Cryopreservation of human embryos and its contribution to in vitro fertilization success rates

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Cryopreservation of human embryos is now a routine procedure in assisted reproductive technologies laboratories. There is no consensus on the superiority of any protocol, and substantial differences exist among centers in day of embryo cryopreservation, freezing method, selection criteria for which embryos to freeze, method of embryo thawing, and endometrial preparation for transfer of frozen-thawed embryos. In the past decade, the number of frozen-thawed embryo transfer cycles per started in vitro fertilization (IVF) cycle increased steadily, and at the same time the percentage of frozen-thawed embryo transfers that resulted in live births increased. Currently, cryopreservation of human embryos is more important than ever for the cumulative pregnancy rate after IVF. Interestingly, success rates after frozen-thawed embryo transfer are now nearing the success rates of fresh embryo transfer. This supports the hypothesis of so called freeze-all strategies in IVF, in which all embryos are frozen and no fresh transfer is conducted, to optimize success rates. High-quality randomized controlled trials should be pursued to find out which cryopreservation protocol is best and whether the time has come to completely abandon fresh transfers. (Fertil Steril® 2014;102:19–26. ©2014 by American Society for Reproductive Medicine.)

**Key Words:** IVF, embryo cryopreservation, endometrial receptivity, slow freezing, vitrification, embryo transfer

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The first pregnancy resulting from transfer of a thawed cryopreserved human embryo was reported in 1983 in Australia (1), and the first live birth following embryo cryopreservation was reported in 1984 in The Netherlands (2). Subsequent to the first successful in vitro fertilization (IVF) treatment in 1978, the need for an effective cryopreservation program arose from rapid development and improvements of assisted reproductive technology (ART) protocols. Initially, all available embryos were transferred in IVF treatments owing to its low success rate, but improvements of clinical and laboratory aspects of IVF led not only to increased pregnancy rates but also to increased risk of multiple pregnancies. To prevent multiple pregnancies, fewer embryos were transferred and the supernumerary embryos cryopreserved for potential future use (3, 4). At that time, selection of embryos became important because the best available embryos had to be transferred fresh owing to the initially low success rates of embryo cryopreservation (5). Since those early days, cryopreservation of supernumerary embryos has become an integral part of IVF treatment. In addition, embryo cryopreservation is applied for women at risk for ovarian hyperstimulation syndrome (6), in embryo donation programs (7, 8), and for fertility preservation in women awaiting cytotoxic treatment (9).

Multiple variables, such as the selection criteria for embryos to be cryopreserved (10), the method of freezing and thawing (11), the synchronization between embryo and endometrial development (12), hormone supplementation during the frozen-thawed embryo transfer cycle (13), and patient characteristics, such as age of the woman (14, 15), determine the efficacy of embryo cryopreservation programs.

In this review, we will discuss the current state of affairs of embryo cryopreservation in IVF treatments and provide supportive evidence for the freeze-all strategy.
arrested state by raising the intra- and extracellular viscosity to a level in which all molecular diffusion and chemical processes are halted. Freezing results in ice nucleation (change of state from liquid to solid around a certain focal point) and subsequent growth of ice crystals that turn water into ice, while all salts become confined to the remaining unfrozen fraction (16). These two phenomena, i.e., ice crystal formation (16) and increased salt concentrations (17), are the main causes of cell damage and possible cell death associated with cryopreservation. To prevent cell damage, cryopreservation protocols aim to dehydrate the intracellular space and as a result minimize intracellular ice formation while keeping the intracellular salt concentrations low (18, 19). Dehydration can be facilitated by adding cryoprotectant agents (CPAs) to the freezing medium. CPAs serve as antifreeze agents by disrupting hydrogen bonds in water. They come in two shapes, namely: 1) permeating CPAs that can enter the cell and directly displace water out of the cell, e.g., dimethyl sulphoxide (DMSO), ethylene glycol (EG), and propanediol (PROH); and 2) nonpermeating CPAs that remain outside the cell and draw water out of the cell by osmosis, e.g., sucrose. An additional effect of permeable CPAs is that they compensate for the increased intracellular salts that could be lethal at high concentrations. The addition of high concentrations of CPAs also lowers the freezing temperature of a solution and thereby reduces intracellular ice formation.

Each cell type has a specific optimal cooling rate determined by its volume-to-surface area ratio and its membrane permeability for water and cryoprotectants. If the cooling rate (decrease in temperature over time) is low, cells have sufficient time to lose water and achieve maximal dehydration, resulting in minimal ice crystals formation. If the cooling rate is high, the time for movement for water out of the cells is limited and ice crystals can be formed.

All cryopreservation methods can basically be divided into slow freeze and vitrification methods. Although slow freezing is likely still the predominant mode of embryo cryopreservation, there has been a major switch to vitrification in recent years. In 1984 the first human live birth occurred from a slow-frozen and subsequently thawed embryo, and the first successful pregnancies and deliveries after vitrification and warming of human cleavage-stage embryos were reported in 1990 (20). Since then, a significant improvement in post-thaw survival rates of human embryos has been reported for both freezing techniques (11).

Slow freezing and vitrification use similar chemicals but differ greatly in the concentration of those chemicals as well as in cooling and warming rates (Table 1). Slow freezing uses relatively low concentrations of CPAs, low cooling rates, and fast warming rates, whereas vitrification uses high CPA concentrations and ultrafast cooling and warming rates. Under slow cooling conditions, dehydration without excessive shrinkage is achieved by exposure to permeating cryoprotectants and exposure time to extracellular hyperosmotic conditions is limited. The principle of vitrification is to reach a glass-like state of the cell without formation of harmful ice crystals (18, 21). Under vitrification conditions, extreme dehydration is achieved by a very short exposure to high concentrations of permeating and nonpermeating cryoprotectants in the context of a high cooling rate. To achieve ultrafast cooling rates, open embryo carriers (e.g., electron microscopy grid, open pulled straw, Cryoloop, McGill Cryoleaf, Hemi-Straw, Cryotop, Cryolock) were designed to allow direct contact of the medium containing the embryo with liquid nitrogen (22, 23).

### EMBRYO CRYOPRESERVATION AND CELLULAR CHANGES

Although implantation potential and resulting pregnancy rates are indicators for efficacy, little is known about the effect of cryopreservation on the physiology and cellular changes of the human embryo. Only a few studies have reported on molecular differences between frozen-thawed embryos and freshly cultured human embryos. For example, increased spindle abnormalities have been demonstrated in post-thaw blastocysts does not per se impair

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### Table 1

<table>
<thead>
<tr>
<th>Differences and similarities between slow cooling and vitrification protocols.</th>
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<tr>
<td><strong>Factor</strong></td>
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<tr>
<td>Cryoprotectant</td>
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<tr>
<td>Concentration of cryoprotectant (initial)</td>
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<td>Incubation time</td>
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<td>Mechanical stress (ice crystal formation)</td>
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<tr>
<td>Programmable freezing equipment</td>
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<tr>
<td>Carrier</td>
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<tr>
<td>Direct contact with LN₂</td>
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*Note: DMSO = dimethylsulfoxide; EM = electron microscopy; LN₂ = liquid nitrogen.*

mitosis or further embryo development. Also, differences in gene expression have been reported between frozen-thawed embryos and fresh embryos (25, 26). Genes involving the apoptosis/stress pathway (BAX), pluripotency pathway (NANOG, SOX2, CDX2), maternal effect genes (ZAR1, EIF1AX, TSC2) were significantly altered in frozen-thawed embryos compared with freshly cultured embryos from the same developmental stage (26).

**TIMING OF EMBRYO CRYOPRESERVATION AND EMBRYO SELECTION CRITERIA**

Embryos can successfully be cryopreserved at all stages of preimplantation development, i.e., the pronuclear (PN; day 1), cleavage (day 2–4), and blastocyst (day 5–6) stages (11). At each stage of development, laboratories tend to formulate quality criteria for an embryo to be considered for cryopreservation. These criteria are often stricter than the criteria used to consider an embryo to be suitable for fresh transfer. It is important to note that if a certain clinic uses very strict criteria, i.e., only top-quality embryos are cryopreserved, their success rates after frozen-thawed embryo transfer will be higher compared with a clinic with lower cut-off criteria for cryopreservation. At the same time, however, they will conduct fewer frozen-thawed embryo cycles per initiated fresh cycle and thus in aggregate the contribution of cryopreservation to their cumulative success rates, i.e., the chance of live birth per initiated cycle including all frozen-thawed embryo transfers from the same cycle, is likely to be lower.

**EMBRYO QUALITY AFTER THAWING**

The development of cryopreserved embryos might be impaired by the effects of cryopreservation or by the warming and thawing process. If warming and thawing occurs too slowly, it could cause cell damage directly or indirectly through the formation of ice crystals. The warming rate, therefore, should be rapid for both the slow freezing and vitrification methods to prevent cell damage during this process. The chance that a cryopreserved embryo will implant is of course associated with its quality after thawing. Embryo quality is most commonly assessed by morphologic evaluation, with fully intact frozen-thawed embryos having a higher developmental and implantation potential than embryos damaged by the cryopreservation method (5, 10, 27–30). However, successful pregnancies have occurred after the transfer of embryos with only a few intact blastomeres, showing retained developmental potential despite substantial freezing damage (31). A real threshold for the lowest proportion of intact cells leading to successful implantations is not clear (31–35). In general, when at least one-half of the blastomeres from an embryo remain intact after thawing, it is considered to be viable (10, 36–39). In principle, it can be argued that frozen-thawed embryos with a chance of implanting should always be transferred and that only those frozen-thawed embryos for which implantation is very unlikely should not. The quality cutoff to conduct a transfer or not varies among centers, varies among different methods of cryopreservation, and depends on the quality of the embryo before cryopreservation. In addition, the necessity to select embryos after thawing is absent, because in general not more embryos are thawed than would be transferred.

Besides post-thaw survival, resumption of mitosis within 24 hours after thawing also has been associated with better implantation potential (40, 41). It is important to note that in principle prolonged culture leads to better selection of embryos and therefore increased implantation rates per embryo transferred, but at the same time to a reduced number of embryos surviving the prolonged in vitro culture and thus potentially to a reduced pregnancy rate per thaw cycle (42).

**FROZEN-TAWED EMBRYO TRANSFER AND ENDOMETRIAL PREPARATION**

The human endometrium undergoes cyclic changes during a menstrual cycle and changes in receptivity in preparation for embryo implantation. This endometrial remodeling is driven by progesterone, and the endometrium is receptive to embryo implantation for only 6–10 days after the LH surge (43, 44). Frozen-thawed embryo transfer should occur in an environment where the development of the embryo and endometrium is temporally synchronized. Both poor embryo quality and poor endometrial receptivity could contribute significantly to the high occurrence of implantation failure (45). Therefore, synchronization of embryo and endometrium development is necessary for successful implantation (46–48).

The possible detrimental effect of ovarian hyperstimulation on endometrial receptivity has been suggested to be intermediated by elevated progesterone. Indeed, progesterone elevation (PE) on the day of hCG administration in fresh embryo transfer cycles, a proxy of endometrial receptivity, has been associated with a decreased probability of pregnancy. More precisely, women with a PE of 0.8–1.1 ng/mL had a lower chance of pregnancy compared with women with progesterone levels <0.8 ng/mL (odds ratio [OR] 0.79, 95% confidence interval [CI] 0.67–0.95). In contrast, no effect of PE on pregnancy chances was observed when embryos that resulted from women exhibiting PE on the day of hCG were cryopreserved and transferred in a subsequent cycle (OR 1.03, 95% CI 0.79–1.34) (49). The same results hold for embryo transfers in donor-recipient cycles (OR 1.18, 95% CI 0.76–1.84) (49).

Frozen-thawed embryo transfer can occur in either a natural menstrual cycle or an artificial cycle with endometrium preparation. Embryo transfer in a natural cycle relies on endogenous sex steroid production from a developing follicle, and timing of embryo transfer is determined by the spontaneous LH surge preceding ovulation or optionally by administering hCG to initiate luteinization. The LH surge is monitored by serum or urine LH levels or with the use of ultrasound monitoring. For both methods, cancellation of embryo transfer can occur because of premature spontaneous ovulation, insufficient development of the dominant follicle, or insufficient endometrium development. Alternatively, in artificial cycles exogenous estrogen and progesterone are administered, either with or without additional GnRH agonists. The main advantage of embryo transfers in artificial cycles is increased patient convenience, with little ultrasound monitoring burden and easier-to-schedule embryo transfers.

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On the other hand, this method is more expensive and may be associated with a higher risk of side effects, such as thromboembolic events (50, 51).

**DIFFERENCES BETWEEN PROTOCOLS AND CLINICAL EVIDENCE**

**Effectiveness**

Consensus is currently lacking on the best practice for embryo cryopreservation. As a result, individual ART centers have developed their own freezing protocols based on their own laboratory experiences and choices instead of an evidence-based freezing protocol. This widespread variability limits effective comparisons of different available protocols, including of method of cryopreservation (slow freezing versus vitrification), optimal timing of cryopreservation and optimal protocol for endometrial preparation for frozen-thawed embryo transfers. Of course, the selection of which cryopreservation method to use will have to be based on the clinical efficacy, safety, and costs of the technique.

The majority of trials evaluating various cryopreservation methods are trials comparing slow freezing and vitrification (52–57). Systematic analysis of these studies showed a higher post-thaw survival rate for vitrification compared with slow freezing for all stages of embryo development, including PN-stage (day 1), cleavage-stage (day 2–4), and blastocyst-stage (day 5–6) embryos (58, 59). However, the number of studies that were included in the meta-analysis was small (n = 7) and the studies were of low quality (58, 59), with some reporting an unclear method of randomization or pseudorandomization (54, 60) and others not being truly randomized studies (52). Also, in five of the seven studies the unit of randomization was embryo instead of patient, so that pregnancy rates or live birth rates were reported per embryo instead of per patient. In this case, no valid conclusions on pregnancy rates per patient per strategy can be drawn because successful pregnancies depend on the selected embryo. Two randomized controlled trials (RCTs) that were published after these meta-analyses showed inconclusive results on the post-thaw survival rate of cleavage-stage (day 2–3) embryos after vitrification or slow freezing (56, 57). In terms of pregnancy rates, no significant differences were reported between slow freezing and vitrification of embryos cryopreserved on day 2–6 of development (56, 58, 59, 61). For PN-stage embryos, pregnancy rates were not reported in any study. Based on these data, no superiority for the method of cryopreservation can be described in terms of post-thaw survival rate of embryos and clinical efficacy.

No RCTs are available that compare the success rates of embryo cryopreservation between embryos cryopreserved at different stages of development. The available data on the effect of timing of embryo cryopreservation are from randomized studies comparing different days of fresh transfer and from retrospective cohort studies (35, 62–64). As a consequence, there is again no consensus about the timing of embryo cryopreservation. Of note, any comparison of the effectiveness of cryopreservation at various stages of development is obscured by differences in culture time. As a general rule of thumb, increased culture time will lead to: 1) better selection of competent embryos and therefore increased success rates per fresh and frozen-thawed embryo transfer (62, 65); as well as 2) fewer embryos surviving in vitro culture up to the point at which transfer or cryopreservation is conducted (66). Illustrative of this is the observation that in RCTs comparing cleavage-stage and blastocyst transfer, blastocyst transfer results in increased pregnancies per transfer, fewer number of cryopreserved embryos, and, in fact, lower cumulative pregnancy rates per patient (i.e., the pregnancy rate per patient including the outcome of both the fresh transfer and possible subsequent frozen-thawed embryo transfers) (64).

Also, whether frozen-thawed embryo transfers should be performed in natural or in artificial cycles is not clear. Furthermore, there is great variability among protocols used in artificial cycles, including variability in the dose, route of administration, duration, and start day of progesterone administration. There is no significant benefit of vaginal over intramuscular or oral progesterone administration, of low-dose vaginal over high-dose vaginal progesterone administration, or of short over long protocols in terms of pregnancy rates (67). Better pregnancy rates have been reported when progesterone administration is started on or after the day of ovum pick-up compared with the day before ovum pick-up (68). For frozen-thawed embryo transfers in natural or artificial cycles, no differences have been reported regarding pregnancy rates or live birth rates (13, 69). Most available data are from retrospective cohorts, and well designed and powered RCTs comparing frozen-thawed embryo transfer in natural cycles with transfer in artificial cycles are lacking. Currently there are three randomized studies (NCT00492934, NCT01780558, and NTR1586) underway comparing both methods of endometrial preparation, including the perceived burden and costs of both treatments being taken into account (70–72).

**Safety**

The main purpose of ART is of course the generation of a pregnancy leading to a healthy live birth. Because of the relatively short experience with ART, little is known about the safety of the technique in terms of the (long-term) health of the children born after ART. For obstetrical and perinatal outcomes, it has been shown that children resulting from ART have poorer outcomes compared with children resulting from spontaneous conceptions, in preterm birth, low birth weight, small for gestational age, cesarean section rate, admission to a neonatal intensive care unit, and perinatal mortality (73–75). Interestingly, data from children born from frozen-thawed embryo transfer cycles show fewer perinatal complications of preterm birth, small for gestational age, low birth weight, and perinatal mortality compared with children born from fresh embryo transfers (76–78). Additionally, outcomes of singletons born after frozen-thawed embryo transfer seem more similar to naturally conceived singletons (79, 80). So far, the etiology of these differences is unknown, although suboptimal endometrial development has been suggested to be a risk factor for the adverse outcomes of ART (81).
Hormonal levels, such as estrogen (82), progesterone (82), and stress hormones, such as cortisol (83), fluctuate throughout the stimulated menstrual cycle and could negatively affect the endometrium as well as the early developing embryo. Proper placentation with fresh embryo transfer may be jeopardized by the supraphysiologic concentrations of estrogen and progesterone, leading to worse perinatal outcomes compared with frozen-thawed embryo transfers in a more neutral physiologic environment. Indeed transfer of fresh embryos has been suggested to lead to more first-trimester loss compared with frozen-thawed embryo transfers (84).

CONTRIBUTION OF CRYOPRESERVATION TO IVF SUCCESS RATES

The general success rates for frozen-thawed embryo transfers have increased in the past few years. The Centers for Disease Control and Prevention (CDC) collected data on ART success rates of all American fertility clinics from 1997 to 2011. According to those data, success rates of both fresh and frozen-thawed embryo transfer cycles (donor eggs not included) have increased over the past 14 years for women of all ages (Figs. 1 and 2) (15). Interestingly, the increase in success rates seems to be greater for frozen-thawed embryos compared with fresh embryos. For example, an increase in live birth rate from 31% to 40% (+29%) for fresh cycles and an increase from 21% to 39% (+86%) for frozen-thawed embryo transfer cycles was reported from 1997 to 2011 for women under the age of 35 years. Unfortunately, the total number of frozen-thawed embryo transfer cycles from 1997 to 2010 was not reported, so Figure 2 displays the live birth rate per frozen-thawed embryo transfer, whereas Figure 1 displays the live birth rate per initiated fresh cycle. Still, it appears that the success rates of frozen-thawed embryo transfer cycles are nearing those of fresh embryo transfer cycles. In addition, the number of frozen-thawed embryo transfers per initiated cycle has increased from 1997 to 2011 from 0.2 to 0.34 (+70%) per initiated cycle for women younger than 35 years (Fig. 3). It is not possible to conclude that these results are solely due to improved cryopreservation protocols. Possible other factors, such as application of better morphologic embryo selection criteria or altered embryo transfer policies, i.e., increased number of single-embryo transfers, could also underlie the improved chance of pregnancy per transferred frozen-thawed embryo and the increase

FIGURE 1
Percentage of all initiated fresh cycles resulting in live births per year from 1997 to 2011 in the United States (adapted from CDC, 2013 [15]). A multiple-infant birth is counted as one live birth.


FIGURE 2
Percentage of frozen-thawed embryo transfers resulting in live births per year from 1997 to 2011 in the United States (adapted from CDC, 2013 [15]). A multiple-infant birth is counted as one live birth.


FIGURE 3
The number of frozen-thawed embryo transfers per the number of fresh cycles per year from 1997 to 2011 in the United States (adapted from CDC, 2013 [15]).

in the number of frozen-thawed embryo transfers per initiated IVF cycle. Despite these uncertainties, the CDC data clearly demonstrate that the relative contribution of frozen-thawed embryo transfer cycles to the total number of live births has increased over the years. In 1997, 12% of all live births in women under the age of 35 years undergoing IVF originated from the transfer of frozen-thawed embryos, and this number doubled to 25% in 2011 (Fig. 4). These data show an increased utilization of frozen-thawed embryo transfer in recent years, with an increasingly important role for frozen-thawed embryo transfer in the success rates of IVF treatments.

FREEZE-ALL STRATEGIES

The main conclusions of the preceding paragraphs, namely: 1) more receptive endometrium in cycles without ovarian stimulation compared with cycles with ovarian stimulation; 2) increased health of children born from the transfer of frozen-thawed embryos compared with children born from cycles with fresh embryo transfer; and 3) increased success rates of frozen-thawed embryo transfer that now almost equal the success rates of fresh embryo transfer (Figs. 1 and 2), have led to the hypothesis of a so-called freeze-all strategy that might lead to increased success rates of IVF/intracytoplasmic sperm injection treatments (85, 86). One small randomized controlled study supports this hypothesis and showed that higher ongoing pregnancy rates were achieved with a freeze-all strategy than with standard IVF cycles with fresh embryo transfers (87). Of note, all available embryos in that study were frozen at the PN stage of development, which is not common in many centers. High-quality studies are needed to evaluate the hypothesis of disengaging the embryo transfer from the cycle with ovarian hyperstimulation to optimize live birth rates. Currently there are four RCTs underway that test this hypothesis (NCT00823121, NCT01841528, NTR3187, and ACTRN12612000422820) (70, 72).

CONCLUSION

The lack of consensus on the superiority of any protocol for the cryopreservation of human embryos results in substantial differences among centers in day of embryo cryopreservation, freezing method, selection criteria for which embryos to freeze, method of embryo thawing, and endometrial preparation for transfer of frozen-thawed embryos. Results from frozen-thawed embryo transfers from the past 14 years show that the utilization of frozen-thawed embryos in IVF treatment has clearly increased. Also, the percentage of frozen-thawed embryo transfers that resulted in live births has increased, and the success rates of frozen-thawed embryo transfers are now nearing those of fresh embryo transfer. As such, cryopreservation and frozen-thawed embryo transfer now contribute more substantially than ever to the cumulative live birth rates of IVF treatments. Together with the data on increased endometrial receptivity in cycles without ovarian hyperstimulation and better health of children born after frozen-thawed embryo transfer, this argues for a freeze-all strategy in IVF, in which embryo transfer is fully disengaged from ovarian hyperstimulation. However, high-quality RCTs should be performed to determine which cryopreservation protocol is best and whether a freeze-all strategy is truly justified in future IVF treatments.

FIGURE 4

The contribution of fresh and frozen-thawed embryo transfers to the number of total live births after assisted reproductive technology per age group from 1997 to 2011 in the United States (adapted from CDC, 2013 [15]). A multiple-infant birth is counted as one live birth.

REFERENCES


12. Groenewoud ER, Cantineau AE, Kollen BJ, Macklon NS, Cohlen BJ. Does elec-


14. van den Abbeel E, Camus M, van Waesberghe L, Devroye P, van Steirteghem AC. Viability of partially damaged human embryos after cryo-


