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A Review Article on Cryopreservation of Human Embryo - Methods, Timing, and Other Considerations for Optimizing Embryo Cryopreservation Program

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ABSTRACT

The contribution of embryo cryopreservation to the birth rate per in vitro fertilization cycle has escalated from a rare subsidy to a vital tool that is called upon to augment the cycle outcome. Embryology laboratories must identify the embryo stage, quality criteria and methodology that will optimize their ability to preserve each embryo's reproductive potential. This chapter reviews the principles of cryopreservation, outcomes based on embryo stage and cryopreservation method and benchmarks that may be employed by the laboratory to measure the performance of their embryo cryopreservation program. Cryopreservation of human embryos from the 2-cell stage up to the morula stage is a safe procedure that has been carried out for the last 25 years. Experience with blastocyst cryopreservation is still limited and pregnancy rates after the use of frozen or thawed blastocysts vary greatly. Vitrification has improved the success of embryo cryopreservation. However, this technique cannot yet be considered a routine procedure. Despite all of the advantages for infertile couples, cryopreservation of human embryos creates severe ethical problems because of the surplus frozen embryos that either have to be destroyed or perhaps used for research. Embryo adoption may provide a solution to imminent medical, ethical and social problems. Cryopreservation of embryos is increasing worldwide. As more data becomes available, scientists are now able to report on outcomes. With better outcomes for women and pregnancies as a result of frozen embryo transfer, they need to consider the implications of using it in routine practice, especially in preference to the current strategy of fresh embryo transfer.

***Keywords**: Cryopreservation; Pregnancy; Blastocyst; Frozen Embryo; Fresh Embryo Transfer; Vitrification; Pronuclear Stage; Slow Freezing; IVF*

Introduction

In 1972, two research groups independently reported the successful cryopreservation of mouse embryos. The following year, the first calf was born from frozen embryos (Wilmut,1973). In the same year, the first human pregnancy was successfully induced from frozen embryos; however, the pregnancy was terminated in the second trimester due to spontaneous abortion. Subsequently, cryopreservation of sperm and embryos (CP) became routine procedures in Human Assisted Reproduction (AR), and Oocyte Cryopreservation (EC) became increasingly popular in clinical practice. In addition, embryo CP has reduced

the number of new embryo transfers and increased the efficiency of IVF cycles. Embryo CP is also a critical tool in canceling embryo transfers (ETs) due to the risk of Ovarian Hyperstimulation, Endometrial Bleeding, Elevated Serum Progesterone Levels on the day of trigger, and other unforeseen events. However, there is still a lot of debate on the optimal stage, protocols and procedures, and Cryoprotective Additives (CPA's) to be used. On average, a Frozen Stored Embryo has a potential of 4% to become a living child, and Cryopreserved Embryos do not exceed 8% to 10% of the total number of BIRs born from AR (de Jong *et al.,* 2002). However, it cannot be denied that the success of zygote/embryo CP has significantly increased the clinical benefit and cumulative conception rate for couples after a single cycle of ovulatory stimulation and IVF in almost 70% of frozen cycles. Patients ranged in age from 31 to 40 years, with 7.5% >41 years. The 'age effect' is evident in the Frozen Embryo Survival Rate (FESR), which has slowly but steadily declined over the past decade as the age of patients increased by an average of 4 years without any alteration in the freezing process. (89% versus 81%; $P < 0.0001$). Frozen cycles are much less likely to be successful over the age of 30, and there's a big difference in success rates over the age of 35 compared to those under 30. The success rate depends on a few things, like how well the freezing process works, which carriers are used (open vs. closed), how often embryos and oocytes are frozen in assisted reproductive programs, how you pick them, and how successful the fresh embryo transfers are. The success rate can be expressed in terms of survival rates, but that's not enough - it's also important to make sure the cells keep working properly.

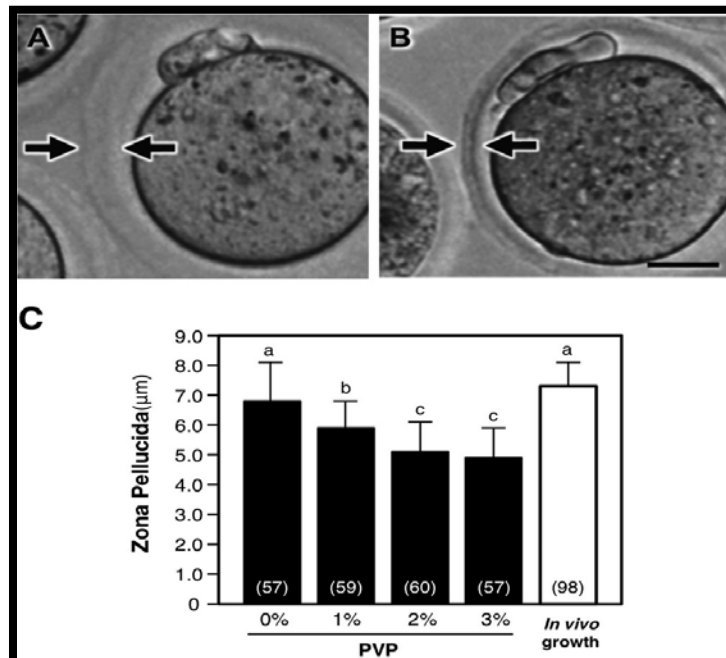

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Figure 1: Comparison of Zona Pellucida Thickness after Growth in Culture Medium Supplemented with Various Concentrations of PVP.

Methodology

Principles of Cryopreservation

Cryopreservation is basically trying to slow down embryos by cooling them down from room temperature (20°C) to 196 °C. This means that during changes in temperature and phase, the embryos are exposed to an environment where they can't survive without help, which puts them at risk for different types of harm, or "cryoinjury". Cryopreservation has previously been extensively discussed in reproductive medicine (Leibo & Pool, 2011). There are four different temperature ranges for the damage that happens during cooling (Nagy *et al.,* 2009).

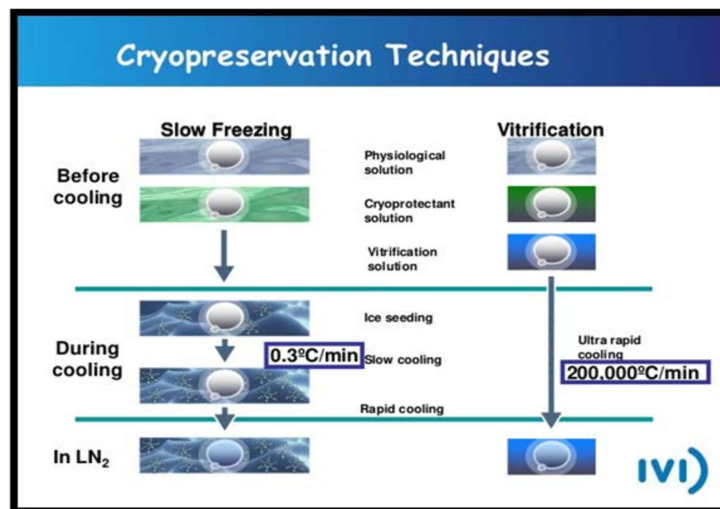

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Figure 2: Techniques of Embryo Cryopreservation

+15 to -5°C

Chilling injury refers to permanent damage that occurs prior to the cells' exposure to freezing temperatures. The lipid bilayer of a cell membrane is fluid and permeable. Phospholipids transition from a liquid to a gel phase as the embryos cool down to -5°C (Ghetler *et al.,* 2005) . When you're cryopreserving embryos from certain mammals that have high levels of lipids, this kind of irreversible harm is a big problem. Oocyte meiotic spindles, microtubules, and cytoplasmic lipid droplets can all be harmed by a chilling injury (Bianchi *et al.,* 2005) . Cryopreservation procedures need to be adapted to the embryonic stage because the ability of mammalian embryos to withstand chilling injuries changes over time (Pedro *et al.,* 2005).

-5 to -80°C

When temperatures go from -5°C to -80°C, ice crystals form on both the inside and outside of cells. These ice crystals can cause damage to the cells, either physically or chemically. Scientists first came up with the idea of a "two-stage hypothesis" for cell damage at these temperatures back in 1972 (Mazur, 1963; Mazur, Leibo & Chu, 1972). Slow cooling causes

extracellular ice to form. When cells cool slowly, the gases in the embryo's environment become hypertonic, reducing the amount of intracellular ice. The hypertonic solution pulls water out of the cell. The "solution effect" destabilizes proteins and damages the cell membrane. However, cells can be damaged when exposed to high levels of electrolytes for long periods of time. Rapid cooling, which prevents water from leaving the cell quickly enough to form lethal intracellular ice, may be equally harmful (Kleinhans & Mazur, 2007).

-50 to -150°C

Solutions can break at temperatures between 50 and 150°C. If the solution is too broken, it can damage big cells or groups of cells, like oocytes or embryos, and it can also damage the cytoplasm or zonal pellucida. The frequency of solution fracture damage has not been published, and this is still a speculative concern (Rall & Meyer, 1989).

-150 to -196°C

Embryos are stored in nitrogen vapours (-190°C) or, in general, in deterrent nitrogen (-196°C). Patients in perpetuity provoke requests like its endless storage leads to subordinate ecchymosis. As of now, there is no proof that anything was harmed while being stored for a period comparable to a human lifetime. Accidental warming is a common cause of cryoinjury, but if storage temperatures are kept stable, chemical reactions stop at -120°C and storage at -196°C prevents thermally driven reactions, so the embryos are essentially suspended in time (Gao & Critser, 2000). Although background ionizing radiation had been raised as a potential danger, the accumulation of direct harm would take centuries (Rall, 2001). Since liquid nitrogen is not sterile and most microorganisms can survive its storage, there is a chance that infectious agents could be transmitted during storage (Bielanski, 2012). Virus from cryopreserved human embryos was transmitted twice, and bovine viral diarrhea virus was transmitted twice after cryopreserved embryo transfer (Drew *et al.,* 2002). Although these diseases cannot be directly linked to embryo cryopreservation and storage, it was demonstrated under experimental conditions that the concern about disease transmission is valid and should be considered when selecting cryopreservation and storage equipment (Bielanski *et al.*, 2000).

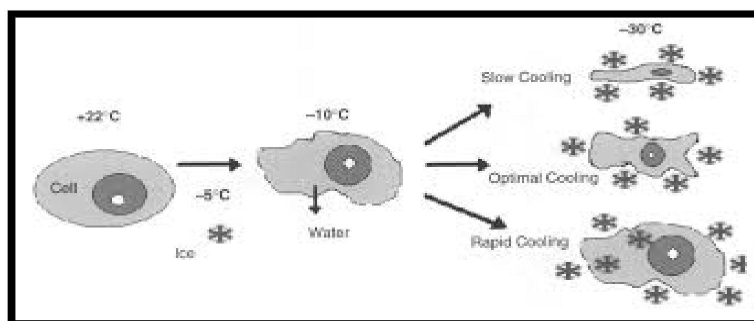

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Figure 3: Mechanisms of Cryoinjury in Living Cells

Warming from -190 to +20°C

The risk of injury during warm-up is the same as during cool-down. Warming has different effects depending on whether intracellular ice builds up or the cells are dehydrated during freezing once intracellular ice develops. Rapid thawing can protect cells by preventing small intracellular ice crystals from recrystallizing into larger, more dangerous ice crystals. In addition to preserving the plasma membrane surrounding the cytoplasm in an apparently viable form, cryopreservation techniques should protect embryos from damage that may not be visible on morphological examination, such as damage to intracellular organelles, the cytoskeleton and cell junctions (Vincent & Johnson, 1992). Despite all these risks to embryo health and survival, they have a remarkable ability to repair or avoid damage using recognized cryopreservation techniques and cryoprotectants, and after thawing, they continue to grow (Van den Abbeel *et al.,* 1997).

Cryoprotectants

Karow defines a cryoprotectant as "any additive that can be introduced into cells prior to freezing and that results in a higher post-thaw survival rate than can be achieved without its addition" and is useful for cryopreservation of cells (Karow, 1974). He has two categories of cryoprotectants (CPAs). Osmotic agents, including small compounds that permeate cell membranes, displace intracellular water, and balance intracellular solutes, and impermeable macromolecular agents like CPA, help maintain the external osmotic gradient and contribute to cellular dehydration. Permeable CPAs include substances such as 1,2-propanedial (PROH), dimethylsulfoxide (DMSO), ethylene glycol (EG), and glycerol. Low molecular weight disaccharides such as sucrose and trehalose are popular choices for sugars used as impermeable CPAs. Impermeable CPA, which is lost after thawing, creates an osmotic gradient that controls the passage of water across cell membranes and helps avoid osmotic shock. For cells to survive cryopreservation, water and invading CPA must be able to cross the cell membrane. The osmotic gradient pushes the highly permeable intracellular water out of the cell, causing the cell to contract, and when the embryo is immersed in a solution containing CPA at hyperosmolarity, the permeable CPA gradually diffuses into the cell. allows you to The ratio of water volume to cell membrane surface area, permeability to water at each stage, and the fact that oocytes and early-stage embryos are less permeable to CPA than morulae and blastocysts Maximize water and CPA exchange (Kasai *et al.*, 1990).The two strategies through which water and entering CPAs invade the cell film are basic dissemination over the lipid bilayer, which is exceptionally temperature-dependent, and through temperature-independent, hydrophilic channels made by proteins called aquaporins. A later ponder found that the kind and amount of aquaporins communicated alter with each formative organization and may influence the layer porousness of CPAs that's interesting to each level (Xiong *et al.,* 2013). A realistic appearance of channel sorts, layer porousness, and alteration in cell measure with each embryonic stage was included within the records of the Alpha agreement assembly on cryopreservation.

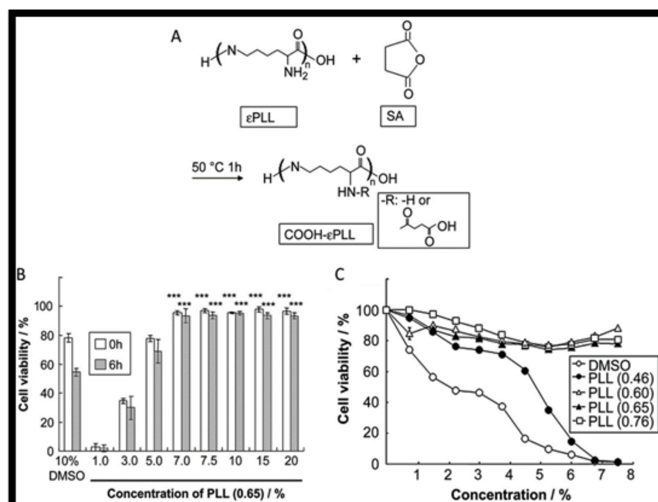

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Figure 4: Development and Application of Cryoprotectants

Results and Discussion

Embryos are frequently vitrified by either slow freezing or quick freezing during cryopreservation. Permeating and nonpermeating CPAs are utilized in both approaches and are eventually reduced to allow for controlled embryonic rehydration during heat. The main differences among the techniques are the duration of CPA exposure, CPA concentration, chilling rate, and warming rate.

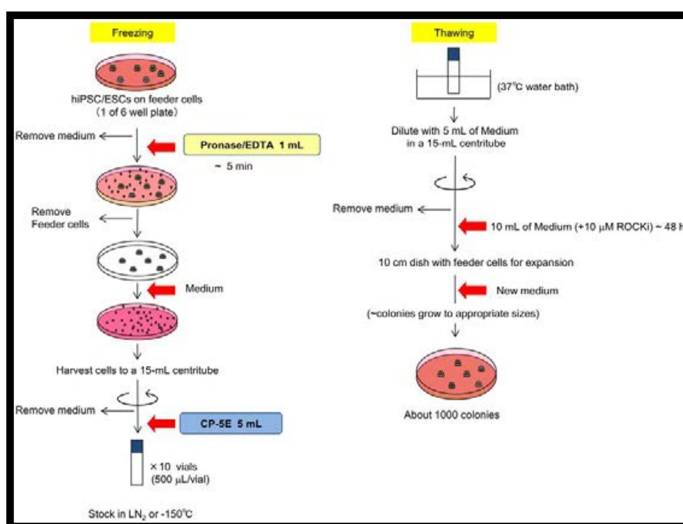

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Figure 5: Scheme Shows the Protocol for the Slow-Freezing Procedure with the Combined use of Pronase/EDTA and Cryopreservation Medium CP-5E (Left) And Rapid Thawing (Right).

Slow Freezing

Moderate hardening, additionally popularly known as unity hardening, addresses intracellular glaze composition and osmotic harm by promoting yell or cautious dosages of CPAs. In 1989, Testart and companions Earlier to hardening, embryos are equilibrated in a hyperosmotic composition holding 1 to 2 M recording CPA (glycerol for blastocysts, PROH or DMSO for pronuclear and gap systematized embryos), accompanying the CPA either in a distinct step over the course of 10 to 20 records or in a step-intelligent habit. Cell decrease is guarded as the water firmly leaves the container and is replaced with each coming CPA. Once balance has been achieved, the container book is rebuilt. The embryos are moved to a mass killing of an ethnic group holding an alliance of 1 to 2 M filtering and 0.2 to 0.3 M nonpermeating CPAs before being introduced into 0.25 mL straws or cryovials and established in a reserved-rate icebox. The embryos are cooled just before 6 to 8 strengths Celsius—just over the point at which something melts from the solution—at a pace of 1 to 2 points per minute. Since the concentrations second-hand for liberal chilling are incompetent to completely prevent intracellular glaze establishment, the aggregation of CPA is raised, all the while abating the process by advancing hailstone composition. Extracellular hail is manually fashioned (plant) by accompanying pre-discouraged grippers by touching the container or hay as certainly from the embryos as it stands proficient. When water freezes, extracellular CPAs and different solutes abridge, forming a new osmotic slope that pulls more water away from the fetus while allowing more CPA in. The hotness of the regulated-rate room is claimed to be loyal for an additional 10 notes to present the embryos with an opportunity to readjust before being very evenly (0.3°C/brief time period) reduced to beneath 30°C. The container is a desire for liquid nitrogen, which is enough intracellular CPA to limit further intracellular hailstone results. The appropriate thawing rate is predetermined to establish the temperature at which chilling stops. Embryos that have undergone cooling at a temperature of 30 to 40°C would have a higher water content and require a higher rate of toasting (200 to 350°C / brief time period) compared to those that have undergone abating at 80°C, and would require a more consistent rehydration rate (25°C for a short time period). In order to control rehydration, the embryos are introduced to an aggregation of nonpermeating CPA (frequently sweet substance), that is to say, two occasions above the aggregation of the definitive cryopreservation resolution. By lowering the standard of osmotic inequality across the container sheath, harsh CPAs have more opportunity to wordy consume the container, and well-absorbed water enters the container at a slower rate (Leibo, 1983).

Vitrification

The term "vitrification" is used to refer to the process of transforming a material into glass; however, it is also used interchangeably to refer to the techniques employed to achieve this transformation. By allowing CPAs to permeate the cell membrane, cell dehydration is accomplished similarly to slow freezing but in a different way. In slow chilling, there is no attempt to assert evenness on either side of the container sheath, the moment of truth for aridity is brief, the aggregation of CPAs is greater, and the rate of chilling is particularly fast.

What keeps cells alive? By drastically increasing viscosity and reducing the amount of time the sample is exposed to temperatures that might lead to chilling damage and the production of ice crystals, vitrification aims to transform liquid into glass. Vitrification is the name given to the process of turning a material into a bottle, but it's more often used to describe the techniques used to manage this change..

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Figure 6: Embryo Vitrification

Cell dehydration is accomplished similarly to slow freezing by allowing CPAs to permeate the cell membrane, but unlike slow chilling, there is no attempt to uphold balance on either side of the container sheet, the moment of truth for aridity is short, the aggregation of CPAs is larger, and the rate of abating is considerably faster. How do cells remain alive? The goal of vitrification is to turn liquid into glass by dramatically increasing viscosity while minimizing the amount of time the sample is exposed to temperatures that might cause chilling damage and ice crystal formation (Fahy *et al.,* 1984). Compared to slow freezing, embryo vitrification procedures are far simpler to use and do not require controlled-rate freezers or other expensive equipment. To make penetrating CPAs, it is usual to join EG and hydrogen with PROH, glycerol, or DMSO. Embryos are exposed to these CPAs in two stages: In order to let intracellular water out of the container and to allow CPA transportation to achieve evenness, initially they are not subjected to a 50% aggregate of the decisive CPA aggregation over 5 to 15 meeting notes. Then, they are only unprotected from the 30 to 40% resolution of CPAs because they should be (about 60 in 2007) (Ghetler *et al.,* 2005). For example, the fetus is cooled at a temperature set per person who carries or carries something, and the sample and liquid nitrogen capacity and resolution arrangement are cooled at 2.5*30*103 °C/short time. The fetus is quickly cooled by taking it out of liquid nitrogen at a temperature of -196°C and filling it with a warmed solution that has reached the ideal temperature. The harsh CPAs must be remote as instantly as likely to humiliate the amount momentary the embryos are unprotected to the poisonous answer. The filtering CPA spreads out slightly from the container, but the toasting answers contain a super-

aggressive aggregation of a non-permeable CPA (usually about 1.0 M of hydrogen) to show osmotic protection and slow down the rate at which the water comes back in. This is the same as thawing later on in steady, chilly weather. The relatively simple vitrification processes used today are the result of years of research into how to cool and bake at fast speeds while still keeping the embryos safe from heat and contamination in liquid nitrogen. After the first human pregnancies and births were reported in the early 1980s, slow refrigeration quickly became the standard for cryopreservation of human embryos. At that time, vitrification wasn't considered possible because of the dangerous CPA concentrations needed to cool large amounts of solution in straws and bottles used for slow freezing. In order to correctly lacquer rodent embryos, Rall and Fahy set a 45-liter blend of CPAs (DMSO, acetamide, propylene glycol, and EG) into a standard 0.25 cc cryostraw before soaking it in liquid nitrogen in 1985. Recognizing the need to lower the detrimental results of singular CPA, this was accomplished (Fahy *et al., 1984*). Despite claims that pups were innate from transplanted embryos that had sustained vitrification, the use of likely perilous DMSO and the temperate toxin acetamide dissuaded investigators from investigating this further vitrification of human embryos. Efforts to replicate this method with cleavage-stage human embryos only resulted in 1/11 surviving (9%). This was thought to be due to the toxic levels of cells "swelling and bursting" after the CPAs were heat-depleting step by step. This was because there wasn't any nonpermeable CPA, but it was probably more likely due to Osmotic Shock in Vitrification Solution, 0.25-0.5 M Nonpermeating Sucrose + 2 M Highly Permeable DMMO) increased embryo survival to 9/11 (82%), but no pregnancies were observed after transfers to six individuals (Kola *et al.,* 1988).

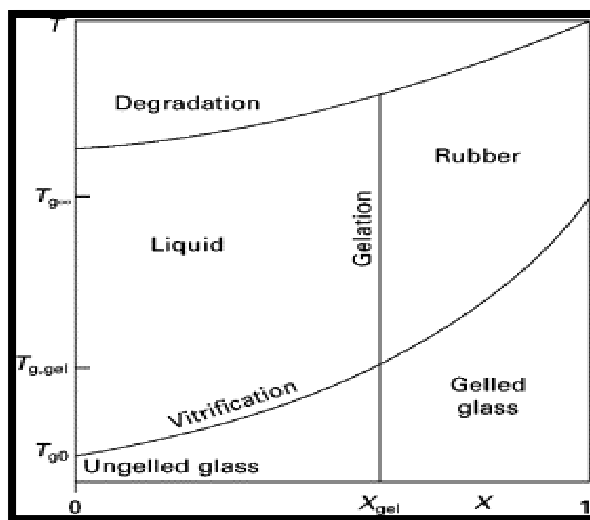

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Figure 7: Vitrification – An Overview

The development of vitrification solutions containing various ratios of piercing and nonpenetrating CPAs was the focus of study over the following ten years, and reports of births after vitrification of animal oocytes and embryos were made (Kasai *et al.,* 1990).

Researchers looking for strategies to cryopreserve oocytes and, due to enhanced culture systems, blastocyst-stage embryos have rekindled interest in vitrification for clinical IVF procedures (Kuleshova *et al.,* 1999).It became very obvious that they needed to make some more changes to speed up cooling and heating to make sure to get consistently high survival rates since both types of cells seem to be really sensitive to cold damage. The most effective way to speed up this process was to reduce the temperature of the sample and the size of the device we were using to contain the embryo (Arav *et al.* 1996). They showed that by cooling and rewarming oocytes and embryos in a range of 0.07 L, or "minimum droplet size," they were able to vitrify these cells at about 50% less CPA concentration than we would need for large-volume vitrification (Arav, 1992). Almost a decade ago, more than 20 devices were created to hold embryos by cooling and heating microvolumes of vitrification solutions, as early papers described the efficient vitrification of embryos in small volumes (Vajta & Nagy, 2006). Clinical IVF facilities are using different vitrification methods to preserve human embryos now that there's more and more proof that embryos survive, develop, and even give birth to healthy babies after vitrification. Different stages of embryos go through different CPAs, but cooling can be done by either directly touching the open system or by touching the closed system with liquid nitrogen. These variables must be considered when selecting and developing a method for use in clinical settings (Valbuena *et al*., 2012).

Finally, the percentage of thawed embryos that result in healthy babies is used to measure the effectiveness of cryopreservation technology. Embryo quality and stage before and after cryopreservation should be considered when comparing the results of cryopreserved embryo transfer with those of non-frozen controls. Since embryo quality criteria may be stricter in cryopreserved embryo transfer than in fresh embryo transfer or embryo stage, the transition time may vary from treatment group to treatment group (Surrey *et al.,* 2010). Because of the virtually infinite combinations of embryo stage, selection criteria, caspase-paired plasma (CPA) type and concentration, device type, cooling and heating rate, and other factors, a comprehensive comparative analysis of reported results in the field of Human Embryo Cryopreservation has been largely unsuccessful. Not many RCTs have been conducted to evaluate the clinical effectiveness of various embryo cryo-reservation methods or to identify the stage of development that maximizes the rate of cumulative zygotic and neonatal growth for a collection cycle (AbdelHafez *et al.,* 2010). Reviewing research that contrasts fresh and frozen embryo transfers is important in determining the impact of cryopreservation on embryos, especially if enough information is provided to establish the number of children per zygote for the oocyte extraction (Ali & Shelton*,* 1993).

Conclusion

Embryo cryopreservation has become an integral part of the IVF process, playing a significant role in the cumulative, one-off increase in live births from conception to egg retrieval following the birth of the initial frozen cut-stage embryo. There has been much debate among IVF doctors about the idea of abandoning all new transfers, as evidence shows that after frozen embryo transfer, live births and newborns are better for singleton

pregnancies than with fresh transfers. A significant change in clinical practice would require large-scale RCTs, as such an approach has many social, clinical, and economic implications. The purpose of this review was to examine the results of different cryopreservation techniques at each stage of development. The best stage and quality of cryopreserved embryos must be determined by programs that are not limited by legal restrictions. The laboratory must be able to evaluate its performance against competitors using key performance indicators provided by the Alpha Society. 47 After gradual freezing and vitrification, pronuclear-stage embryos exhibit comparable results, according to this review. In general, vitrification promotes better blastocyst and cleavage-stage embryo survival rates. Some facilities have tailored their slow-freezing procedure to provide similarly high implantation and survival rates per thawed embryo at both phases. The observation of decreased metabolism in embryos at the cleavage stage following gradual freezing suggests that vitrification could be a preferable option. 119 If the survival and implantation rates for both procedures are the same for blastocyst-stage embryos, the choice boils down to staffing, equipment, and the cryopreservation device. Slow freezing takes significantly longer than vitrification, but when large numbers of embryos are ready for cryopreservation, vitrification can take longer. Even though vitrification only takes a tiny amount of liquid nitrogen and only takes about an hour to get from the fridge to storing the embryos, slow freezing takes a lot of expensive equipment to keep up and makes it harder to get rid of the embryos. Plus, it needs to be kept cold. And since glazing can be contaminated, it has to be done in a closed environment kept cold because of the possibility of contamination, glazing must be done in closed equipment. By using HSV straws produced excellent results despite slower cooling and warming rates. Other sealed glass devices are available, but the results of these devices were not included in the discussion because the formulas for the glass solutions were not disclosed. Validation studies and training programs must be created before any new method is used in the lab to guarantee that performance will meet standards. These metrics will offer performance evaluations in a timely manner.

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