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Tribute

Does the developmental stage at freeze impact on clinical results post-thaw?

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Abstract

The value of cryopreserving prezygotes, pre-embryos or blastocysts for future thaw and transfer is an important consideration of every IVF program. The convergence of two factors, a higher pregnancy rate and a lower multiple gestation rate, can be managed effectively through the establishment of a successful cryopreservation programme. In this article, freezing and thawing results from pronuclear oocytes, pre-embryos, and blastocysts are compared.

Keywords: *blastocyst, cryopreservation, freeze, thaw*

Introduction

Twenty years have passed since the very first pregnancy from a thawed human pre-embryo was reported in the world (Trounson and Mohr, 1983). During this time, most IVF programmes have embraced cryobiology in order to augment clinical pregnancy from a single ovarian stimulation attempt. As ovulation induction protocols have improved, allowing the recruitment of multiple healthy oocytes, so has the need grown to manage their numbers responsibly. It is usual today to harvest in excess of 10, or sometimes even 20, mature oocytes from a woman. Before freezing techniques were routinely used in the laboratory, a woman producing so many gametes would be forced to either limit the number inseminated or risk having to discard healthy pre-embryos, since only two to four could be transferred safely to the uterus after fertilization. Neither was an attractive option.

It is now apparent that pregnancy after thawing is nearly equal to the transfer of fresh pre-embryos, at least in some programmes. When the cumulative effect of adding thawed pregnancies (only from cycles failing to become pregnant following fresh transfer) to fresh pregnancies is examined, delivery outcomes are significantly enhanced (see below: calculating pregnancy potential from prezgyote, pre-embryo, and blastocyst stages) (Veeck *et al.*, 1993). Additionally, patients at risk of ovarian hyperstimulation syndrome (OHSS) are managed more effectively by freezing all conceptuses upfront, thereby reducing, although not eliminating, the likelihood of adverse clinical symptoms once pregnancy is established (Queenan *et al.*, 1997).

Cryoprotectants and cryobiology

The primary goal in establishing an appropriate freezing protocol is to do as little damage as possible while exposing conceptuses to non-physiological ultra-low temperatures. Cryopreservation protocols essentially freeze-dry or dehydrate pre-embryos to prevent intracellular ice from forming.

The formation of intracellular ice crystals can mechanically damage specimens by disrupting and displacing organelles, or slicing through membranes. This is why freezing techniques use cryoprotective agents to control ice formation at critical temperatures. It has been shown that when human cells are placed into a medium that contains an intracellular cryoprotective agent, intracellular water readily exits the cell as a result of the higher extracellular concentration of cryoprotectant. This causes some cell shrinkage until osmotic equilibrium is reached by the slower diffusion of the cryoprotectant into the cell (Mazur, 1984). Once equilibrium is reached, the cell resumes a normal appearance. The rate of

permeation of cryoprotectant and water is dependent on temperature; equilibrium is achieved faster at higher temperatures.

Cryoprotectants are also beneficial in their ability to lower the freezing point of a solution. Solutions may remain unfrozen at –5°C to –15°C because of super-cooling (cooling to well below the freezing point without extracellular ice formation). When solutions super-cool, cells do not dehydrate appropriately, since there is no increase in osmotic pressure from the formation of extracellular ice crystals. To prevent super-cooling, an ice crystal is introduced in a controlled fashion in a process call seeding. This contributes to intracellular dehydration as water leaves the cell to achieve equilibrium with the extracellular environment (Whittingham, 1977; Mazur, 1984). If the rate of cooling is too rapid, water cannot pass quickly enough from cells, and as the temperature continues to drop, it reaches a point when the intracellular solute concentration is not high enough to prevent the formation of ice crystals. Human prezygotes, pre-embryos, and blastocysts, which hold substantial intracellular water, are usually cooled at slow rates below the seeding temperature (0.3°/min) to permit adequate dehydration.

Membrane permeability by cryoprotectants varies between developmental stages. As such, it has been found that some cryoprotective agents are suitable for freezing early stages of development, while others are more suitable for freezing later stages. Dimethylsulphoxide (DMSO) and 1,2-propanediol (PROH) are frequently used for freezing early cleavage stage pre-embryos and propylene glycol (glycerol) is commonly used for blastocysts. All three intracellular agents have fairly small molecules that permeate cell membranes easily. In addition to these, there are several extracellular substances that help dehydrate and protect cells. The most frequently used is sucrose, which possesses large, non-permeating molecules and exerts an osmotic effect to aid in accelerated cell dehydration. Sucrose cannot be used alone but is often used in conjunction with standard permeating, intracellular cryoprotectants.

During the freezing procedure, all chemical reactions within cells should be suspended. Under extremely cold liquid nitrogen storage conditions (–196°C), it is estimated that it would take hundreds, or even thousands, of years before background ionizing radiation causes significant damage to stored cells.

If cooling is terminated at relatively high temperatures (over –40°C), cells carry more intracellular ice than if cooled longer to lower temperatures (around –80°C). In order to protect cells, thawing must be carried out rapidly to induce rapid ice dispersal. Conversely, samples cooled to around –80°C should be thawed more slowly to allow for gradual rehydration (Schneider, 1986). If water re-enters cells too rapidly, they may swell or burst. It is common to expose frozen specimens to progressively lower concentrations of cryoprotectant to ensure that it is slowly and gently removed.

Vitrification

The concept of vitrification is to protect cells by completely avoiding all ice crystal formation. To accomplish this, cryoprotective solutes must be increased to 40% (w/v) or higher. DMSO is frequently used, but PROH, ethylene glycol, and other agents have been tested. Because high concentrations of these cryoprotectants are toxic at room temperature, they are generally introduced at 0°C. Samples may be plunged directly into liquid nitrogen without needing to induce a seed crystal; the viscosity is so great that solutions solidify into glasslike states. Unfortunately, vitrified specimens must be thawed in ice water, which is fairly inconvenient (Quinn and Kerin, 1986; Friedler *et al.*, 1987, 1988). Although the procedure has been slow to gain acceptance for routine human cryopreservation, several live births have been recently reported, most involving blastocysts (Choi *et al.*, 2000; Yokota *et al.*, 2000, 2001; Mukaida *et al.*, 2001). One novel modification to standard vitrification techniques has been to reduce the fluid content within the blastocyst before freezing (Vanderzwalmen *et al.*, 2002). In this report, it was noted that the efficiency of freezing was negatively correlated to the expansion of the blastocele, and therefore, the investigators postulated that blastocele ice crystal formation was causing damage during the cooling process. They proceeded to artificially reduce the blastocele volume before freezing by inserting a needle into the cavity until contraction occurred. Using this method, survival rates were much improved over controls, pregnancy rates showed a positive trend, and implantation rates were significantly higher.

Current materials and methods

Most prezygote and pre-embryo freezing protocols used today are based loosely on the original work from the mid-1980s by Jacques Testart and his colleagues (Testart *et al.*, 1986a,b). PROH and DMSO are commonly used as cryoprotectants, with or without sucrose. On the other hand, most blastocyst freezing protocols have evolved from the published work of Yves Ménézo and use glycerol with sucrose (Ménézo *et al.*, 1992, 1993). Cornell methods utilize PROH for prezygote and pre-embryo stages, and glycerol for blastocysts; sucrose aids cell dehydration for pre-embryos and blastocysts. All specimens have been frozen in sterile cryovials within a cryoprotectant medium volume of 0.3 ml. A Planer Series III biological freezer is utilized. The Cornell protocols have been amended from the early published work in several ways to fit our current needs. Modifications include: 1) the base medium is a phase I sequential formulation, modified by HEPES buffers; 2) extra macromolecules (protein) are added in the form of 0.5 g/l HSA (5% HSA solution) and ~16% Plasmanate; and, 3) for blastocysts, the freezing cryoprotectant concentration is elevated to 10% and additional dilutions are included for the thawing process.

Prezygote freezing

Specimens were loaded directly into cryovials containing 1.5 mol/l PROH and equilibrated for 30 min at room temperature. Within the biological freezer, cryovials were cooled at a rate of –1.0°C/min until –6.5°C. They were then held for 5 min, manual seeding was performed, and they were held for an additional 5 min. Cooling was continued at a rate of –0.5°C/min to –80°C. Cryovials were then plunged into liquid nitrogen.

Prezygote thawing

Cryovials were placed in a pre-cooled biological freezer at –100°C. They were then held for 5 min. Warming was accomplished at +8.0°C/min until room temperature was reached. Prezygotes were removed from cryovials and held for 5 min before being taken through decreasing concentrations of cryoprotective medium: 1.0 mol/l PROH for 3 min, 0.75 mol/l PROH for 3 min, 0.5 mol/l PROH for 3 min, 0.25 mol/l PROH for 3 min, 0.0 mol/l PROH for 3 min. Specimens were washed thoroughly and incubated overnight. Survival was defined as cleavage to at least the 2-cell stage.

Pre-embryo freezing

Pre-embryos were exposed to increasing concentrations of cryoprotective medium at room temperature: 0.5 mol/l PROH for 5 min, 1.0 mol/l PROH for 5 min, 1.5 mol/l PROH for 10 min, 1.5 mol/l PROH/sucrose for 10 s. They were then loaded into cryovials containing 1.5 mol/l PROH/sucrose. Cryovials were equilibrated for 15 min at room temperature before being cooled at a rate of –2.0°C/min until –7.0°C. They were held for 5 min, manual seeding was performed, and they were held for an additional 5 min. Cooling was continued at a rate of –0.3°C/min until –30°C. Cryovials were then plunged into liquid nitrogen.

Pre-embryo thawing

Cryovials were warmed in a 30°C water bath for 30–90 s and then held for 5 min at room temperature before pre-embryos were removed. Pre-embryos were taken through decreasing concentrations of cryoprotective medium: 1.0 mol/l PROH/sucrose for 3 min, 0.75 mol/l PROH/sucrose for 3 min, 0.5 mol/l PROH/sucrose for 3 min, 0.25 mol/l PROH/sucrose for 3 min, 0.0 mol/l PROH/sucrose for 3 min. Specimens were then washed thoroughly and incubated until intrauterine transfer.

Blastocyst freezing

Blastocysts were exposed to two concentrations of cryoprotective medium at room temperature: 5% glycerol solution for 10 min and 10% glycerol/0.2 mol/l sucrose solution for 10 min. They were then loaded into cryovials and cooled at a rate of –2.0°C/min until –7.0°C. Cryovials were held for 5 min, manual seeding was performed, and they were held for an additional 10 min. Cooling was continued at –0.3°C/min until –38°C. Cryovials were then plunged into liquid nitrogen.

Blastocyst thawing

Cryovials were thawed at room temperature for 60 s before being warmed in a 30°C waterbath for 30–90 s (until all ice was removed). Blastocysts were removed from the cryovials and taken through decreasing concentrations of cryoprotective medium: 10% glycerol + 0.4 mol/l sucrose for 30 s, 5% glycerol + 0.4 mol/l sucrose solution for 3 min, 0.4 mol/l sucrose solution (no glycerol) for 3 min, 0.2 mol/l sucrose solution (no glycerol) for 2 min and 0.1 mol/l sucrose solution (no glycerol) for 1 min. Specimens were then washed thoroughly and incubated until transfer. Blastocysts frozen on day 5 were incubated overnight; blastocysts frozen on day 6 were transferred the same day as thawing.

Replacement strategies in Cornell

At Cornell, frozen–thawed conceptuses are replaced in either natural or programmed cycles. Natural cycles are not supplemented with progesterone unless there is an overwhelming reason to do so, and all women are treated in a prophylactic manner for 4 days with antibiotics and corticosteroids.

Natural cycle replacement (used in ovulatory cycles with normal concentrations of luteal phase progesterone)

Supplemental progesterone was not administered unless medically indicated or unless the patient experienced a previous pregnancy failure using a non-supplemented protocol. If administered, 200 mg micronized progesterone was given vaginally b.i.d. or t.i.d. and continued until a negative pregnancy test 14 days after replacement or through week 12 if pregnant (weaned down starting weeks 9–10). Medrol (16 mg/day; Pharmacia Corporation, Peapack, NJ, USA) and tetracycline (250 mg, q.i.d.) were administered for 4 days, beginning on the day of the LH surge.

Prezygotes were thawed on the day of ovulation or the next day (1 day after LH peak and/or day of oestradiol dip) and transferred on the day after thaw.

Pre-embryos were thawed 1 day after ovulation (2 days after LH peak and/or day after oestradiol dip) and transferred on the day of thaw.

Blastocysts were thawed 4 days after the LH peak and transferred on the following day (day 5 blastocysts) or thawed 5 days after LH peak and transferred on the same day (day 6 blastocysts).

Programmed cycle replacement (adequate suppression confirmed on day 2 of cycle)

Luteal suppression was accomplished using 0.2 mg gonadotrophin-releasing hormone agonist (GnRH-a). This dosage was reduced to 0.1 mg starting on the predetermined day 1 of the cycle and maintained until day 15.

Transdermal oestrogen patches (Climara 0.1 mg patch; Berlex Laboratories, Montville, NJ, USA) were administered as follows: days 1–4, 0.1 mg every other day; days 5–8, 0.2 mg every other day; days 9–10, 0.3 mg every other day (depending on oestradiol concentrations); days 11–14, 0.4 mg every other day; days 15+, 0.2 mg (two patches every other day, 7 weeks).

Progesterone (50 mg i.m.) was administered beginning on day 15 through 12 weeks gestation (weaned down starting week 9–10, depending on serum concentrations). Medrol (16 mg/day) and tetracycline (250 mg, q.i.d.) were administered beginning on day 15 for 4 days. Prezygotes were thawed on

day 16 and transferred the following day; pre-embryos were thawed on day 17 and transferred the same day; blastocysts frozen on day 5 were thawed on day 19 and transferred the following day; blastocysts frozen on day 6 were thawed on day 20 and transferred the same day.

Observations: does one developmental stage freeze better than another?

Prezygotes

Freezing success with the pronuclear stage has spanned more than a decade and has culminated in thousands of births. It is thought that the prezygote's lack of a spindle in large part explains its excellent survival and potential for implantation. Being single-celled, it is easy to determine whether or not a prezygote has survived thawing; when its membrane is not intact, the cell appears flattened and usually dark in colour. Left in culture for 15–24 h, the healthy pronucleate oocyte enters into syngamy, completes the fertilization process, and proceeds to the first cleavage. Cell division is therefore the true indicator of survival; fewer than 5% of prezygotes appearing healthy immediately after thaw fail to complete their first mitosis.

Despite the good results achieved after freezing this stage, there are also certain disadvantages. Because prezygotes are frozen before cleavage occurs, there are few reliable indicators to assist in determining that the cell possesses the capacity for continued growth. Consequently, prezygotes with limited potentiality are sometimes frozen. It is disappointing to freeze a large number of prezygotes for a woman only to find that the ones not frozen arrest in culture or exhibit abnormal morphological characteristics by days 2 or 3. In such cases, the patient might have been better served if freezing had been delayed. Furthermore, it is important to begin freezing the prezygote before breakdown of pronuclei, since entrance into syngamy negatively impacts results. This urgency to start freezing procedures may be inconvenient for some programmes without adequate staffing.

The morphology of the thawed prezygote is generally similar to its pre-freeze appearance, but occasionally, the cytoplasm appears clearer and organelle accumulation around pronuclear structures is reduced after thaw. Also, it is not uncommon to see nucleoli scattered randomly within pronuclear structures post-thaw despite an alignment at pronuclear junctions before freezing.

Pre-embryos

Like pronuclear oocytes, pre-embryos freeze well and implant at acceptable rates after thaw and transfer. Almost any cleavage-stage specimen can be frozen successfully, from 2-cell to blastocyst. Freezing the pre-embryo is fairly convenient because, unlike the prezygote, there are no urgent timing considerations. In addition, information is known about both morphology and growth rate, allowing the selection of potentially viable conceptuses for either fresh transfer or storage. It has become extremely common in the last decade to choose pre-embryos with the best morphology for fresh transfer and to freeze others with acceptable morphology only after fresh selection has been made.

Sometimes survival after thaw is difficult to evaluate because not all blastomeres endure the rigours of freezing and thawing. Dying blastomeres may be present amongst living ones, but these can be removed easily by aspirating them out through an artificial hole in the zona pellucida. Generally, a pre-embryo possessing >50% viable blastomeres upon thaw is considered a survivor. There is no convincing evidence to suggest that the loss of one or two blastomeres is detrimental to 8-celled human or mouse pre-embryos (Veiga *et al.*, 1987; Hartshorne *et al.*, 1991; Rulicke and Autenried, 1995). Nonetheless, it has been reported that fully intact pre-embryos demonstrate higher implantation rates than do partially intact ones (Van den Abbeel *et al.*, 1997).

Blastocysts

Blastocysts have the advantage of possessing many cells. The loss of a few during freezing and thawing will not compromise the integrity of the entire specimen. This may be one reason why blastocysts have been frozen and thawed so successfully over the years in domestic animals for both research and commercial purposes. Blastocyst cryopreservation in the human was first reported by Cohen *et al.* (1985), using glycerol in a series of 10 increasing concentrations. Following that initial report, blastocyst freezing was only occasionally incorporated into clinical protocols because of the difficulties involved with maintaining high rates of blastocyst development *in vitro*.

Through the 1990s, reports of clinical pregnancy after blastocyst thaw fell in the range of 10–30% per transfer (Kaufman *et al.*, 1995), percentages not significantly better than results with earlier stages. Although several groups reported freezing blastocysts quite successfully, early attempts often relied on co-culture systems to support pre-embryo growth (Ménézo *et al.*, 1992; Freitas *et al.*, 1994; Ménézo and Ben Khalifa, 1995). Today, the availability of sequential media has led to a dramatic increase in the practice of blastocyst freezing and pregnancy rates well over 50% are being realized following the replacement of thawed blastocysts.

Few reports have been published detailing the efficiency of blastocyst freezing after culture in sequential media. Langley *et al.* describe a comparison of thawed day 3 pre-embryos versus blastocysts during a 30-month period (Langley *et al.*, 2001). In this study, the survival rate was higher for blastocysts and the implantation rate was doubled (21.9 versus 10.1%, 72 blastocyst cycles). In 2002, Behr *et al.* reported a 36% clinical pregnancy rate and 16% implantation rate for thawed blastocysts from 64 cycles (Behr *et al.*, 2002). Given these few peer-reviewed reports generated after extended culture in sequential media, there may not be adequate evidence to support the concept that blastocysts are now optimal for human freezing trials.

Nonetheless, the Cornell programme has benefited greatly from the adoption of blastocyst freezing protocols (**Table 1**). While acceptable clinical pregnancy rates of nearly 40% were realized after freezing and thawing cleavage-stage pre-embryos in more than 700 cycles, much higher rates were

Table 1. Survival, pregnancy and implantation by stage of development[g].

Stage transferred	*Survived/thawed (%)*	*Clinical pregnancies/transfer (%)*	*Implanted/no. transferred (%)*
Prezygotes only	1101/1441 (76.4)	99/235 (42.1)[a]	170/997 (17.10)[d]
Pre-embryos only	1646/2093 (78.6)	153/409 (37.4)[b]	231/1516 (15.2)[e]
Blastocysts only	196/254 (77.2)	52/81 (64.2)[c]	67/174 (38.5)[f]
Mixed thaw and transfer	411/561 (73.3)	36/69 (52.2)	72/367 (19.6)

[a–f]$P < 0.05$ in favour of blastocysts for comparisons a versus c, b versus c, d versus f, and e versus f.
[g]Prezygotes and pre-embryos frozen January 1995 to June 2002; blastocysts frozen July 2000 to June 2002.

established using blastocysts (>60%) without any concomitant drop in the number or proportion of patients having conceptuses frozen. Nearly one in four women under age 40 have had blastocysts frozen after undergoing day 3 transfers, and 60% of women undergoing day 5 transfers have had at least one blastocyst cryopreserved on day 5 or day 6. Two-thousand blastocysts have been frozen in just over 2 years, though less than one-fifth have been thawed since so many of the patients involved have not returned for a second child after becoming pregnant from their fresh cycles.

Most of the blastocysts frozen in the Cornell programme are generated following the fresh transfer of day 3 conceptuses. After intrauterine transfer, remaining viable pre-embryos are examined each day for 2 or 3 additional days to evaluate their suitability for freezing. This has been termed the post-transfer observation period. Blastocysts forming on either day 5 or day 6 are cryopreserved for future use. Only rarely and under special circumstances have day 7 conceptuses been frozen. Extended culture is carried out using a complex, non-commercial sequential medium prepared weekly on-site. Pre-embryos are grown in phase I of this medium for days 1–3 and in phase II for days 4–6. All pre-embryos are cultured under conditions of 5.5% CO_2 in air at 37.2°C.

The survival rate for thawed blastocysts is very stable at 77%. Clinical pregnancy per cycle with blastocysts thawed and replaced is 64%; the ongoing or delivered rate is 54% and the implantation rate is 39% (**Table 1**). Pregnancy rates are not different whether blastocysts are replaced in either natural or programmed cycles. Furthermore, pregnancy rates with blastocysts are stable across all maternal ages; 14/21 women (67%) over the age of 40 have established clinical pregnancies although their miscarriage rate is more than double that observed for younger women (24% miscarriage; 43% ongoing).

It is generally assumed that blastocysts that develop in a timely manner *in vitro* are of better quality than those that develop more slowly. However, a retrospective review of blastocyst thaw outcomes from Cornell demonstrates otherwise. As stated earlier, blastocysts have been frozen on either day 5 or day 6 depending on their speed of growth *in vitro*. Day 5 frozen blastocysts are thawed the day before transfer, while day 6 blastocysts are thawed the morning before transfer is carried out. In a recent abstract for the ASRM, pregnancy outcomes were analysed in 81 consecutive patients returning for thawed blastocysts over a 2 year period (Clarke *et al.*, 2002). Thirty-nine patients received a transfer of day 5 frozen–thawed blastocysts and 42 patients underwent transfer with day 6 blastocysts. There were no significant group differences in patient age (34.0 versus 35.0 respectively), average number of blastocysts transferred (2.3 versus 2.0), or morphology of the blastocysts after thawing. No significant differences were found in the post-thaw survival rates (73.4 versus 80.5%), clinical pregnancy rates (63.2 versus 63.4%), or ongoing pregnancy rates (55.3 versus 53.7%), nor were differences observed in implantation rates (39.8 versus 39.5%).

While it is logical to assume that pre-embryos reaching the blastocyst stage faster (day 5) would be 'healthier' than their day 6 counterparts, these data and the data of others suggest that rate of development may not be crucial to subsequent post-thaw success (Behr *et al.*, 2002). Surprisingly, this is in direct conflict to reports of fresh transfer using day 5 and day 6 blastocysts, where pregnancy was observed to be significantly lower with the slower-growing conceptuses (Shapiro *et al.*, 2001). Also, in contrast to our work, Marek *et al.* carried out a study comparing outcomes from 127 thawed blastocyst cycles where blastocysts were frozen on day 5 or day 6 (Marek *et al.*, 2000). Survival rates post-thaw were good for both groups, but the clinical pregnancy rate per thaw (50 versus 29% respectively), ongoing pregnancy rate per thaw (43 versus 23%), and implantation rate (34 versus 15%) were all significantly lower for day 6 blastocysts. Why these results are so different from our own is not clear.

Comparative results of differing developmental stages at freeze

Results of prezygote, pre-embryo, and blastocyst thaw trials are detailed in **Table 1**. Statistical significance was determined by chi-square analysis.

In this retrospective analysis, blastocysts demonstrated similar survival, but were associated with statistically higher clinical pregnancy and implantation rates when compared with earlier stages.

Calculating pregnancy potential from prezgyote, pre-embryo, and blastocyst stages

Of the many tribulations associated with running a cryopreservation programme, one of the most frustrating is that embryologists cannot reap the fruits of their labour

(pregnancy after thawing) until months or years have passed. It is common for patients to wait for some time before returning for a thaw attempt after a negative fresh cycle, or to delay 2 or more years after the birth of a child. This situation gives rise to special problems in tracking results during a given freezing period and makes it difficult to identify the efficiency of a new protocol.

There are three common ways to analyse freezing/thawing results. These include calculating pregnancy rate per thaw attempt, pregnancy rates per cycle with transfer of thawed conceptuses, and calculating an *augmented* pregnancy rate per cycle with freezing based on fresh pregnancy plus thawed pregnancy. This latter method has been discussed in detail by Dr Howard Jones in numerous publications (Veeck *et al.*, 1993; Jones *et al.*, 1995, 1997a,b; Schnorr *et al.*, 2000).
In the last analysis, *augmented* pregnancy rate refers to the actual cumulative pregnancy rate achieved by patients upon combining pregnancies established from both fresh and thawed transfers:
(i) The *base* fresh pregnancy rate is defined as the number of clinical pregnancies established after the transfer of non-cryopreserved pre-embryos over the number of non-cryopreserved (fresh) transfer cycles, i.e. $(250/500) \times 100 = 50\%$.
(ii) The *augmented* pregnancy rate is defined as the actual number of clinical pregnancies generated by the transfer of non-cryopreserved pre-embryos *plus* the actual number of clinical pregnancies generated by the transfer of thawed pre-embryos in cycles failing to become pregnant with fresh transfer, over the number of transfer cycles, i.e. $(250/500 + 125/500) \times 100 = 75\%$.
(iii) A *projected* augmented pregnancy rate can defined as the actual number of clinical pregnancies generated by the transfer of non-cryopreserved pre-embryos *plus* the actual number of clinical pregnancies generated by the transfer of thawed pre-embryos in cycles failing to become pregnant with fresh transfer, *plus* the number of clinical pregnancies expected from the potential transfer of conceptuses still in cryostorage for patients not yet pregnant from fresh or thawed attempts (this last calculation uses the thawed pregnancy rate established to date) over the total number of cycles with a transfer, i.e. $(250/500 + 125/500 + 25/500) \times 100 = 80\%$. The validity of reporting this last *projected* cumulative pregnancy rate is open to criticism because of its reliance on past performance and assumptions that future results will be similar.

Using the augmented pregnancy model described here, Cornell results are shown in **Table 2** where blastocysts appear to be an optimal stage for freezing.

Children born following cryopreservation and thawing

Cryopreservation has no apparent negative impact on perinatal outcome and does not appear to affect adversely the growth or health of children during infancy or early childhood (Wennerholm *et al.*, 1998). Furthermore, the available data do not indicate an elevation in congenital malformations for children born after freeze–thaw procedures (Wada *et al.*, 1994; Tarlatzis and Grimbizis, 1999; Wennerholm, 2000). While it remains unclear if freezing poses long-term risks to children so conceived, there is no direct evidence thus far to raise concern.

Quality control considerations

Quality control procedures for cryopreservation include all those necessary for routine IVF (maintenance and documentation of temperature, environment, toxicity, and sterility). Extra measures extend further to maintaining daily biological freezer chart recordings, logging exact seeding temperatures, and regular freezing/thawing of abnormally fertilized conceptuses to provide instant feedback on acceptable survival rates. By consistent adherence to these basic techniques, one may feel relatively assured that what is being frozen today will not present a problem in the future after thawing. In addition, biological freezers must be checked regularly under a preventative maintenance agreement for accuracy of temperature and function.

Hazard plans

Disaster prevention is the key to a successful freezing strategy. Techniques should be designed to eliminate unexpected problems during actual freezing and thawing procedures and should cover any potential loss of stored specimens or records. Not unusual in large programmes, many thousands of cryopreserved conceptuses may be housed in storage tanks; explosion, fire, flood, and chemical hazards are of utmost concern because of the enormous responsibility and liability that such storage entails. The following suggestions may prevent future unforeseen problems:
(i) Establish a written hazard plan for unexpected disaster. At Cornell, security staff are introduced to the laboratories, informed of the necessity of moving storage tanks to a safe area in the event of after-hours hazard, and provided with a written list of emergency procedures and pertinent phone numbers. These phone and pager numbers are updated on a routine basis.
(ii) A second biological freezer should be available as backup in case of primary machine malfunction.

Table 2. Cumulative pregnancies, January 1995 to June 2002.

	Prezygotes only frozen (%)	*Pre-embryos only frozen (%)*	*Blastocysts only frozen (%)*	*Mixed frozen (%)*
Fresh base clinical pregnancies/transfer	63.8	68.2	73.9	78.4
True augmented cumulative	70.9	75.6	80.2	86.0
Projected augmented cumulative	71.2	76.9	85.2	87.8

(iii) Biological freezers should be kept on emergency backup power and/or may be connected to an uninterrupted power supply to prevent loss or spiking of power during use.
(iv) Embryologists should know to distrust the gauges of delivered liquid nitrogen tanks; for safety, a secondary liquid nitrogen source should be available.
(v) Perform regular inspections of cryostorage tanks. Assess the integrity of vacuum seals and measure liquid nitrogen levels; document. Establish a routine plan for topping off liquid nitrogen levels in storage tanks, even if tanks possess a 'self-fill' mechanism. Never reuse liquid nitrogen or transfer from one source to another.
(vi) Record cryostorage locations on original patient documents and duplicate storage information using at least one other method (computerized database, hand-written log, or other). For safety, keep copies of these records at two different and separate locations.

Benefits

The value of cryopreserving prezygotes, pre-embryos, or blastocysts for future thaw and transfer is an important consideration of every IVF program. The techniques offer many benefits related to increasing the number of healthy pre-embryos available for current or future transfer. While a higher pregnancy rate is often correlated to a greater number of transferred pre-embryos, the incidence of multiple pregnancy increases as well. The convergence of two factors, a higher pregnancy rate and a lower multiple gestation rate, can be managed effectively through the establishment of a successful cryopreservation program.

Addendum

This lecture summary was prepared for a symposium that honoured Doctors Howard and Georgeanna Jones. While the text supplied here does not relay the affection and gratitude that was expressed for these individuals during the actual presentation, their enduring guidance and leadership was acknowledged and understood by all in attendance.

Two of the greatest lessons learned from Drs Howard and Georgeanna Jones are given below:

GSJ: 'Remember that when things don't go as well as expected, never point a finger at someone else, rather always assume first that the problem might lie in your own work.'

HWJ: 'An enormous mistake is made by those who believe they will elevate themselves through demeaning others. The same applies to the foolish person who believes he will remain on top by refusing to share knowledge and methods.'

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