permeating cryoprotectants also stabilize intracellular solutes which otherwise would be lethal in a hyperosmotic state. A similar dehydration effect is mimicked in the extracellular environment with temperature reduction promoting further dehydration. Dehydration is dependent upon the rate of temperature depression and limited by the cell permeability to water [35, 86].

Non-permeating cryoprotectants are generally higher molecular weight polymers (e.g., sucrose, polyethylene glycol, polyvinylpyrrolidone, ficoll, dextran). These agents mimic the dehydration mechanism of penetrating cryoprotectants but remain outside of the cell. Generally, less toxic than penetrating cryoprotectants at the same concentration a successful vitrification strategy is to create a mixture of non-toxic level of permeating cryoprotectant(s) by the addition of non-permeating cryoprotectant. Interestingly as toxic effects of permeating cryoprotectants have been shown to be at least somewhat biochemical and unique in action, total molarity of a mixture may not be a reliable indicator of cryosolution embryotoxicity [53].

For application, CPAs are contained within a “carrier” solution that will help keep cells alive during cryopreservation. They act to provide osmotic and physiological support and avoid deviations from isotonicity, which could result in dehydration or swelling and burst of cells. It is important to note that the efficacies of carrier solutions are unpredictable and vary based on the individual or mixture of CPAs present [19].

Concern of long-term putative effects of these chemicals has paved the way for investigation into and application of extracted or modified natural biological agents, which are evolutionarily found in extreme environments. While conventional cryoprotectants interact with water, the application of uniquely acting, naturally based complementary agents, is an attractive proposition.

Biological anti-freeze molecules of sorts (e.g., cyclohexanediol and polyvinyl alcohol) [87, 88] selectively adsorb to the surface of ice crystals inhibiting ice crystal growth and ice re-crystallization. Ice blockers, including polyvinyl alcohol and polyglycerol (i.e., X-1000 and Z-1000) [89], specific to vitrification solutions act to prevent ice crystal formation. Together, these agents may also play a role in potentially damaging re-crystallization of ice growth during warming [90].

Ice-nucleating agents act to achieve deliberate ice growth in defined sites. This phenomenon is largely important in intact tissues and organs where integrated cell cooperation is essential to normal function. Unlike small tissue sections, organs are unable to effectively absorb cryoprotectant solution by simply soaking in a solution. In the case of whole organs, introduction of cryoprotectants by perfusion (through existing vasculature) is necessary. As
perfusion, due to capillary distribution and time requirement of CPA diffusion, may not be equivalent throughout a larger structure, random ice crystal growth can be lethal simply by mechanical disruption. By achieving deliberate ice growth in specific sites, the damaging effects of super-cooling and likewise intracellular ice formation can be mediated and potentially avoided.

Lastly as organisms synthesize solutes and metabolites in response to cold survival strategies (e.g., trehalose [91], glycerol [92], polyols [93, 94]), understanding how biological structures interact with these mixtures may offer added benefits to current freezing regimes.

5. Slow freezing versus vitrification

To compare slow freeze technology to vitrification, the efficiencies of cryopreservation must take into consideration several factors: (i) disparities in embryo quality between the “best” freshly transferred embryo and subsequent frozen embryos; (ii) lab-specific criteria for embryo cryopreservation may foster higher implantation rates by discarding some reproductive potential of lesser quality embryos; (iii) cryo-survival should be defined in terms of complete or partial survival and (iv) post-thaw selection criteria for the transfer of cryopreserved embryos.

Therefore, a randomized control study comparing slow freeze and vitrification protocols would require standardization of protocol under optimal conditions with sibling specimens. To add more complexity in comparative analysis are individual case variations, including age discrepancies, effects of hormonal stimulation, supplementation, and endometrial priming, all of which must be taken into account.

Despite these challenges in reviewing evidence based data, as a generality the technique of vitrification has been preferentially adopted over the more traditional approach of slow cooling.

Vitrification of oocytes [95, 96] and embryos of all stages has been shown to be superior to slow freezing [6]. A large amount of clinical data suggest that one of the major consequences of the intracellular damage to embryos from slow “conventional” freezing is decreased survival as well as diminished implantation potential and outcomes when compared to vitrification [97–99]. Despite lower survival rate, there are some data that suggest similar if not improved implantation rate with slow freeze technology with fully intact good quality day 3 embryos [100]. It is known that embryo survival is not an all or none phenomenon, and therefore, comparison should be stratified on a similar quality basis.

The lack of homogeneity in some reported data is anticipated and may be due to laboratory practice or clinic-specific differences, as with other ART procedures.

As the majority of early vitrification was with cleavage stage embryos, it was recognized that failure to develop to an expanded blastocyst stage was largely a consequence of chromosomal compromise and inability to lead to a successful outcome. A bifurcated movement to karyotype
embryos through pre-gestational genetic screening and cryopreserve blastocysts rather than their cleavage stage counterparts is advantageous in the identification of embryo competence and in reducing risk of miscarriage and chromosomal defects [101, 102]. This practice is important in that common morphological parameters of blastocyst scoring are not related to chromosomal status [101] and particularly for women of advancing maternal age [103].

Over the past decade, with vitrification, it has become a standard of practice to expect a post-thaw survival of >90% [104, 105] and implantation and pregnancy potentials marginally equivalent to fresh embryos [106–109].

Studies reveal longer gestational periods and heavier and healthier babies born as a result of frozen embryos compared to their fresh counterparts [110]. It is not clear whether this is related to cryotechnique or maternal factors [111]; but confirms the value of vitrification.

Reports of increased post-revitalization implantation potential over fresh counterparts [112] may be a consequence of staggered embryo transfers in which embryo procurement and implantation are performed in separate cycles. In this scenario, optimal synchronization and endometrial receptivity may be achieved in contrast to the impact of high levels of hormones present in harvesting cycles [113].

Success rates with vitrification supporting this revolutionary technology are not limited to gametes and embryos; however, extend to gonadal tissues, and non-reproductive applications including cornea [114], brain [115], heart [116], vascular [117] tissues, and cartilage [118]. The permeation of larger tissue sections and even whole organs, e.g., ovaries [119–122], shows promise in transplantation. Efficacy and potential of vitrification technologies as demonstrated through such a broad spectrum of applications justify its utility and warrant further investigation into enhanced cryopreservation potential.

Though the safety and efficacy of cryopreservation technologies is largely supported by current success rate, however, some degree of uncertainty and challenge remains.

Human embryonic stem cells (hESCs) have been established from isolated inner cell masses and more recently from single blastomeres obtained from cell stage embryos [123, 124]. The systemic reporting of chromosomal abnormalities and the recurrent manner in which they appear highlights the importance of understanding the underlying source [125]. In part, these changes are ascribed to the cryopreservation method, “adaptive pressure to” or “lab-specific variations in” cell culture [126–128] or are simply inherent to the cell itself [129–131]. Similarly, IVF embryos may be associated with increased risk of epigenetic abnormalities. At least in the case of hESCs, for cell line stability and quality assurance, the safety and efficacies of different cytogenetic methodologies have been assessed as they relate to genomic integrity and chromosomal stability [132]. As chromosomal instability is largely related to carcinogenesis, similar investigation into embryo cryopreservation methods may provide insight into the quality and safety of established cryopreservation protocols. Understandably, in as much as embryonic culture periods are acute in length (as compared to hESCs), still, the long-term effects of even small epigenetic changes are unknown.
6. Trends in embryo storage

Worldwide, 1.5 million ART cycles are performed each year and this number continues to rise [133]. Up to a third of patients who undergo IVF have supernumerary embryos that are cryogenically stored. A case is made for the perpetually increasing reserve of embryos and more importantly those of patients lost to follow up. For reasons undescribed here, these embryos are termed “abandoned”.

At least in the USA, estimates provide that there are up to 1.4 million abandoned embryos [134] and though unknown it is safe to assume this number dwarfs the combined worldwide total. Even with permitting signed patient consents, clinics are hesitant to act and discard unclaimed embryos, largely because of the lack of regulatory guidance [135], leaving clinics vulnerable to unanticipated legal ramifications. Recently, several solutions have been proposed.

Consideration of imposing strict time limits on storage, outlining relevant responsibilities of fertility clinics and patients, and clarifying absolute guidelines related to unrestricted utility of embryos in terms of donation for third party, teaching or research use must be defined within a strict legal framework. Above all adequate long-term storage facilities are lacking.

A somewhat limited solution supporting non-fertile couples in conception and familial continuity is embryo donation. It is noteworthy to mention that cryopreservation has indirectly found a place in a larger market in terms of the transport and exchange of all types of biological samples. Initial concern over the sensitivity of microscopic volumes employed in vitrification to potentially shifting conditions during shipment has largely been overcome by advanced vapor shipping dewars, temperature monitoring, and precautionary handling.

Specific to embryo donation, ethical and genetic consequences of donation to related and unrelated parties, including offspring, must be carefully considered. In addition, given lack of restrictions on storage time limits, decades old donated embryos, thus far, may provide relatively antiquated genetics which ultimately interfere with the natural evolution of the population.

Similarly, in the case of multiple embryos donated to different parties, even a marginal risk of unsuspecting, related siblings, procreating by chance may have devastating consequences for the developing fetus. This is even more likely if embryos are donated to a clinic within the same geographical region. As such, advanced screening methods prior to procreation and/or pre-natal testing may be of benefit.

Contrary to this line of thought, in a separate context, in animal and cell line research laboratory settings, recessive gene expression and cell line mutations confer an advantage for study purposes and cryopreservation provides cell line stability against undesired changes induced by adverse events or long-term culture [136].
7. The thawing process

Given the worldwide exchange of reproductive cells, which is practically commonplace, IVF labs generally house a repository of embryos cryopreserved with various methodologies and formulations. The core responsibility of clinics is to ensure safety and best practice outcomes, and this requires staff training and laboratory access to a myriad of cryopreservation formulations and techniques.

In actual practice, IVF laboratories may permit revitalization of embryos using readily available thaw solutions/protocols. This is in contrast to purchasing the specific formulations matched to the cryopreservation solutions that the cells were frozen in. The impact of such mix and match freeze/thaw practices on embryos/oocytes is largely unexplored and may be quite significant given the unique actions of CPAs and unpredictability of carrier solutions as previously described. Observed success may be due to the robustness of certain reproductive cells and this may provide some artificial confidence in this regard. Prior to such validation cross-use of combinations of cryoprotectant solutions should be approached with caution.

The development of cryopreservation techniques has had immense impact across many disciplines, most notably reproductive medicine. While significant advances have been made, further advances are needed in the changing landscape of fertility. Cryopreservation has allowed IVF to evolve into a medical procedure that is efficient, safe, readily accessible, and relatively affordable.

8. Oocyte cryopreservation

While the cryopreservation of cleavage stage embryos has been a proven method for 20 years, more recently, all pre-implantation stages of embryos, including oocytes, were shown to be successfully frozen. Recent resurgence into oocyte freezing makes this application a noteworthy aspect of cryopreservation as it applies to clinical reproductive medicine.

Oocyte cryopreservation was initially focused on fertility preservation of females undergoing gonadotoxic treatments. Further application circumvented restrictions imposed on embryo freezing, which were largely a consequence of ethical, moral and legal boundaries barring embryo cryopreservation. Similarly, viewed as insurance for individuals of advanced reproductive age, oocyte banking for non-medical purposes, otherwise termed social egg freezing, supports future fertility potential in an increasingly growing group.

Women delaying childbearing until age 35 are a growing group. A trend citing increasing pregnancy rates in women in their thirties and forties is attributable to first births rather than subsequent birth, and is more pronounced in women of higher education. Psychosocial issues supporting delayed conception include parental financial stability, decreased marital discord, and increased behavioral and cognitive test scores of offspring. These are compounded with increased risk to mother and fetus including prenatal care requirements, fetal distress, preterm
birth, neonatal intensive care admission, and morbidity to women of advanced maternal age [137–140].

In the USA, first birth rates for women aged 35–39 generally increased from the mid-1970s to 2012, while steady increases for women aged 40–44 began later in the early-1980s [141]. While the trend is not as dramatic in Canada, over the past two decades the average age for births to first time mothers in 2011 had risen to over 30, the oldest age on record. The year 2015 marks the first year that the average age of British women having children has passed 30 for the first time. More women over 35 are now first time mothers than the under 25s in marked contrast with the pattern as recently as 5 years ago [142]. This trend of women waiting longer to have children is consistent across race and ethnicity [143].

The high success rate with egg donation confirms that egg quality, rather than uterine factors associated with age, is the primary barrier to pregnancy in older women [144]. Progressively by early 40s to age 43, the chance of becoming pregnant through IVF exponentially decreases to near 5% and by age 45, the use of donor eggs is the only reasonable alternative. Despite these dismal outcomes, many couples or single women in their early 40s will choose to accept the lower chance of becoming pregnant and use their own eggs.

Egg freezing for preservation of fertility shows promise for success. Age remains a problem faced by women interested in using elective egg freezing. As the age of women undergoing egg freezing increases, the outcomes of assisted reproductive technology cycles utilizing their frozen eggs become less favorable.

A non-discriminatory cost-basis analysis of otherwise healthy 25-year-old women foregoing fertility until 40 revealed oocyte cryopreservation as cost-effective if IVF cycles exceeded $22,000 [145]. A hypothetical decision tree surrounding elective oocyte cryopreservation with procreation attempt at 3, 5, or 7 years after initial decision reveals greatest improvement in probability of live birth occurring if oocytes are banked at 37; noting an additional $29,000 cost per live birth in this group otherwise. However, highest probability of live birth was achieved with oocyte cryopreservation <34 years of age with no cost benefit observed for 25–30 year old age range delaying pregnancy to 40 years of age [146]. A separate analysis cites 36 years of age as the upper cut-point of non-donor oocyte cryopreservation for “success versus failure”, with vitrification technology superior to slow freeze methodology [147]. Although an absolute value may not be identified for childbearing based on individual factors and resources, success probabilities at 42 years of age declining to <5% may safely advocate against oocyte cryopreservation for women >42 years of age. These models may not be reflective of all patient populations including elective, infertile, and cancer patients pursuing oocyte freezing, and individualized analyses may provide a more discriminatory framework.

Fertility preservation for (non-)medical reasons is controversial and becoming increasingly common [148]. Ethicists have upheld women’s reproductive freedom while pointing out that the so-called social freezing merely postpones social problems, rather than solving them. The real challenge is two-fold. There is a clear lack of information and inadequate regulation.
Success rates of frozen oocytes vary among clinics, and this is reflected in conflicting statistics and the lack of a scientifically sound framework for patient education. Reports are as low as 10 and as high as 60% success rate. Access to data in establishing clinic-specific reliable predictors is lacking as individual clinics are limited in critical mass numbers to effectively determine the feasibility of this relatively new technological offering.

Though it has been reported that rates of survival, fertilization, and implantation of “young” cryopreserved oocytes fertilized with ICSI are comparable to those of fresh oocytes [149], limits imposed by nature are a constraint lending to advance planning as egg quality decline begins at age 30 and increases significantly after age 35. With respect to aging, this technique of suspending the biological clock aiming to reconcile “personal and professional timelines” must align with current limits of scientific technologies and should be critically discussed on a case-by-case basis. Critics warn of bio-objectification [150], where women could be even considered unaware victims of “a commercially exploitative context, thus undermining rather than expanding reproductive autonomy” [151].

An acceptable degree of success allowed regulatory bodies providing ART oversight including ASRM, CFAS to lift the experimental designation of oocyte cryopreservation; albeit with limited guidance. Still the majority of health care companies have yet to support elective oocyte cryopreservation for purposes other than medical necessity. Select companies are leaders in providing paid benefits for social egg freezing [152]. Mollifying procreation with career casts light on the authenticity of this offering as the employment organization benefits by not prioritizing the adjustment of the social framework of the employment organization to incorporate motherhood. Rather opinions suggest these companies seek “a productive, not a reproductive, workforce” [153].

This controversial interaction between technology and society shifts the attention from a medical procedure to a social phenomenon, which needs to be analyzed within a regulatory framework of bioethics, biopolicy, bioeconomy, and biolaw [154] with unbiased, validated reporting. In this regard, men and women can make educated choices in life decisions to harmonize personal, professional needs [155], and pregnancy.

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References


[14] Luyet BJ, Gehenio PM. Life and Death at Low Temperatures. Luyet BJ, editor: Nor- 
mandy, Mo., Biodynamica; 1940.


[16] Mousson A. A few facts concerning the melting and freezing of the water. An Pfyaft 
1858;105:161–74.

[17] Burton EF, Oliver WF. The crystal structure of ice at low temperatures. Proc Roy Soc 


[21] Fahy GM, Levy DI, Ali SE. Some emerging principles underlying the physical proper-

[22] Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by 

504.

[24] Critser JK, Arneson BW, Aaker DV, Ball GD. Cryopreservation of hamster oocytes: 
effects of vitrification or freezing on human sperm penetration of zona-free hamster 


[26] Van Blerkom J. Maturation at high frequency of germinal-vesicle-stage mouse oocytes 
after cryopreservation: alterations in cytoplasmic, nuclear, nucleolar and chromosomai 


[40] Lassalle B, Testart J, Renard JP. Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. Fertil Steril 1985 Nov;44(5):645–51.


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Quan GB, Wu SS, Lan ZG, Yang HY, Shao QY, Hong QH. The effects of 1,4-cyclohexa-

Wowk B, Fahy GM. Inhibition of bacterial ice nucleation by polyglycerol polymers.

Badrzadeh H, Najmabadi S, Paymani R, Macaso T, Azadbadi Z, Ahmady A. Super cool
X-1000 and Super cool Z-1000, two ice blockers, and their effect on vitrification/

O’Neil L, Paynter SJ, Fuller BJ, Shaw RW, DeVries AL. Vitrification of mature mouse
oocytes in a 6 M Me2SO solution supplemented with antifreeze glycoproteins: the effect

Block W. Water or ice?--the challenge for invertebrate cold survival. Sci Prog 2003;86(Pt

Ring RA, Tesar D. Adaptations to cold in Canadian Arctic insects. Cryobiology 1981
Apr;18(2):199–211.

Hochachka P, Somero G. Biochemical Adaptation. Mechanism and Process in Physio-
2002.

Storey KB, Baust JG, Buescher P. Determination of water "bound" by soluble subcellular
components during low-temperature acclimation in the gall fly larva, Eurosta solida-

Cobo A, Diaz C. Clinical application of oocyte vitrification: a systematic review and

cryopreservation with slow freezing versus vitrification. Results from the National

vs. slow cooling protocol using embryos cryopreserved in the 5th or 6th day after oocyte

Li Z, Wang YA, Ledger W, Edgar DH, Sullivan EA. Clinical outcomes following
cryopreservation of blastocysts by vitrification or slow freezing: a population-based

Wang XL, Zhang X, Qin YQ, Hao DY, Shi HR. Outcomes of day 3 embryo transfer with
29(9):883–9.

Zhu HY, Xue YM, Yang LY, Jiang LY, Ling C, Tong XM, et al. Slow freezing should not
be totally substituted by vitrification when applied to day 3 embryo cryopreservation:


Matthews TJ, Hamilton BE. First births to older women continue to rise. NCHS Data Brief 2014 May(152):1–8.
[142] Bingham J. Middle age mothers overtake the young in Britain's maternity wards. The Telegraph. 2015.
[143] Bichell RE. Average Age Of First-Time Moms Keeps Climbing In The U.S. NPR. 2016.