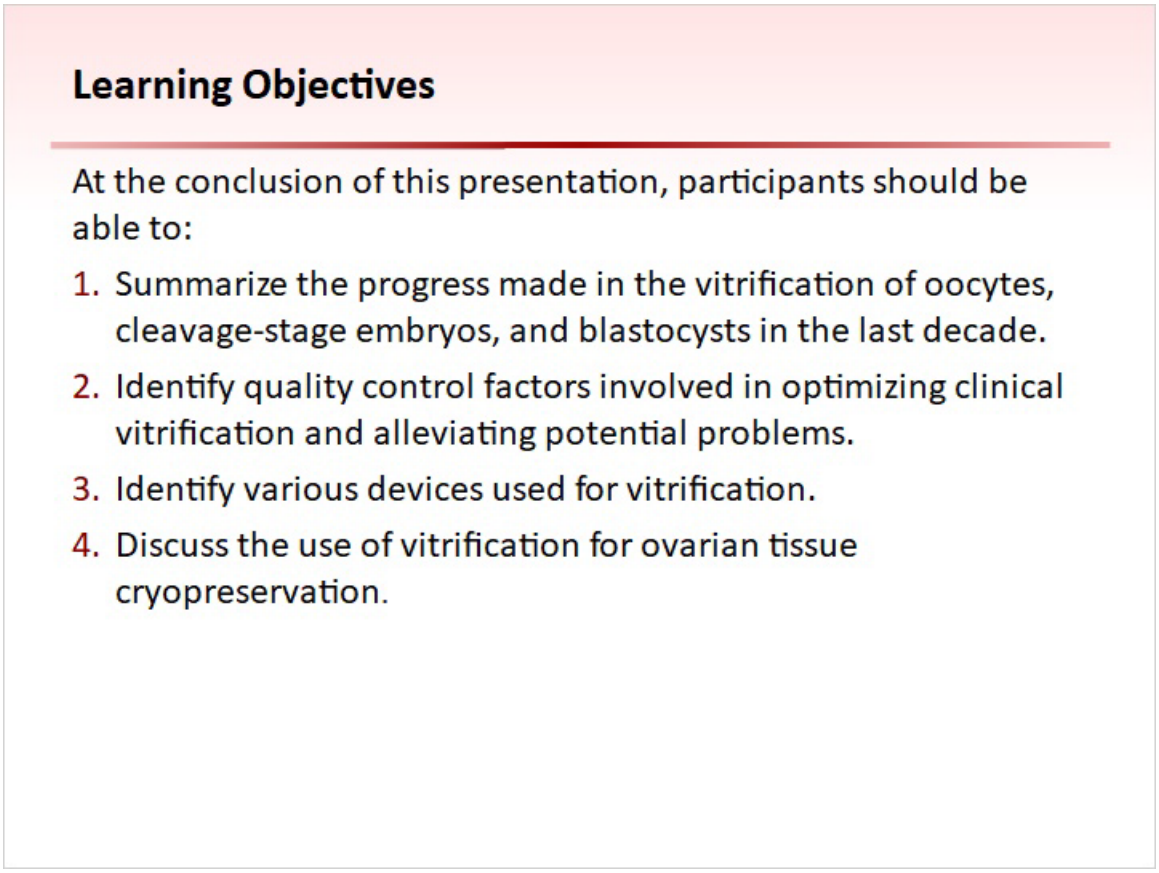
LABCC100 Lesson 25

1.1 Vitrification of Oocytes, Embryos, and Tissues

Notes:

Welcome to the American Society for Reproductive Medicine's eLearning modules. The subject of this presentation is Vitrification of Oocytes, Embryos, and Tissues.

1.2 Learning Objectives

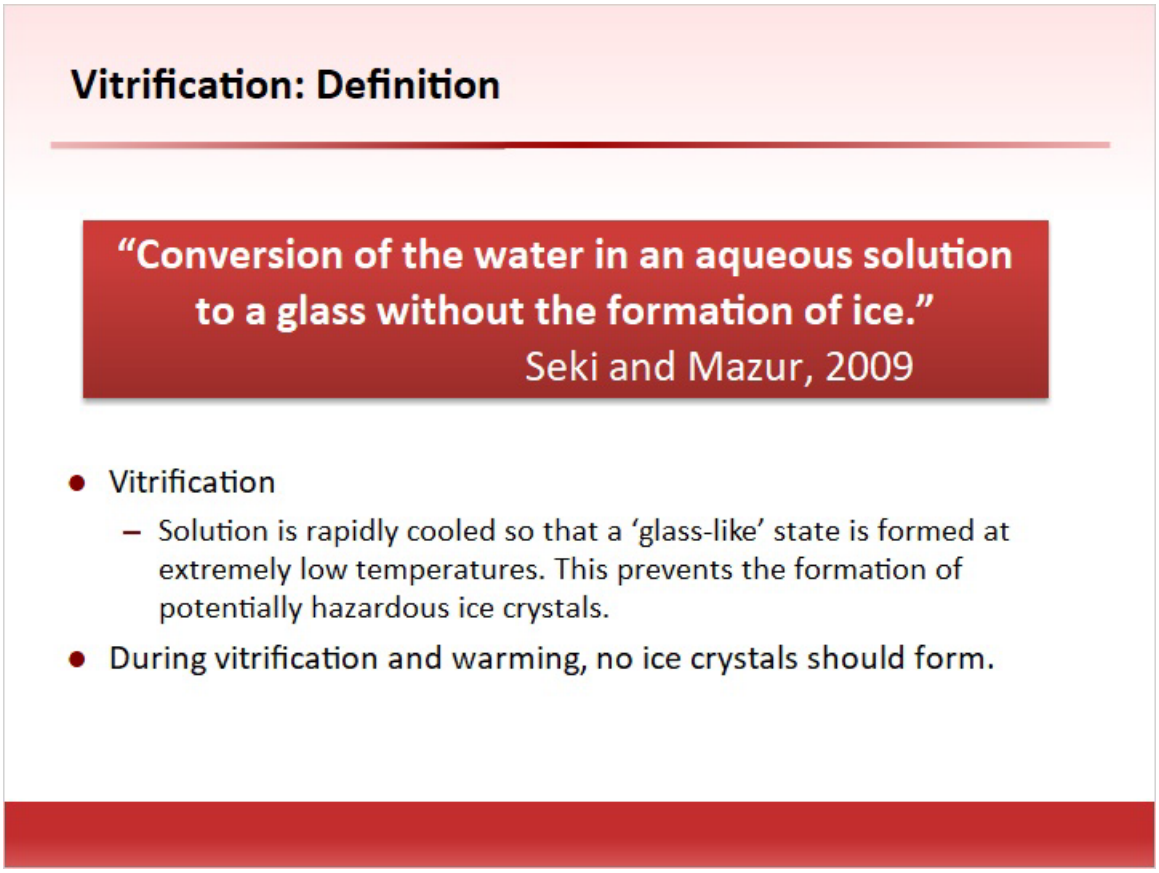


Notes:

At the conclusion of this presentation, participants should be able to:

1. Summarize the progress made in the vitrification of oocytes, cleavage-stage embryos, and blastocysts in the last decade.
2. Identify quality control factors involved in optimizing clinical vitrification and alleviating potential problems.
3. Identify various devices used for vitrification.
4. Discuss the use of vitrification for ovarian tissue cryopreservation.

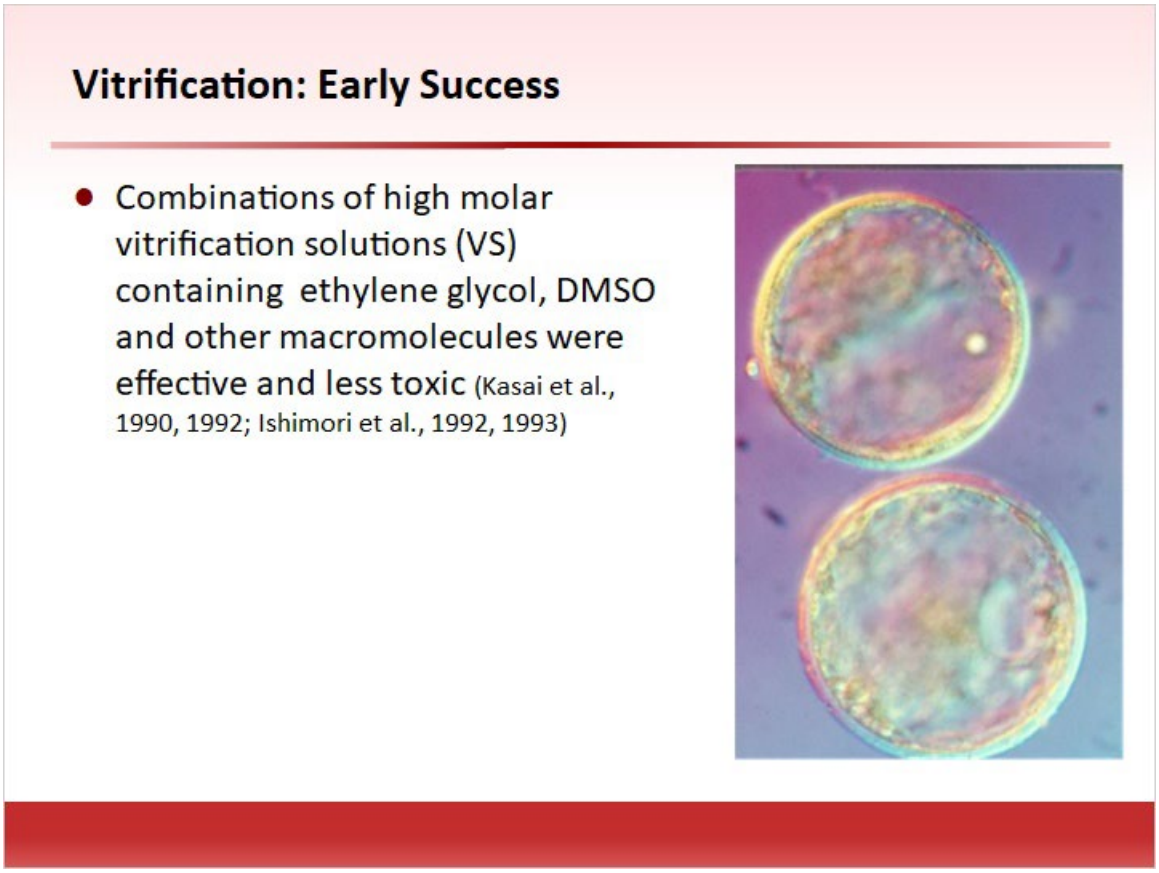
1.3 Vitrification: Definition



Notes:

Although there are several methods for cryopreserving cells, oocytes and embryos primarily are cryopreserved by either what is known as 'slow-freezing' or by vitrification. Vitrification is defined as the process of the conversion of water in an aqueous solution to a glass without the formation of ice. Other methodologies will not be discussed here. There are notable differences between slow-freezing and vitrification. In the slow-freeze method, as the temperature is lowered, water will crystallize outside the cell. This will osmotically draw water from within the cell as the extracellular solute concentration increases. In contrast, during vitrification the solution is rapidly cooled so that a 'glass-like' state is formed at extremely low temperatures. This prevents the formation of potentially hazardous ice crystals. During vitrification and warming, no ice crystals should form.

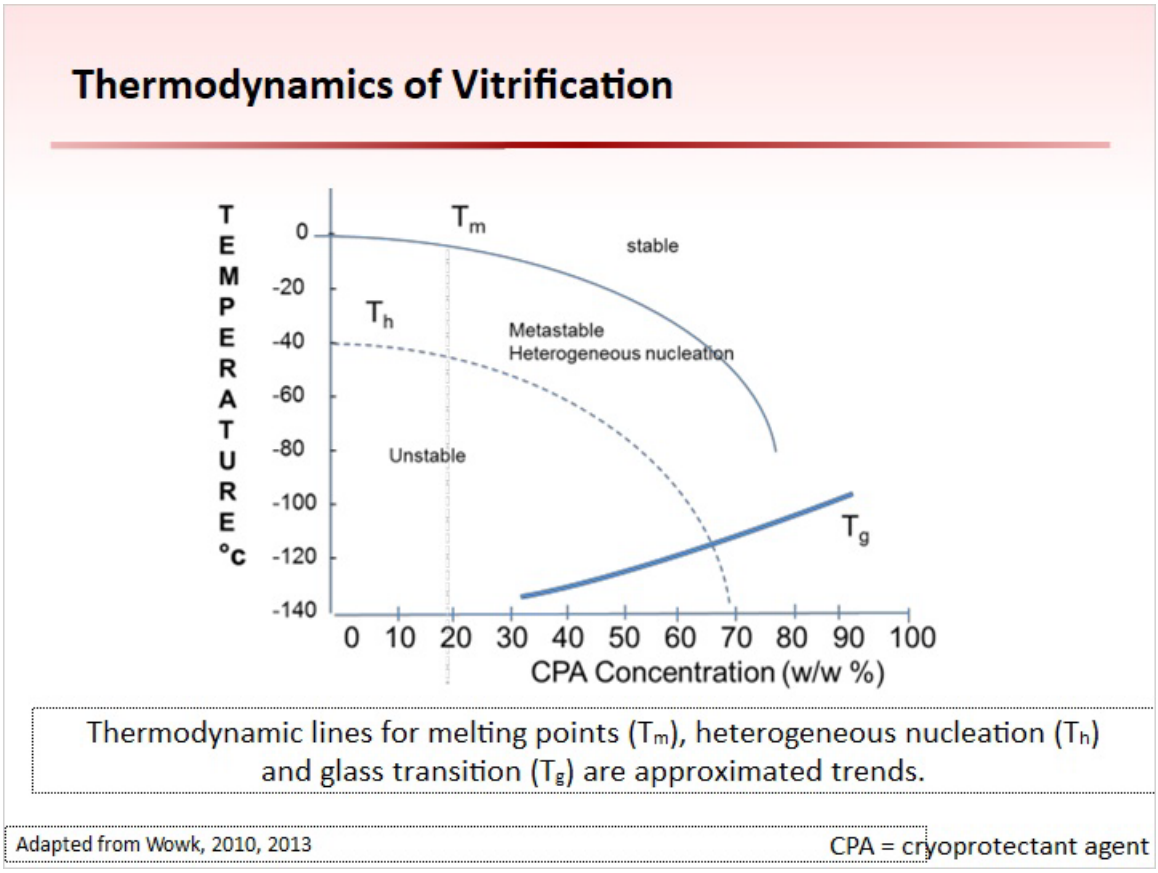
1.4 Vitrification: Early Success



Notes:

Early attempts at vitrification revealed that an effective combination of vitrification solutions composed of ethylene glycol, DMSO, and other macromolecules reduced the potential cytotoxicity compared with the use of individual cryoprotective agents. The added molecules served to increase solution viscosity and increase the potential efficiency of forming a glass-like solid devoid of ice formation under rapid cooling and warming.

1.5 Thermodynamics of Vitrification



Thermodynamic lines for melting points (T_m), heterogeneous nucleation (T_h) and glass transition (T_g) are approximated trends.

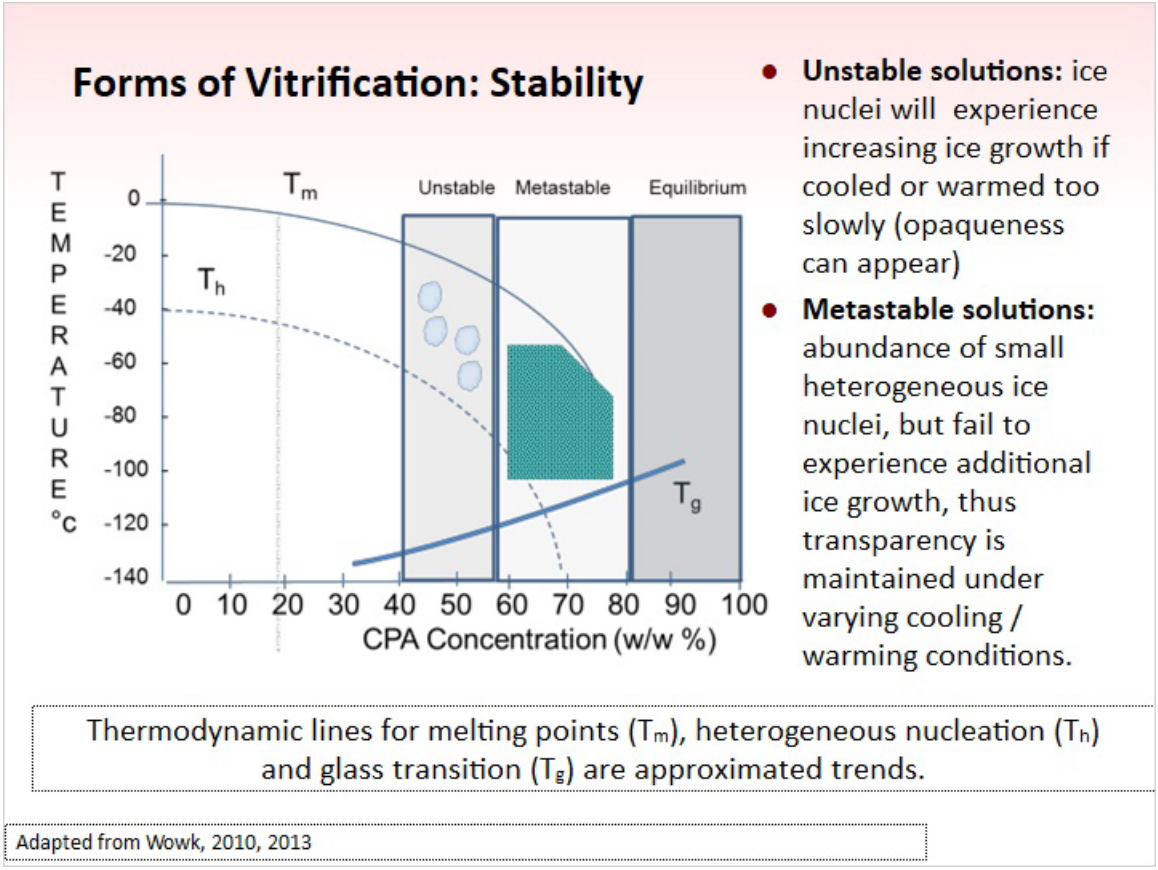
Adapted from Wowk, 2010, 2013

CPA = cryoprotectant agent

Notes:

The complex thermodynamics involved in vitrification depend on the rates of cooling and subsequently warming, with respect to the total concentration of cryoprotectant. In understanding vitrification, a few thermodynamics concepts are shown on the Phase Diagram. $T_{m,}$ as shown in the upper thin blue line is equal to the temperature where ice melting occurs; T_h as shown in the dashed line is equal to the temperature where heterogeneous nucleation of chemical impurities in solution occurs; depending on the relative size of the impurity particle, a curve can be drawn. Below that curve, homogeneous nucleation and spontaneous ice formation can occur, down to a temperature where glass formation (T_g) can occur in solution with approximately 30% cryoprotectant agent or more (thick line). A line of stability can be drawn at approximately 60% concentration of cryoprotectant where T_m is essentially equivocal to heterogeneous ice nucleation, thus reducing or preventing ice growth. Furthermore, T_h approaches T_g suggesting that spontaneous ice formation is not a risk. Therefore, for practical purposes one can refer to vitrification in terms of being STABLE, METASTABLE or UNSTABLE, depending on the concentration of cryoprotectant.

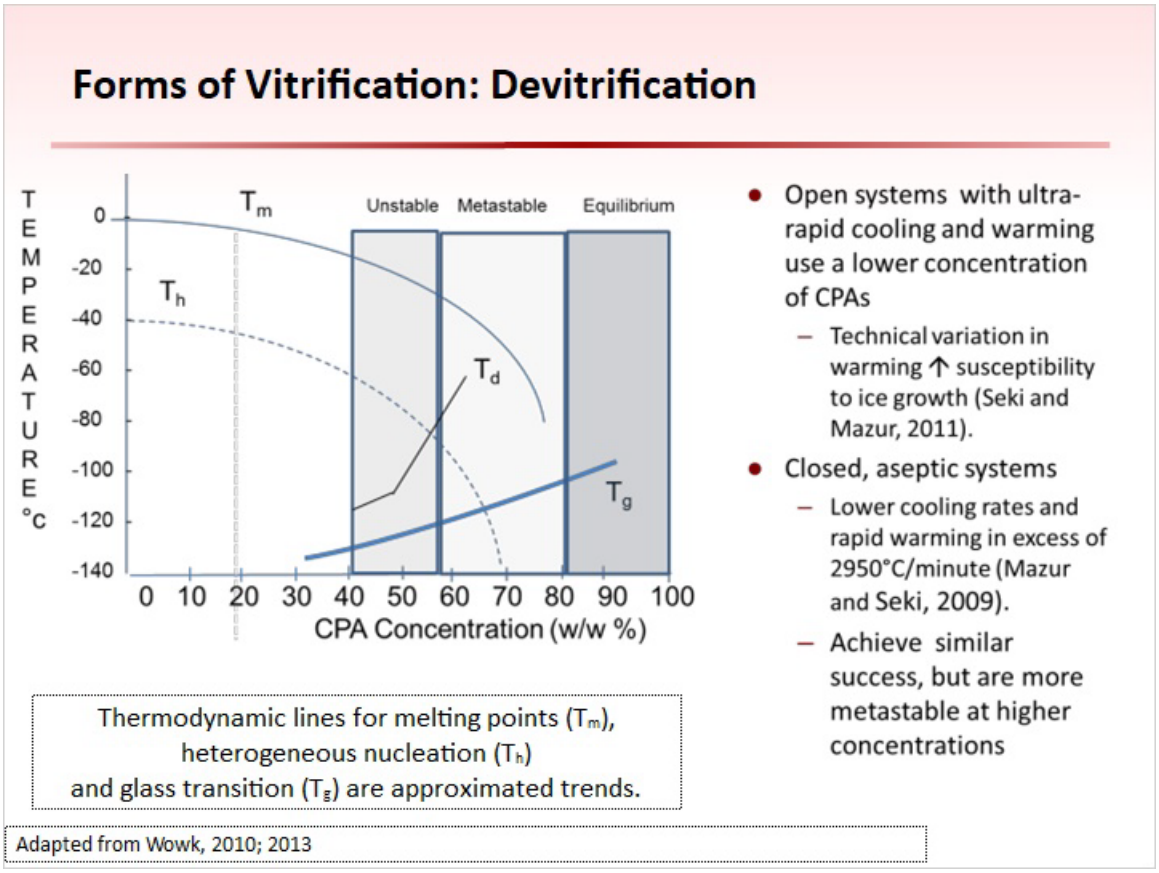
1.6 Forms of Vitrification: Stability



Notes:

Under a low experimental warming rate (e.g., 40°C/minute), heterogeneous ice nucleation of impure solution particles can be visualized and serve as a template for progressive ice growth (i.e., recrystallization), relative to the total concentration of cryoprotectant and warming rate. In metastable solutions, diffuse micro-ice nuclei can be observed. However, because they are smaller than a wavelength of light, the solution remains transparent. These solutions are not dependent on rapid cooling and/or warming to minimize recrystallization. The unstable solutions may form fewer ice nuclei in number, but the nuclei are larger and susceptible to further growth unless rapidly warmed. The opaque characteristic is a sign of ice formation rather than the desired 'glass-like' solution. Both the type of vitrification device and volume of solution used influence cooling and warming rates. This is of particular concern with "unstable non-equilibrium" vitrification.

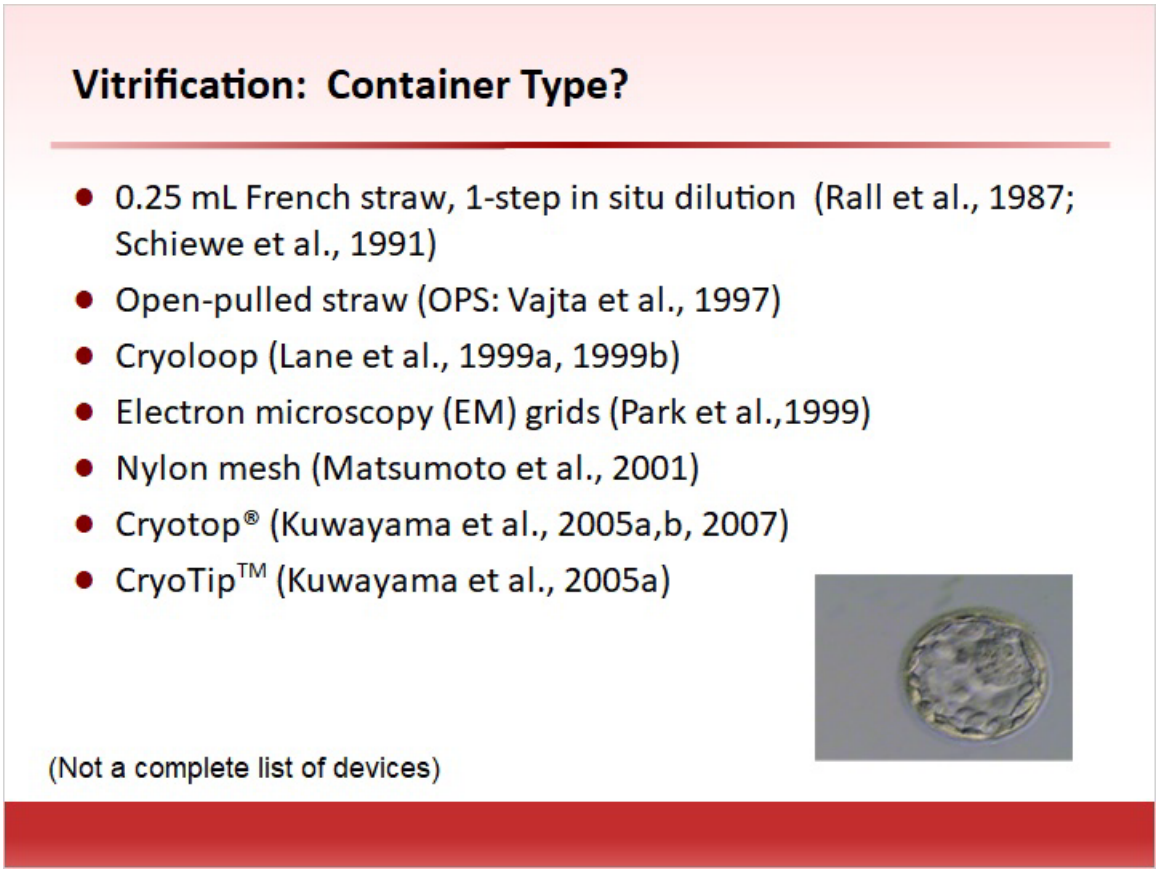
1.7 Forms of Vitrification: Devitrification



Notes:

This phase diagram defines zones of vitrification stability. It also introduces the concept of devitrification, which was defined by Seki and Mazur (2009) as “the conversion of a glass to a highly viscous supercooled liquid.” Crystallization of ice nuclei is associated with the devitrification process where the supercooled liquid is converted to ice during warming. The latter formation of ice is referred to as “recrystallization.” Within the metastable range, the rate of T_d outruns spontaneous ice formation (i.e., unstable homogeneous nucleation). The survival of oocytes and embryos vitrified in the “unstable” zone is highly dependent on warming rates to minimize the physically injurious effects of recrystallization.

1.8 Vitrification: Container Type?



Notes:

As knowledge of vitrification thermodynamics improved, new low-volume container systems and devices were developed. One of the earliest was a variation of the 0.25 mL straw that was heat-pulled on one end to over a 200% reduction in the inner diameter. The cryoloop device (Lane et al., 1999) was developed for x-ray crystallography experimentation and adapted to secure an embryo in a thin film of cryoprotectant, creating ultrarapid cooling conditions while stored in the hull of a cryovial. Other thin droplet systems were devised using electron microscopy grids or nylon mesh, but ultimately found limited use. The Cryotop® device, a plastic stick/open blade method, holds a microdroplet of vitrification solution with the embryo on its flattened end (i.e., the blade). Depending on the amount of excess solution extracted, cooling rates could be significantly lowered (10,000-20,000°C/min) compared with straw devices. It was popular early on among noncryobiologists who believed that ultrarapid cooling rates were essential to vitrification of embryos and oocytes. Several mimicking devices were subsequently developed and used in clinical application. The first commercial device that met the US Food and Drug Administration's (FDA) Current Good Manufacturing Practice (cGMP) regulations was a "closed system" that was sealed closed on both ends. However, meeting FDA approval did not

necessarily mean there were not inherent flaws in the system.

1.9 Vitrification Devices

Notes:

Over 25 different vitrification device systems have been developed for the experimental or clinical vitrification of embryos. Shown here are commercialized and noncommercial systems that have been used in clinical assisted reproductive technology (ART). These included open and closed systems, as well as aseptic-closed systems. The latter are double-container systems that prevent the actual vitrification device from coming into direct contact with liquid nitrogen, thus preventing any possible cross-contamination in storage. That issue will be addressed later.

1.10 Storage Systems and Devices: 1980s-2000s

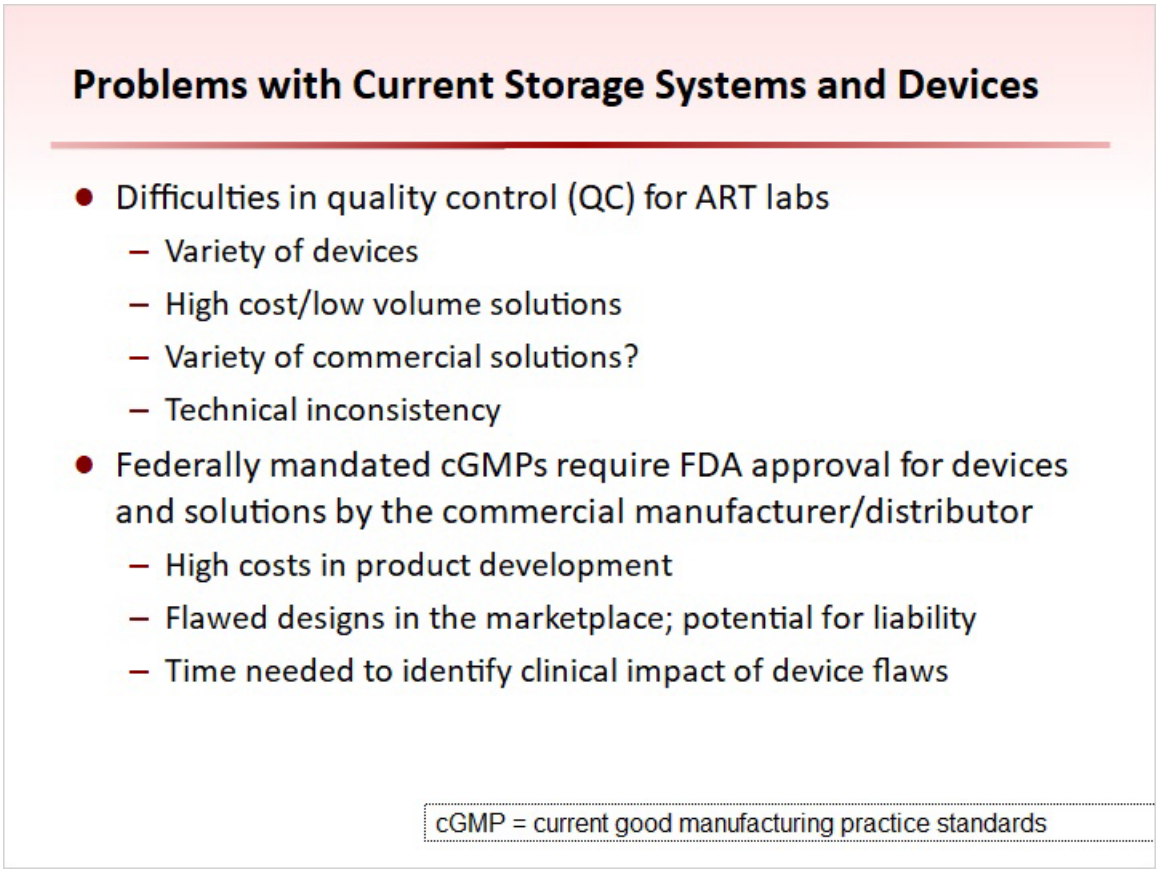
Storage Systems and Devices: 1980s-2000s

- Straws and vials used commonly for standard cryopreservation storage
- Straws
  - High capacity storage, uniform cooling/warming, sealed container, ease of handling
- CBS™ High Security Embryo Straw introduced in 2003
  - Sterile, secure storage, tamperproof, internalized labeling

Notes:

Two container systems (straws and vials) were used successfully for over two decades. Straws became the preferred device because of their high storage potential, uniform cooling/warming (high surface to volume ratio), ease of handling, and the ability to be heat-sealed as a closed container. A significant improvement for slow freezing of embryos was the development of the CBS™ High Security Embryo Straw in 2003. Because of its reliable weld seals (automated without technical variation), aseptic storage, and tamperproof, internalized, and dual-colored labels, it also became the container of choice for noncommercial aseptic vitrification systems.

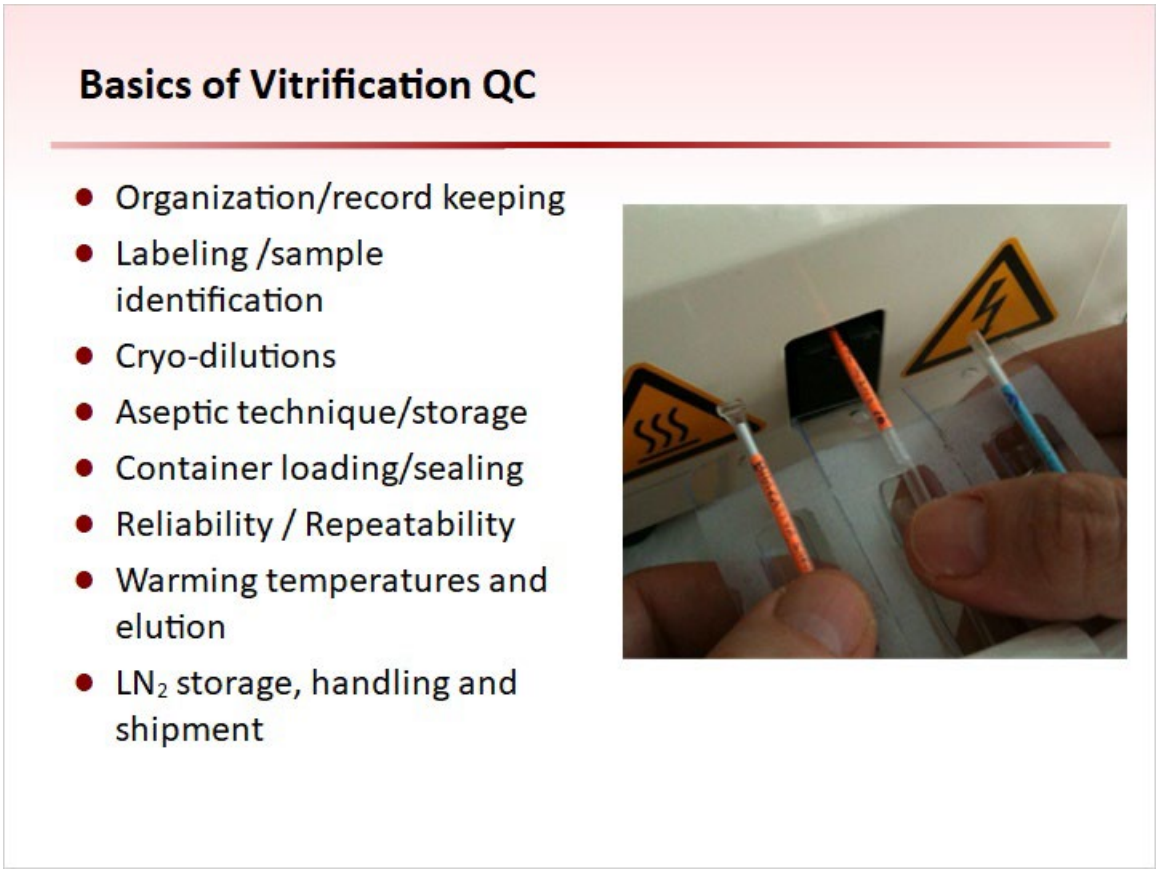
1.11 Problems with Current Storage Systems and Devices



Notes:

More recently, the commercial development of a wide variety of vitrification devices and solutions has created substantial difficulties in quality control (QC) for ART labs. This has created technical inconsistencies between laboratories, and, depending on the device and the learning curve, technical variation within laboratories as well. Because of FDA-mandated cGMPs, high development costs were incurred to market the devices. This resulted in manufacturer reluctance to make device modifications in response to potential QC flaws, some of which have created potential liability issues when used by laboratories unfamiliar with the device. Other devices experienced changes that were positive, such as colorized plastic wands and the use of translucent plastics that improved user reliability for identifying samples and their contents (embryo or oocytes) quickly, easily, and efficiently. The clinical impact of device flaws can take months or years to be fully appreciated since the cryopreserved material must be warmed and used.

1.12 Basics of Vitrification QC



Notes:

The basic QC factors needed to make vitrification a consistent, efficient, reliable, and effective ART procedure include the following:

1) **Organization** – Having paperwork and/or computer -records as well as vitrification set-up fully prepared prior to initiating vitrification.

2) **Labeling** – Ease, accuracy, and the reliability of sample labeling and identification are of critical importance. An ideal system includes a dual color system with duplicate labels or **a** tamperproof label system.

3) **Cryo-dilutions** – Distinct dilution steps so that solution purity is maintained and precisely timed without variation.

4) **Aseptic technique and possible aseptic storage** – although viral cross-contamination between embryonic samples in liquid nitrogen storage has never been reported, potential long-term exposure to various contaminants in cryostorage justifies the development and use of aseptic closed devices if their effectiveness can be validated in contrast to an open system.

5) **Container sealing** –Variation among technicians can depend on the type of sealer used (e.g., is training required?), and the plastic composition and diameter of the straw. For example, CBS™ straws have a polymer resin that welds together (not by heat) with an automated sealer

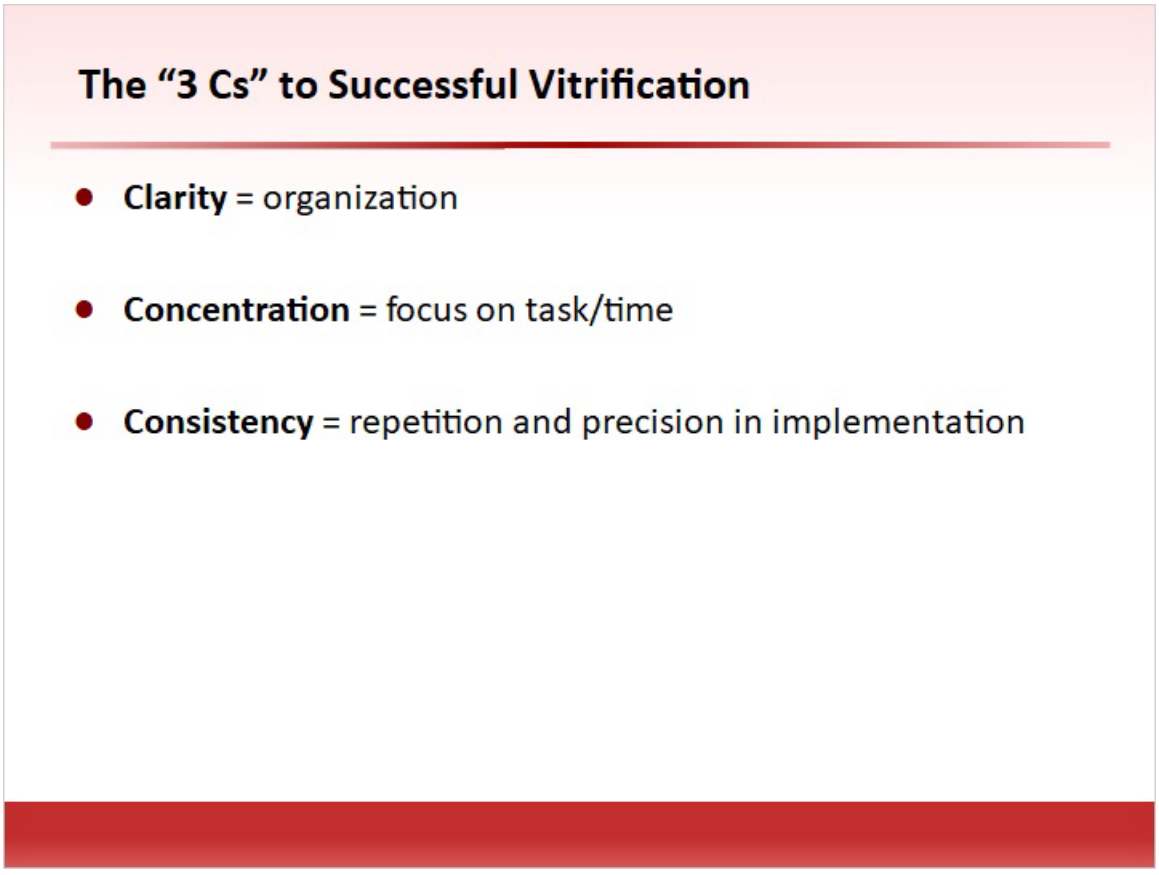
that eliminates technical variation;

6) **Reliability/Repeatability** – Is the vitrification system simple and easily repeatable in its daily application among all technicians? Can embryos/oocytes be consistently recovered without a risk of loss?

7) **Warming temperatures** – Is the warming potential of the device compatible with the vitrification solution used to ensure optimum survival and developmental competence?

8) **Liquid nitrogen storage, handling and shipment** – Because of the potential adverse effects of uncontrolled/suboptimal warming of vitrification microdevices, the ability to maintain specimens below –150°C conditions during storage, identification verification, and possible shipping and receiving, is of paramount importance.

1.13 The "3 Cs" to Successful Vitrification

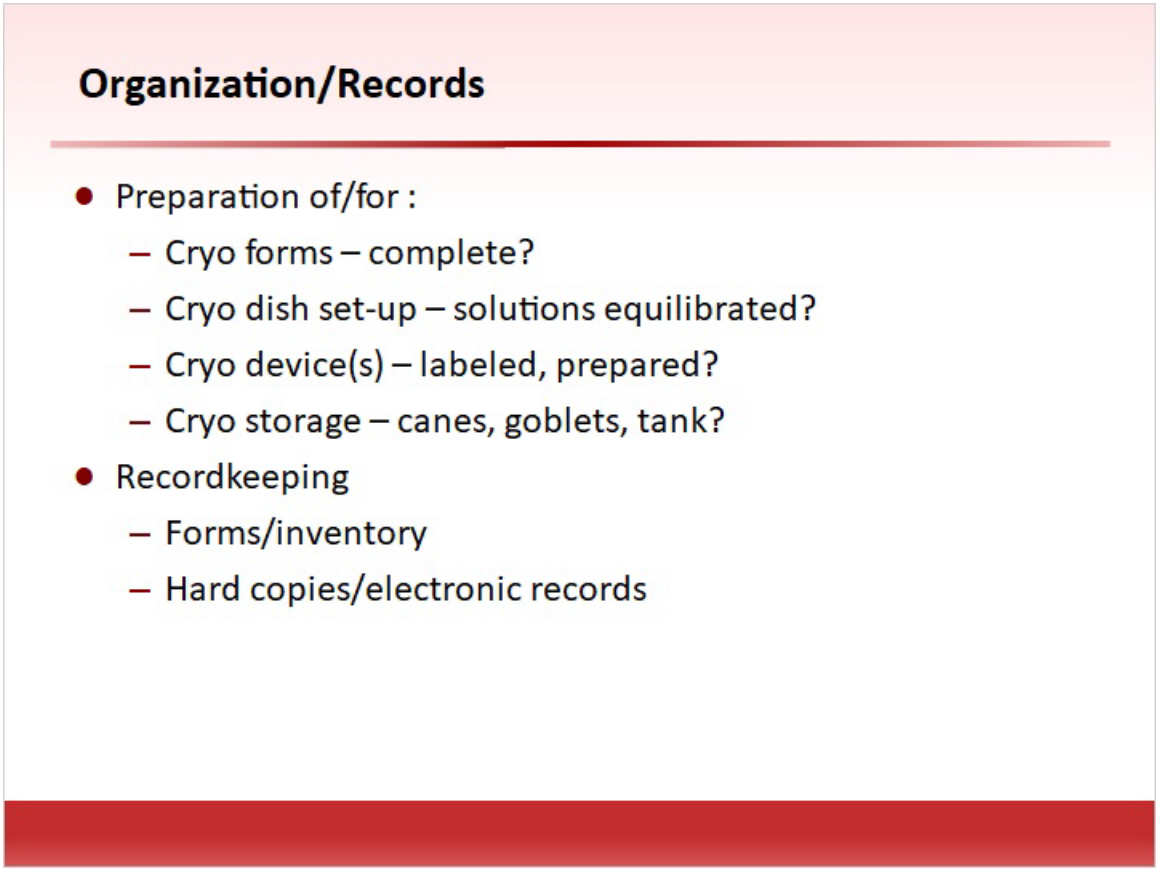


Notes:

In the individual application of a vitrification procedure, it is of critical importance that an embryologist has the 3 Cs:
1) Clarity of mind – be methodical and organized with attention to detail; 2) Concentrate on the procedure and step at hand and be focused and anticipate timed events; and 3)

Consistency over time – repetition and precision in implementation of each step. The application of these standards for laboratory personnel influences the overall success and limits variation within a vitrification program.

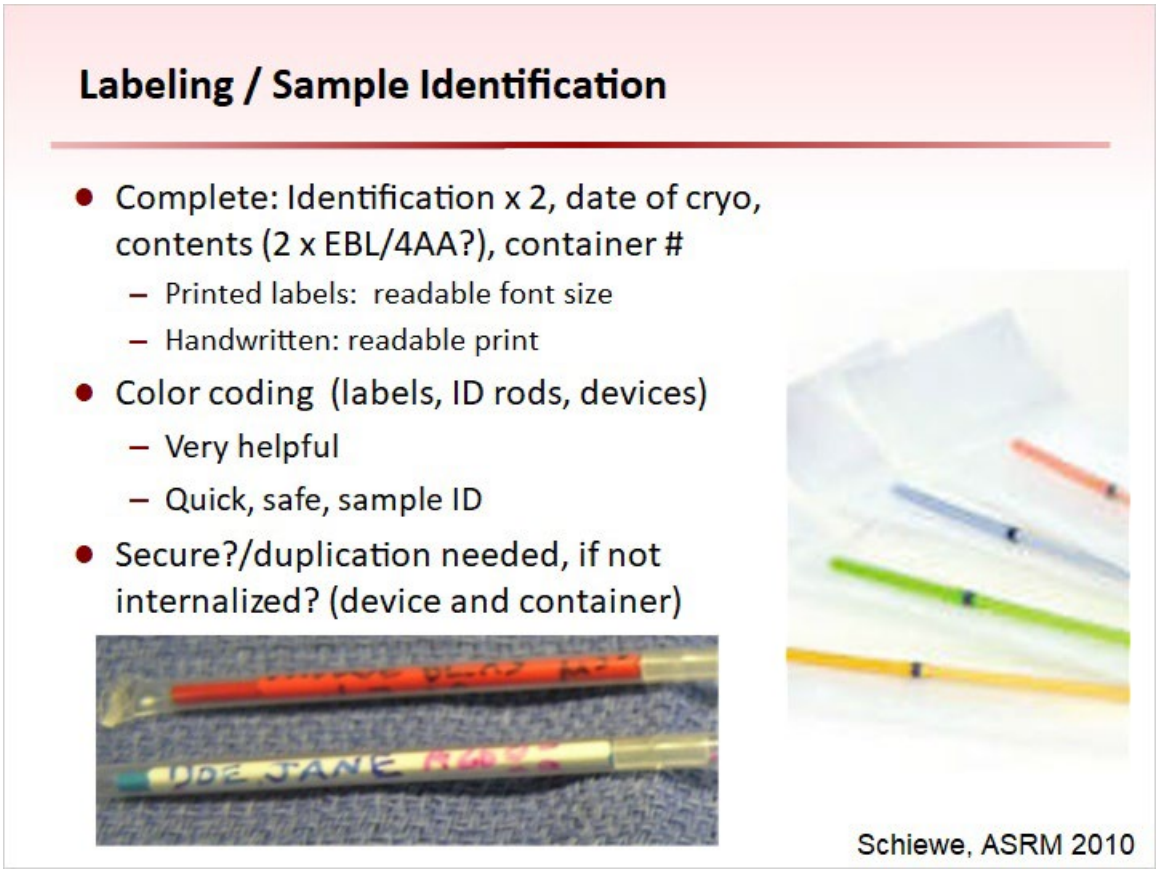
1.14 Organization/Records



Notes:

Listed here are things to consider and do before initiating the actual vitrification procedure. Events during the procedure are timed events and occur quickly, requiring precision.

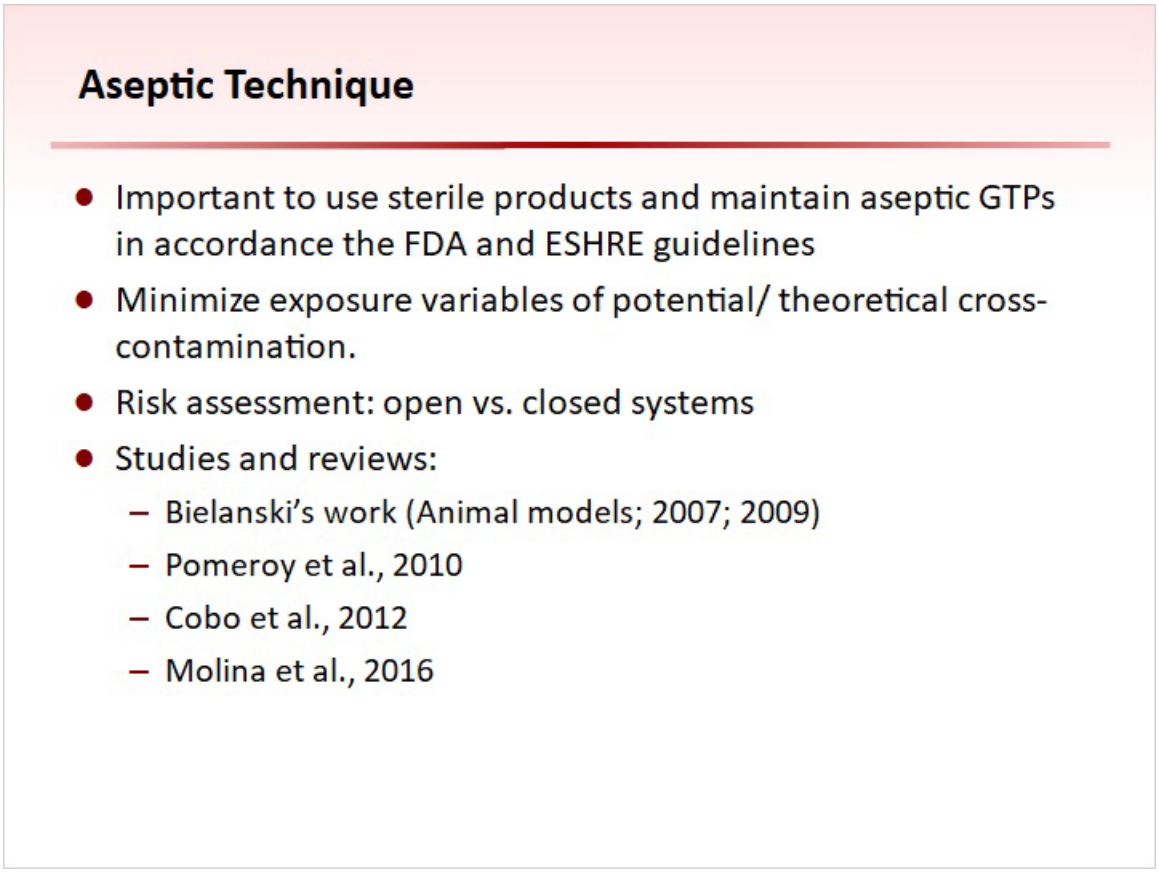
1.15 Labeling / Sample Identification



Notes:

Clear, well positioned, identifiable labels on vitrified sample are a key to optimal storage and handling of specimens. A good label includes complete identification by two different methods, the date of cryopreservation, the contents and the container number. Printed labels should have a readable font size while handwritten labels should have legible print. Many manufacturers offer color coded labels or devices that can serve as a double-check. Is the label secure? Can the sample be identified if the label was removed?

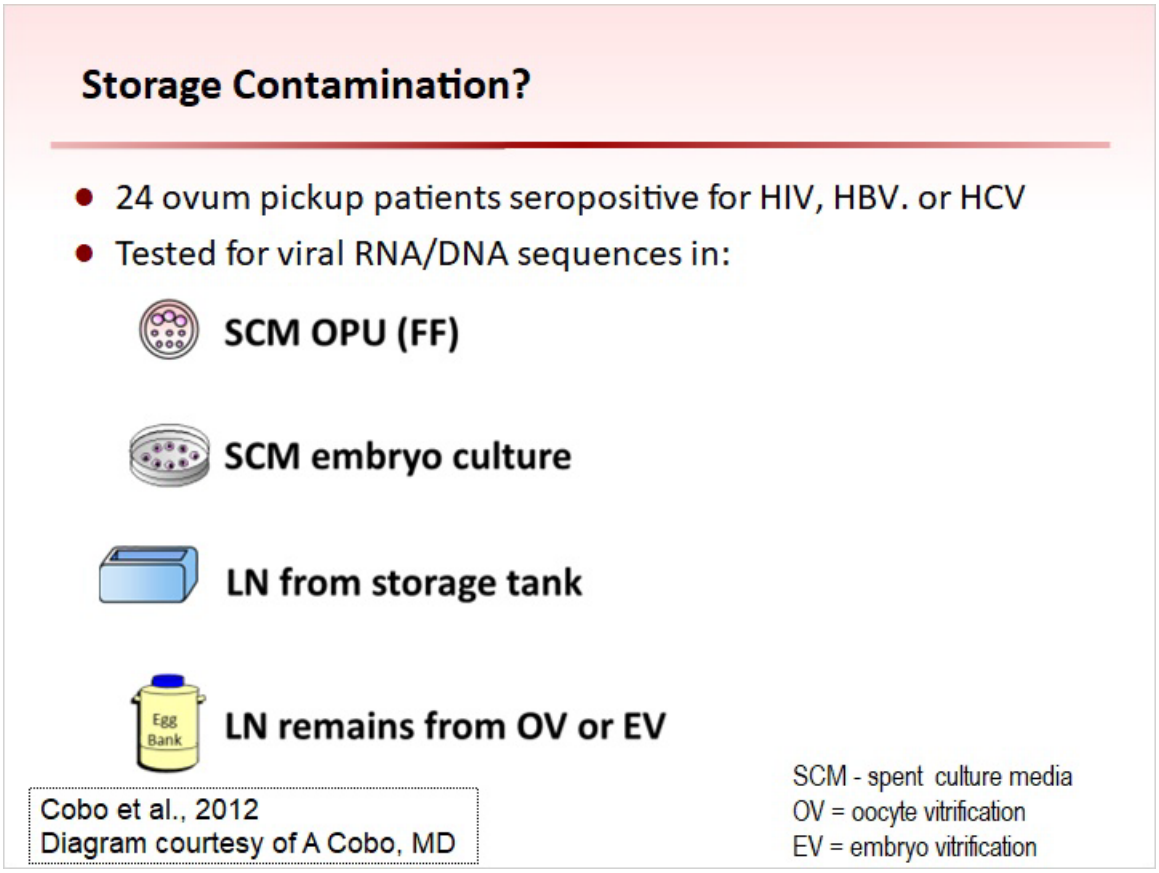
1.16 Aseptic Technique



Notes:

The zona pellucida of embryos and oocytes is a porous, layer network of interwoven glycoproteins. Therefore, it is theoretically susceptible to the cross-contamination of infectious agents and other microbial contaminants (Bielanski, 2007). While there has never been a verified case of viral cross-contamination of semen, embryos, or ova via liquid nitrogen storage (Pomeroy et al., 2010), it is clear that those who are convinced that ultra-rapid cooling rates are a necessity to vitrification are willing to accept risks. Based on Bielanski's studies, liquid nitrogen storage tanks accumulate and preserve microbial agents as well as gametes and embryos. In turn, open-storage devices, and to a lesser extent closed-sealed devices, may harbor pathogens and other contaminants that could be released into subsequent sucrose solutions upon warming.

1.17 Storage Contamination?

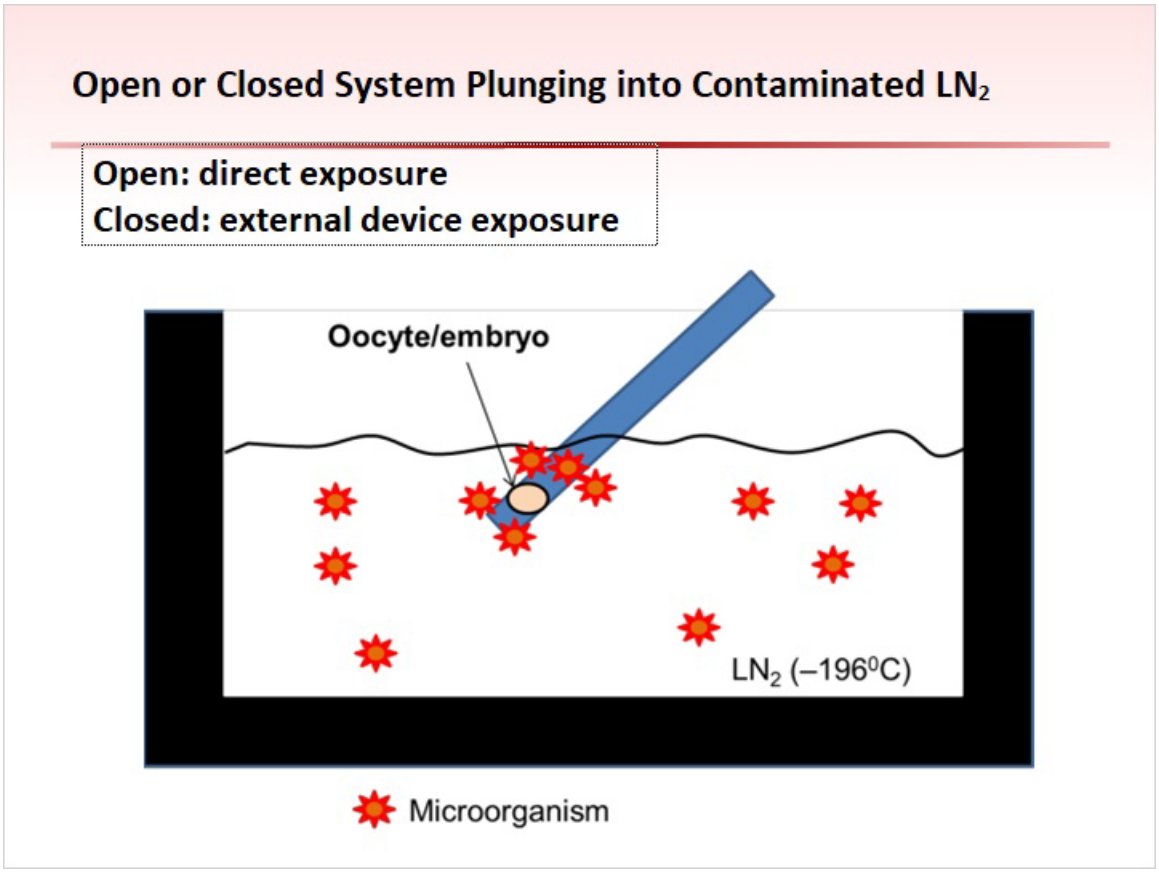


Notes:

Closed aseptic devices maintain the gamete/embryo-containing device in an aseptic air space without any contact with liquid nitrogen. The study by Cobo and coworkers demonstrated in 63 samples from 24 patients that viral sequences were not detectable via reverse transcription and subsequent real-time polymerase chain reaction (PCR), after culture and vitrification of oocytes/embryos from patients seropositive for HIV and hepatitis B and C, using an open device for vitrification.

The study failed to detect viral RNA or DNA sequences in spent culture media from follicular fluid, after culture, or post-vitrification/storage of oocytes/embryos from patients seropositive for HIV and hepatitis B, or hepatitis C. They concluded that there was no risk of cross-contamination among seropositive ART patients, even when using an open device for vitrification.

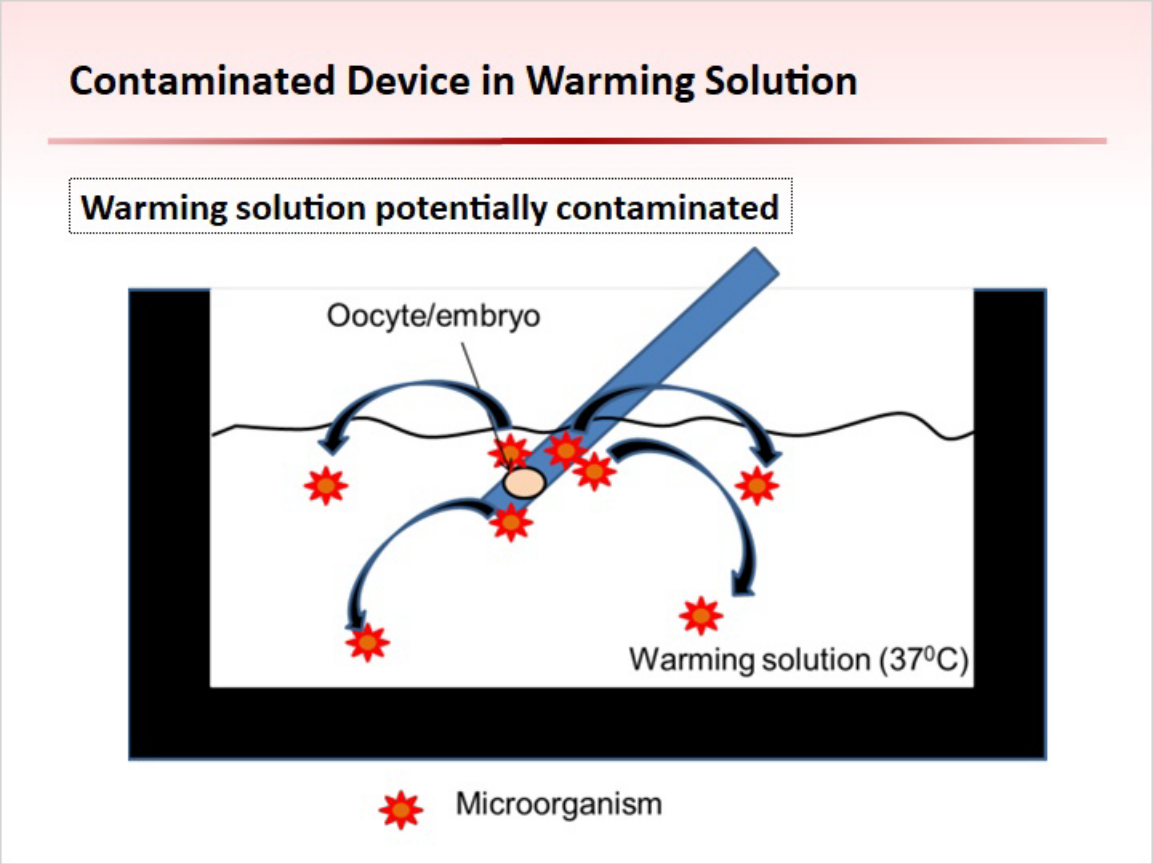
1.18 Open or Closed System Plunging into Contaminated LN2



Notes:

The next several slides illustrate theoretical examples of how contamination might be introduced. As an open or closed device is plunged into liquid nitrogen, microorganisms theoretically can attach to the device. An open container could allow the vitrified material to have direct exposure while a closed container will have external device exposure.

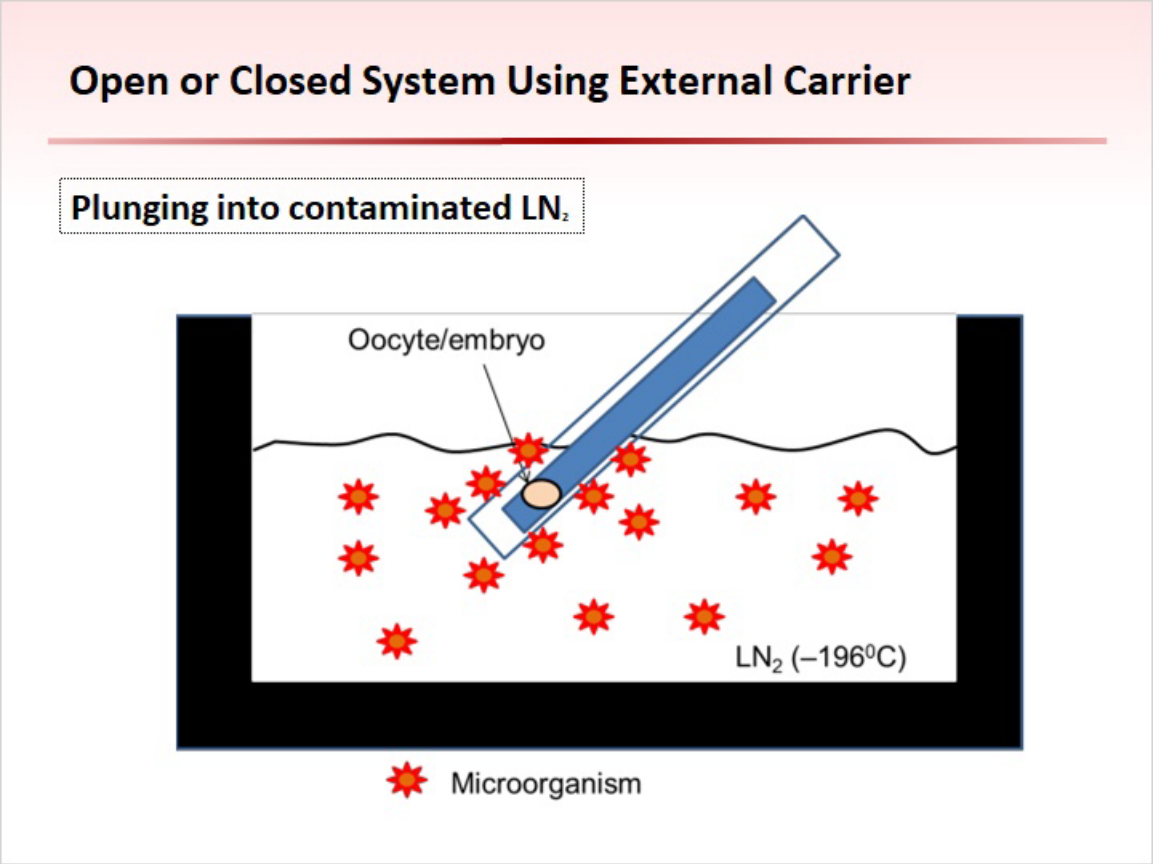
1.19 Contaminated Device in Warming Solution



Notes:

When that device is moved to the warming solution, any attached microorganisms could also be transferred to the warming solution.

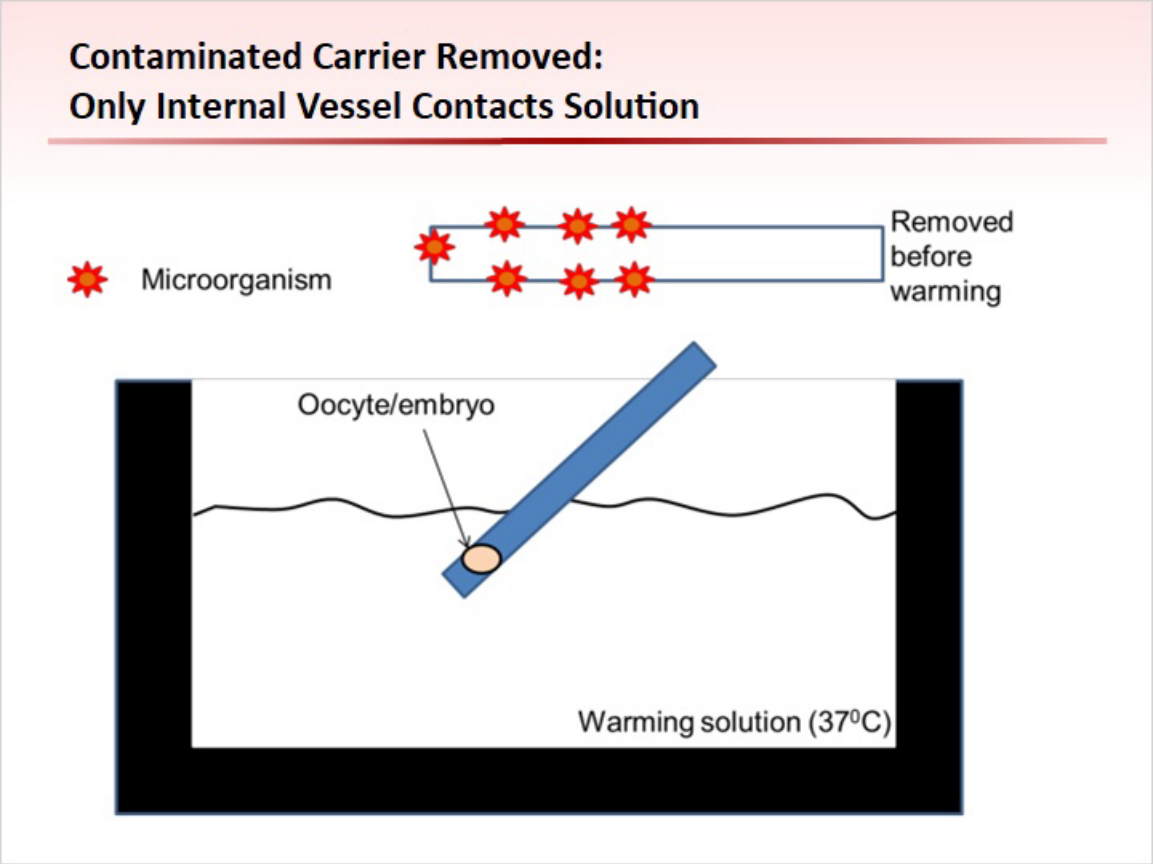
1.20 Open or Closed System Using External Carrier



Notes:

Here is an example of an open or closed device with an external closed container. As noted previously, the microorganisms could attach to the external container.

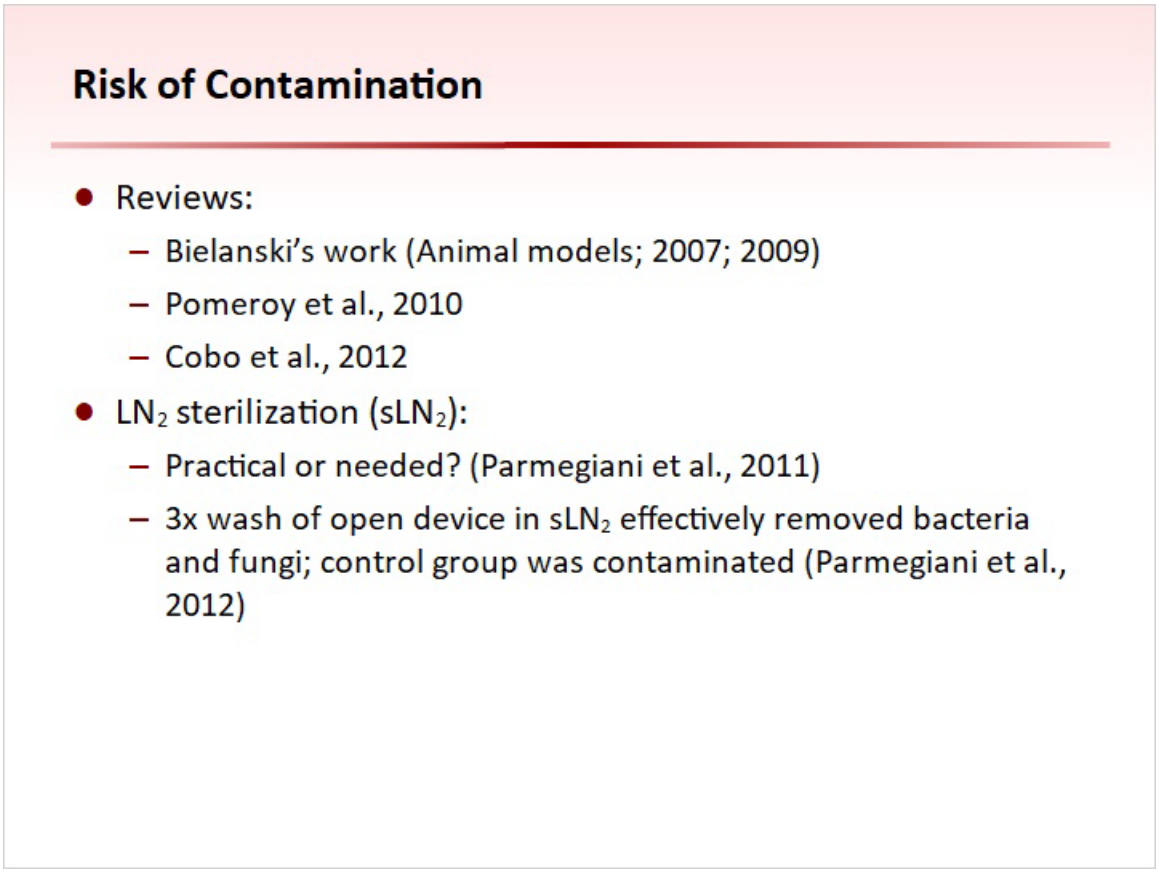
1.21 Contaminated Carrier Removed:



Notes:

However, in this example, the external container is removed before warming and only the 'clean' device that was not directly exposed to the microorganisms is placed in the warming solution. Although these illustrations are theoretical, it is important to reiterate that there has not been any documented evidence that such contamination has occurred in human cells.

1.22 Risk of Contamination

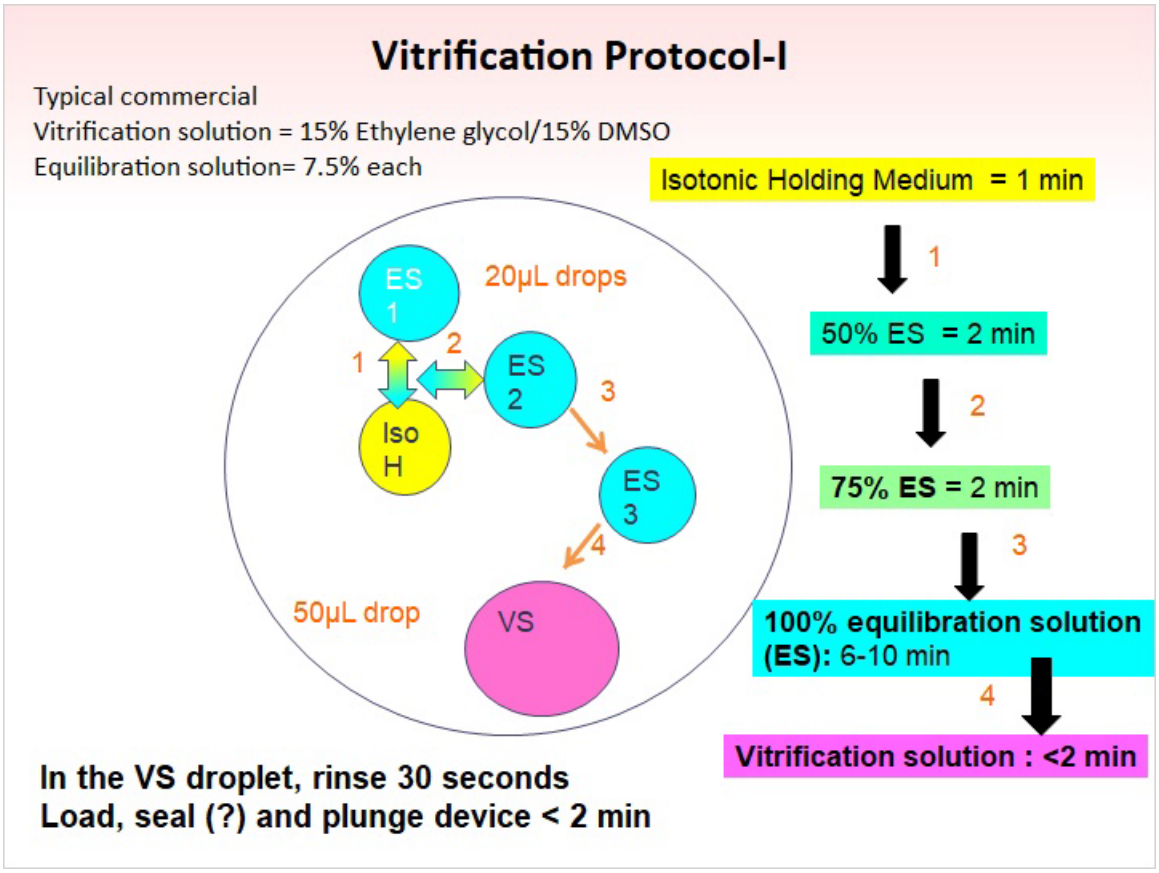


Notes:

The risk of contamination to the culture medium and/or gametes and embryos under clinical conditions is remote. However, it has been shown to be a real risk. This risk could be eliminated by prewarming and serial rinsing of the open device in ultraviolet (UV) sterilized liquid nitrogen.

Just as few programs appear willing to convert and/or adapt to the routine use of liquid nitrogen vapor storage tanks, the same is likely true for the filtration or sterilization of liquid nitrogen under standard lab conditions. Considering the FDA's current good manufacturing practice regulations mandate that vitrification devices be effectively sealed, it seems logical that similar storage requirements may one day be instituted, especially once randomized controlled trials demonstrate their competency.

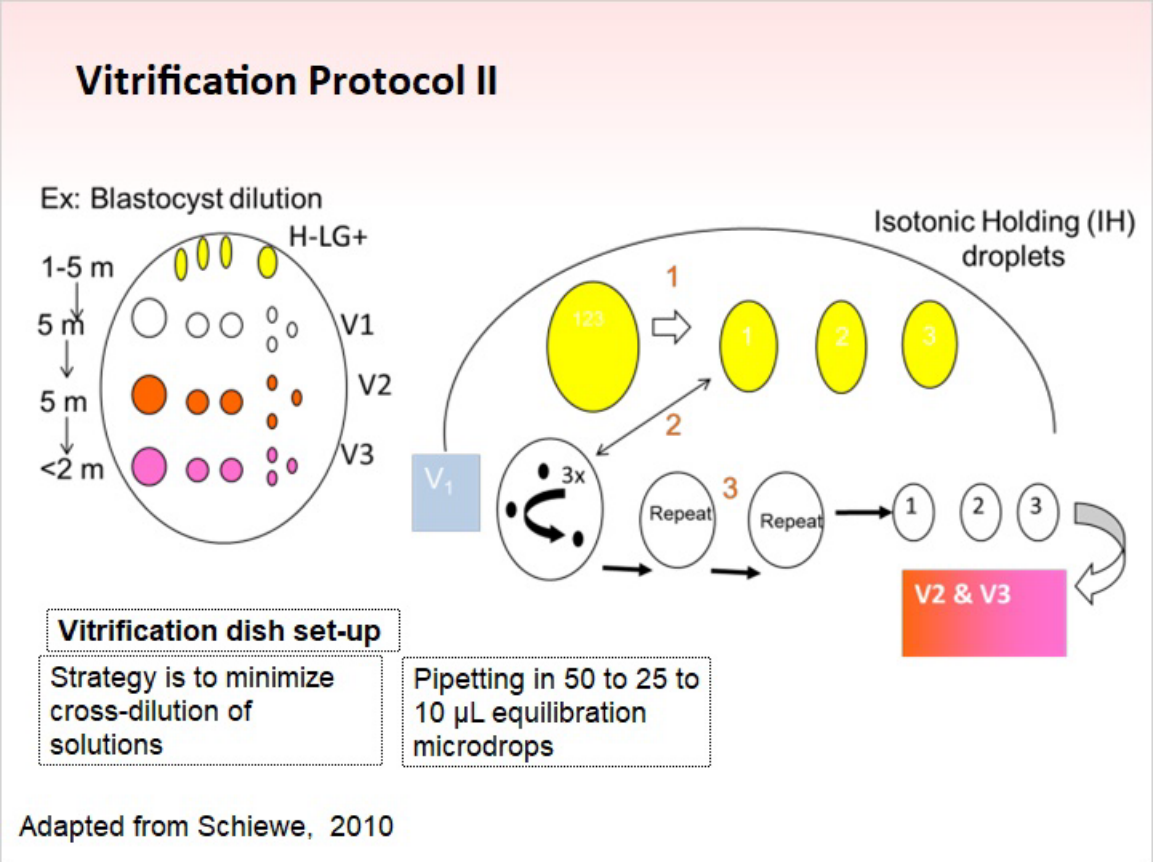
1.23 Vitrification Protocol-I



Notes:

Several different vitrification protocols exist in the ART field. One popular method involves the gradual mixing of equilibration solution (ES) with the initial isotonic holding medium in 2 steps at ambient temperature, before equilibration in 100% ES for 6 to 10 minutes prior to final dilution and device loading in the vitrification solution (VS). It is always a good idea to rinse or clear the pipette between solutions to minimize cross-contamination for repeated use situations. This method allows the embryologist to work with 3 to 4 groups of individual embryos in a 15-minute interval.

1.24 Vitrification Protocol II

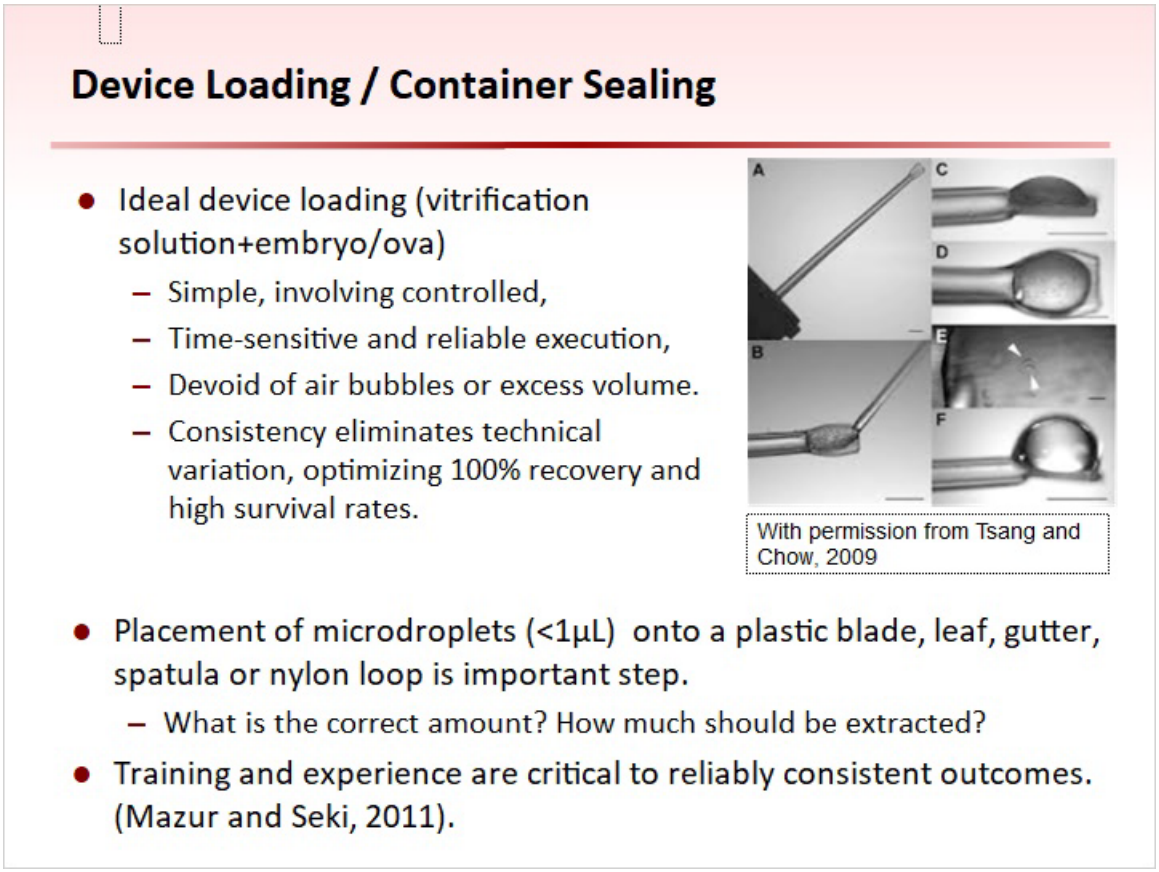


Notes:

This alternative blastocyst dilution approach maintains precise timing using 3 distinct vitrification solutions (V1, V2, V3). In this dilution scheme, individual isotonic holding droplets are placed on the upper edge of a 100 mm petri dish. Embryos are initially pipetted into a large (25-50µL) droplet of isotonic holding medium (IH), which accumulates oil residue from the culture dish. The embryo is then moved into individual IH drops (10-20µL) with a clean pipette, where they await dilution into V1. A pipette (e.g. preset at 3µL) is filled with V1 and then used to pick up embryo #1. In doing so, half of the contents are expelled on top of and around the embryo. The embryo is immediately picked up in a minimal amount of V1-mixed IH medium. The remaining volume is aspirated into the pipette (with V1) creating an initial in situ dilution with V1 and then released into the first V1 droplet. The embryo(s) are moved in 3 points of each droplet before repeating the process twice. After 30-40 seconds of pipetting, the embryo(s) are moved to the final equilibration droplet (total of 5 minutes in both V1 and repeated into V2; one droplet/device). Finally, the embryo(s) are diluted into the final V3 solution over a 1 to 1.5 minute exposure time before loading, sealing, and plunging the device into liquid nitrogen. Using this method, 4-5 devices (i.e., embryo groups) can be prepared in a 15-minute interval, with holding and wash droplets maintaining their

relative purity between groups if meticulous and thoughtful pipetting is performed (rinsing and clearing between steps). An alternative procedure for oocytes involves 5-10 minutes in V1, 2.5 minutes in V2 and 1-1.5 minutes in V3 , allowing 2 groups of up to 5 ova each per container.

1.25 Device Loading / Container Sealing

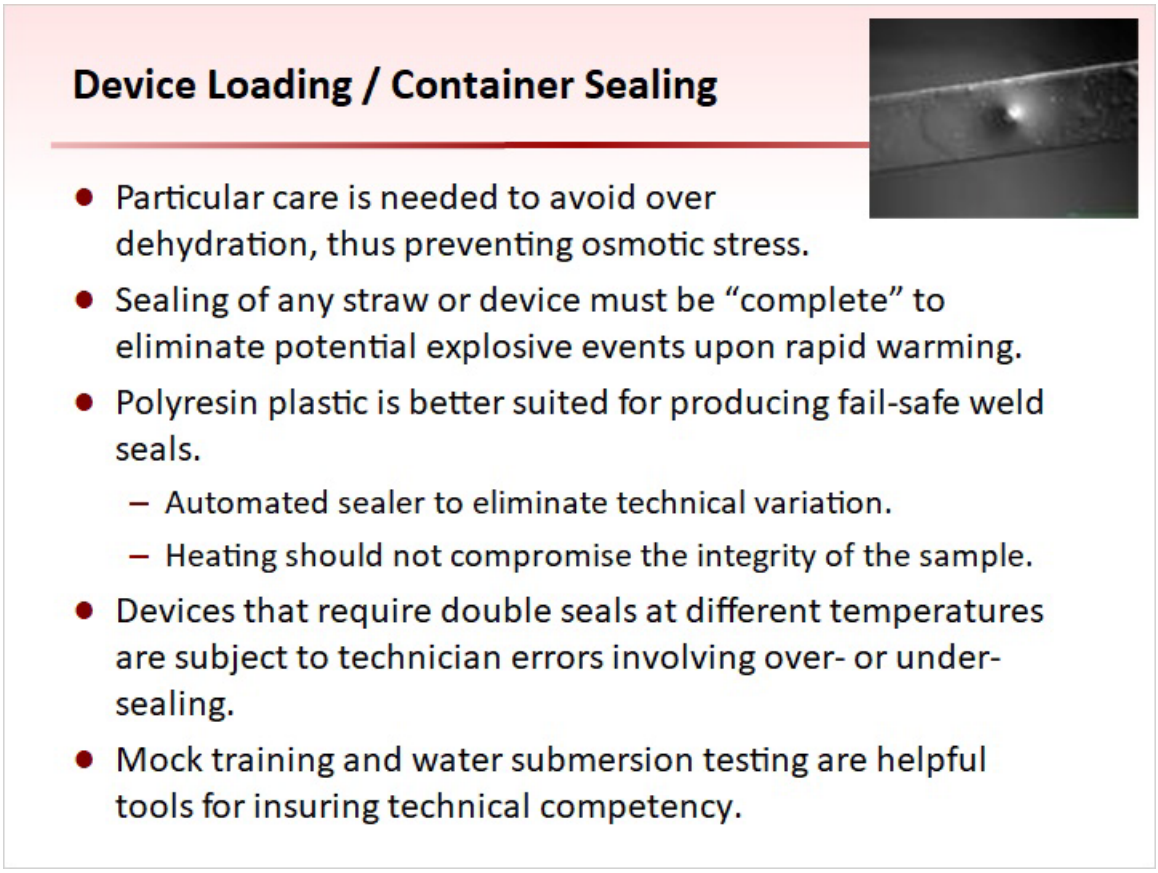


Notes:

An ideal device should allow for a repeatable volume of vitrification solution with an embryo/ova to be loaded simply in a time-sensitive, reliable, controlled manner, devoid of air bubbles. The goal is to eliminate technical variation, while optimizing 100% recovery and high survival rates. Recovery rates should not be neglected, as they represent a potential design flaw to a device, and create a serious liability risk to the manufacturer and to the users.
Systems that rely on the placement of microdroplets (<1µL) onto a plastic blade, leaf, gutter, spatula, or nylon loop are vulnerable to variation in the amount of residual solution extracted from around the specimen. Training and experience are critical to reducing technical variation and reliably consistent outcomes. The drive to attain super-rapid cooling rates (>50,000°C/minute) through maximal

extraction of vitrification solution comes at a risk of osmotic injury and reduced tolerance of the device to slower warming rates.

1.26 Device Loading / Container Sealing

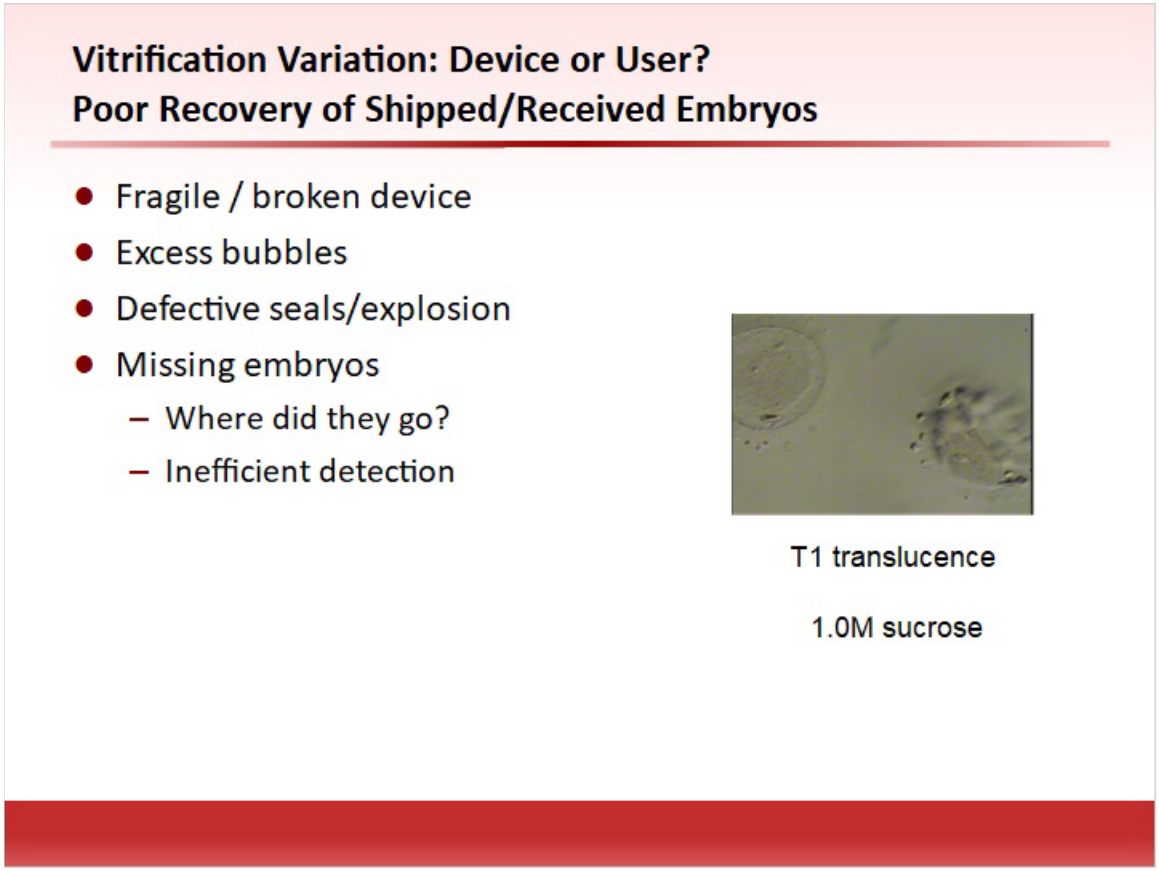


Notes:

After the material is placed on the device surface, the excess volume can be pipetted off. When extracting residual vitrification solution volume from the device surface, care must be to taken to: 1) not pipette an embryo/egg accidentally; 2) not remove too much volume causing excessive dehydration/injury; and 3) not take too much time being overly precise.
When using straws to load the vitrification solution or internalized devices (i.e., aseptic vitrification), the system becomes "closed" by a heat sealer each end. Care must be taken to ensure the completeness of seal. Standard polypropylene straws that are heat crimped or melted together may appear sealed but can be susceptible to subzero thermal expansion and become leaky. Therefore, precise standardized technique, heat settings and training are required to ensure reliable, consistent seal. Straws that are sealed within a liquid nitrogen vapor environment are vulnerable to incomplete seals. Testing the patency of a seal, in

training, is easily accomplished by applying a water submersion test as discussed in the module on cryopreservation in this series. If the seal is incomplete, an air space may appear in the seal or actual bubbling may be observed.
Straws made of polyresin plastic form complete “weld seals” and minimize technician variation when using an automated sealer. Some vitrification devices require sealing of the actual pipette. Be aware of the location of the embryo/eggs relative to the source of heat used to seal. Care must be taken to use the correct heat intensity based on the material and outer diameter being sealed. Overheating can cause the device to be bent or damaged.

1.27 Vitrification Variation: Device or User?

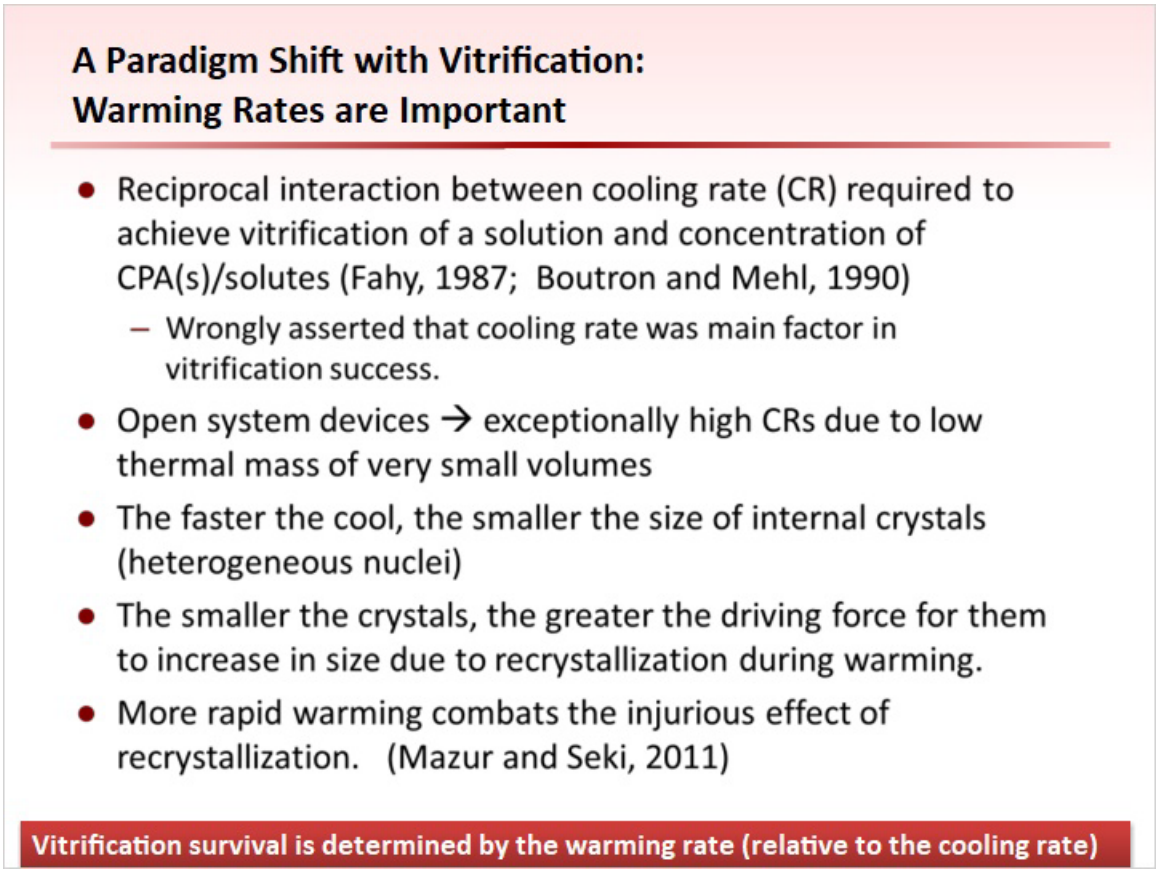


Notes:

Failure to recover an embryo or egg(s) can present serious problems, especially if the sample was vitrified by another lab and/or the receiving lab is unfamiliar with a given device. Was the device jarred pre- or post-vitrification? Was the specimen accidentally aspirated pre-vitrification or simply not identified post-warming? Failure to identify eggs can be particularly problematic in devices that do not allow direct visualization of

the eggs on or in the device, because they can become highly translucent in 1.0 M sucrose solutions. Aseptic closed devices essentially have outer straws encasing the device inside. Additional information on shipping/receiving vitrified material is covered in the module on management of cryopreserved gametes and embryos.

1.28 A Paradigm Shift with Vitrification:



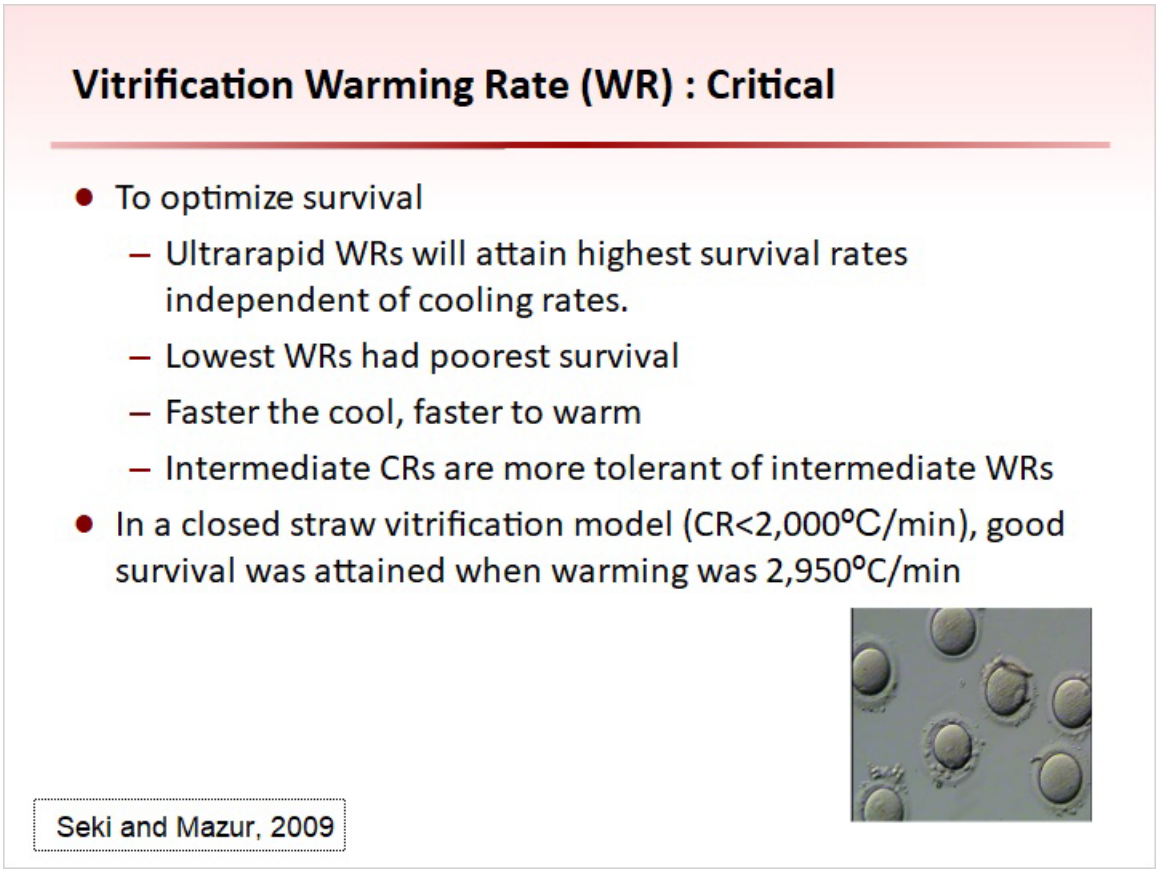
Notes:

A strong reciprocal interaction exists between the cooling rate required to achieve vitrification of a solution and the concentration of cryoprotectant solutes. In turn, it had been initially asserted by some that cooling rates were the main factor in vitrification success.

This led to open-system devices being created that could achieve exceptionally high cooling rates due to the low thermal mass of their very small volumes. The faster the cool, the smaller the size of the internal crystals (heterogeneous nuclei); the smaller the crystals, the greater the driving force for them to increase in size due to recrystallization during warming. Thus, one needs to warm more rapidly to combat the injurious effect of recrystallization. This led to subsequent findings that vitrification survival is determined

by the warming rate, relative to the cooling rate.

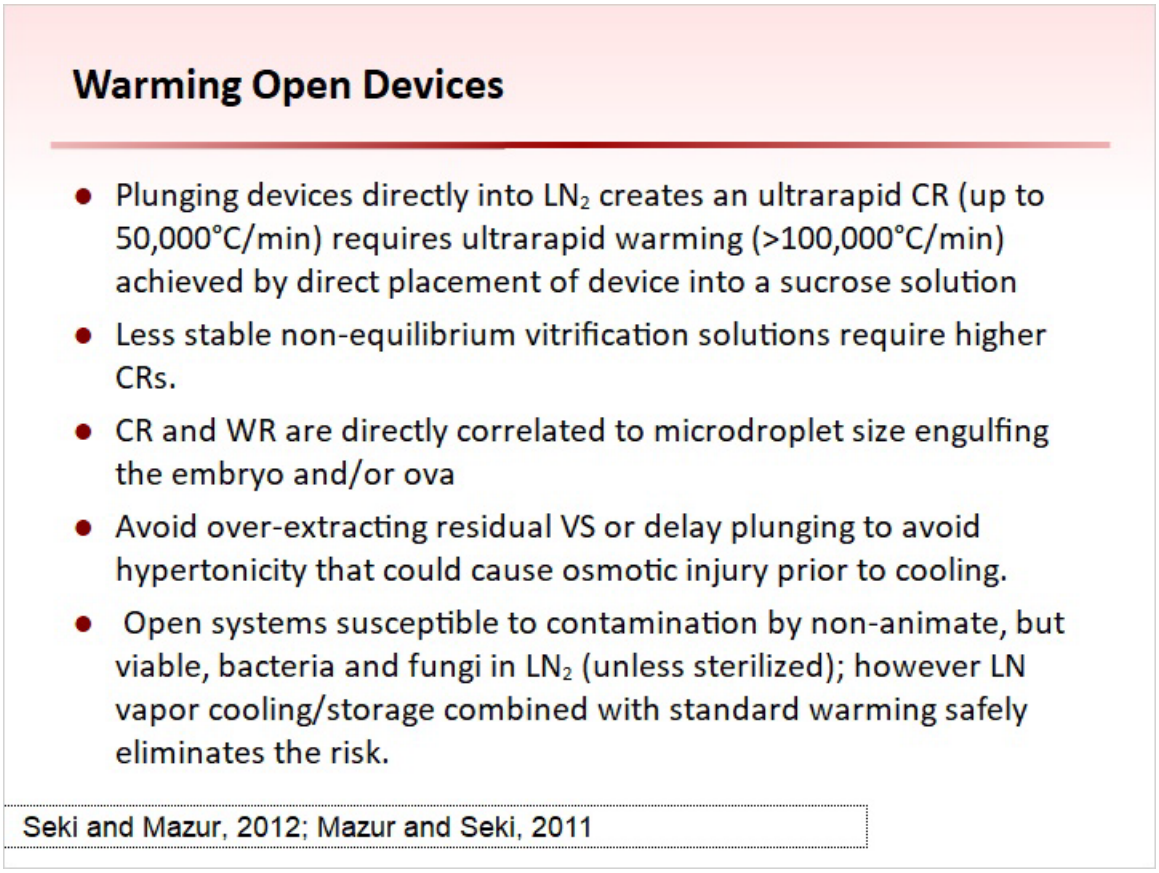
1.29 Vitrification Warming Rate (WR) : Critical



Notes:

Using a nonequilibriium, unstable vitrification solution model, it was demonstrated that ultrarapid warming is the key determinant that overrides conditions created at any cooling rate. However, the problem is complex, in that a closed device that achieves intermediate cooling rates performs well with an intermediate warming rate ≥2,950°C/min, whereas an open system utilizing high cooling rates experiences decreased survival at lower warming rates.

1.30 Warming Open Devices



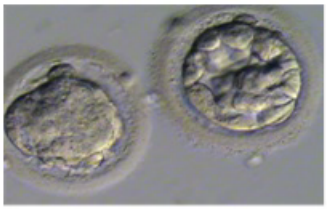
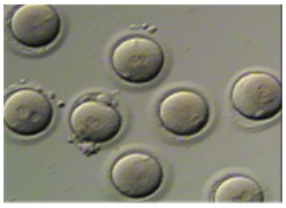
Notes:

Plunging devices directly into LN_2 creates an ultrarapid cooling rate (up to 50,000ºC/minute) which requires ultrarapid warming (>100,000ºC/minute) achieved by direct placement of the device into a sucrose solution. Less stable non-equilibrium vitrification solutions require higher cooling rates. Cooling rates and warming rates are directly correlated to the microdroplet size engulfing the embryo and/or ova. Care must be taken not to over-extract residual vitrification solution or to delay plunging, to avoid hypertonicity that could cause osmotic injury prior to cooling. This comes with practice. Open systems are susceptible to contamination by nonanimate, but viable, bacteria and fungi in LN_2 (unless sterilized); however LN_2 vapor cooling/storage combined with standard warming safely eliminates the risk.

1.31 Universal Elution of Vitrified Embryos and Oocytes

Universal Elution of Vitrified Embryos and Oocytes

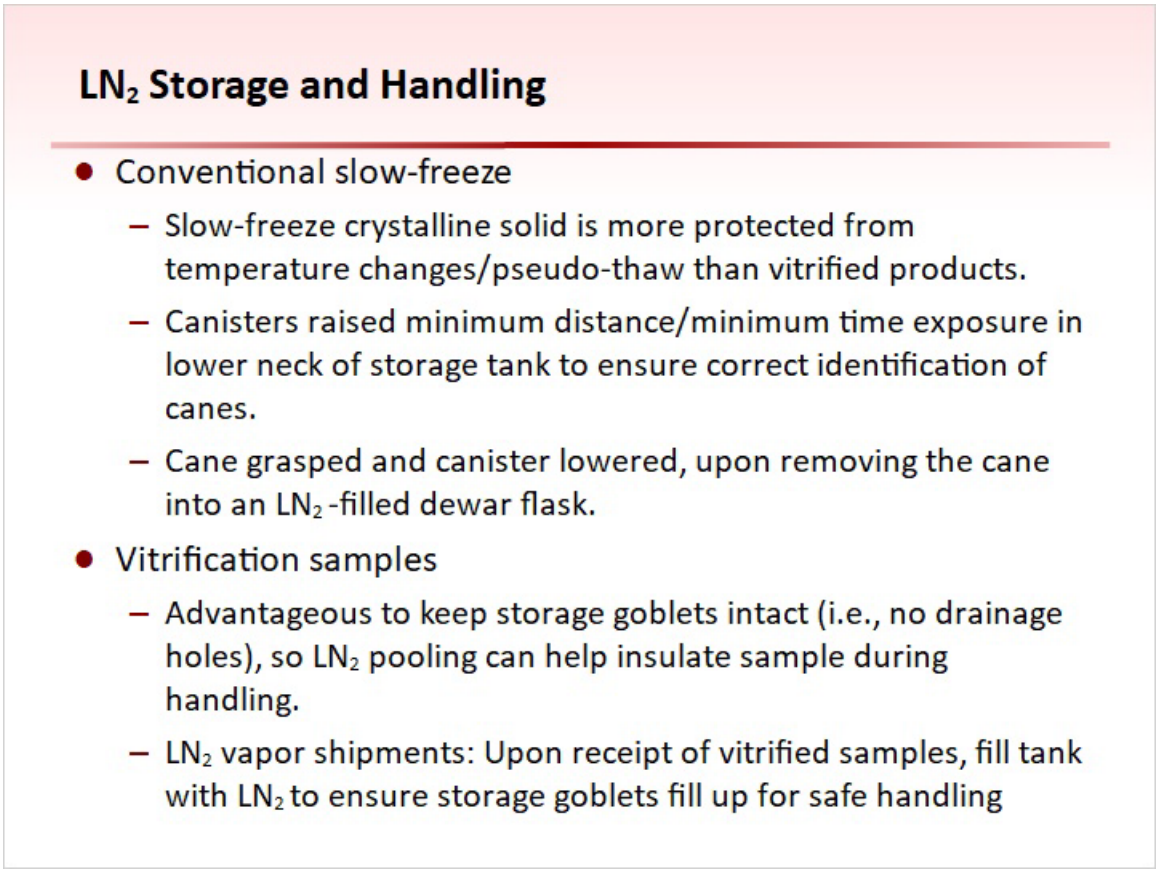
- Use of 1.0M sucrose step-down (0.5M to 0.25M) elution procedures theoretically can be applied to any vitrified eggs/embryos (Parmegiani et al., 2014) with a high level of efficacy, in the absence of procedural stock solutions.

Notes:

Because so many different vitrification devices and commercial solutions exist, it is important to recognize that 1.0M sucrose is an effective “universal” warming solution for vitrified, as well as slow-freeze oocytes and embryos of various stages. It is not financially feasible or practical to maintain various thawing solutions for slow-freeze and vitrified samples, nor is it needed. A 3-step, or possibly 4-step (+0.125M), elution (under ambient temperatures) in decreasing concentrations of sucrose at 3 minute intervals prior to final equilibration in isotonic medium is sufficient to optimize post-thaw survival and viability.

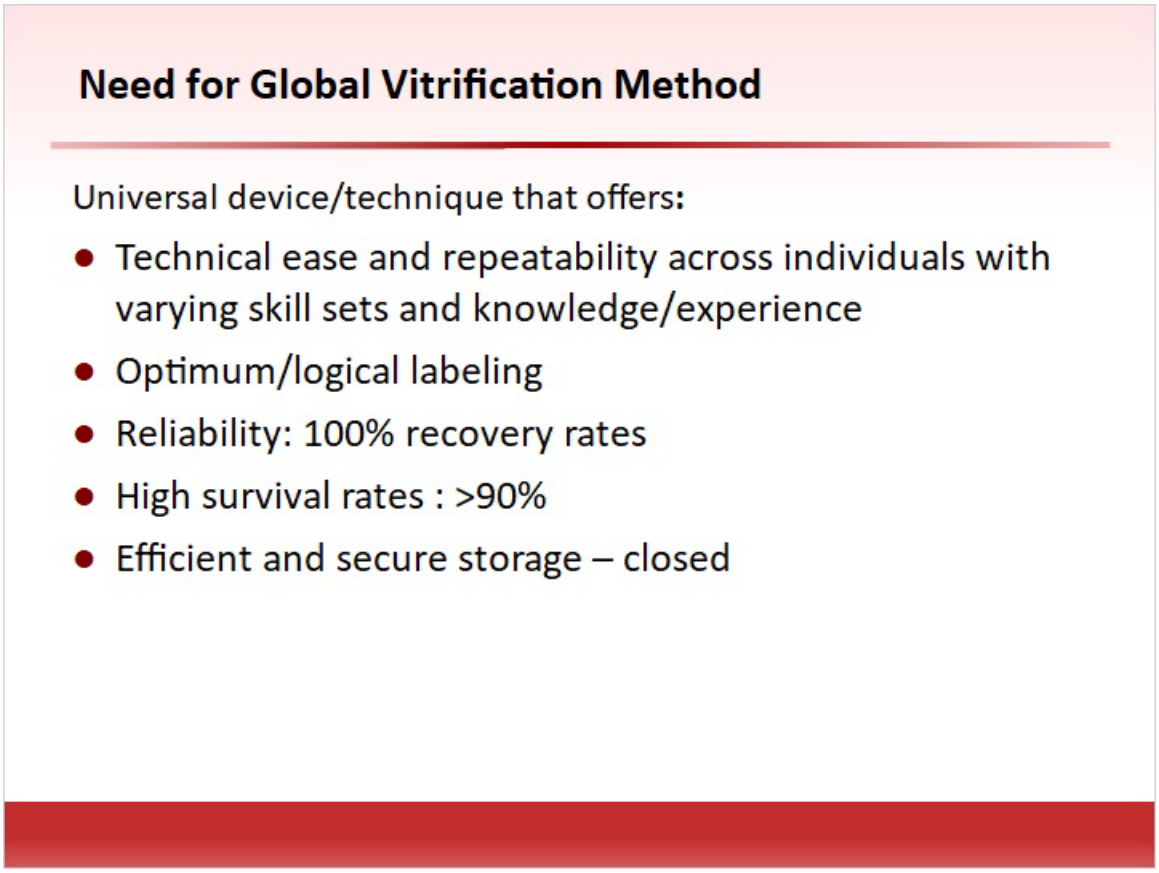
1.32 LN2 Storage and Handling



Notes:

Liquid nitrogen handling is different for samples that are vitrified as compared with those that were slow-frozen.
Slow-freeze crystalline solid is more protected from temperature changes or a pseudo-thaw than are vitrified products. Canisters should be raised a minimum distance and exposed for minimal time in the lower neck of a storage tank to ensure correct identification of a cane. The cane should then be grasped and the canister lowered quickly upon removing the cane into an liquid nitrogen filled dewar flask. For vitrified samples, it is advantageous to keep storage goblets intact (i.e., without drainage holes), so liquid nitrogen pooling in the goblets can help insulate the sample during handling. Upon receipt of vitrified samples, always refill the tank with LN_2 to ensure that the storage goblets fill up for safe handling.

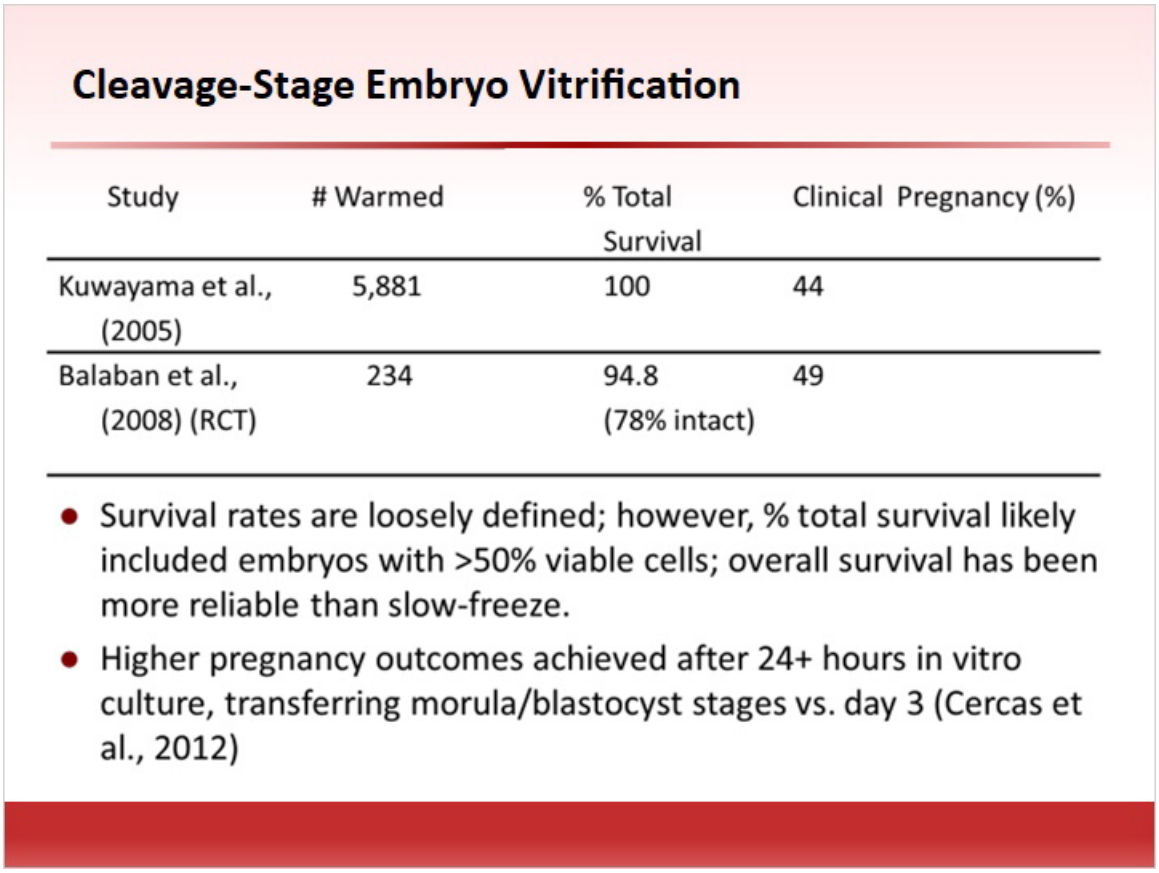
1.33 Need for Global Vitrification Method



Notes:

There is a need for a global vitrification method with a universal device and technique. This would ideally offer technical ease and repeatability across individuals with varying skill sets and knowledge/experience, optimum/logical labeling, reliability (100% recovery rates), high survival rates (>90%), and efficient and secure storage (closed?).

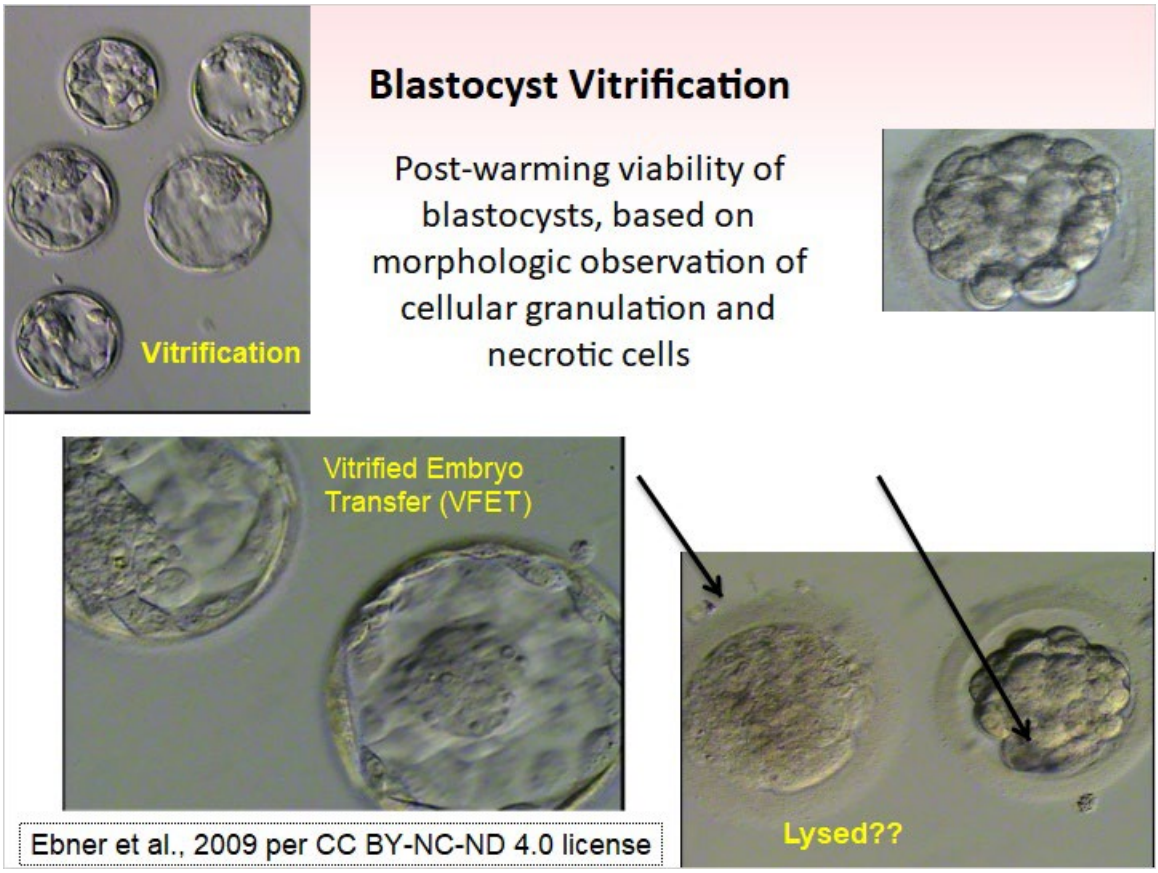
1.34 Cleavage-Stage Embryo Vitrification



Notes:

Early in its clinical application, Kuwayama and co-workers demonstrated the effectiveness of cryopreserving cleavage stage embryos by vitrification in open and closed systems. Survival was loosely defined, and based on slow-freeze history, likely encompassed any embryo with ≥50% of its original cell number. Subsequently, Balaban (2008) more accurately assessed the overall effectiveness of embryo vitrification, finding that 78% were completely intact, while the remaining experienced one or more degenerate cells, not exceeding 50% of the total cell number. Comparable pregnancy rates were attained in the two studies. Another study found advantages with greater than 24 hours of in vitro culture, allowing for the selection and transfer of morula or blastocyst-stage embryos, with both pregnancy and implantation rates (68.7%, 64.7%, respectively) being significantly higher than day-3 transfers (33.3%).

1.35 Blastocyst Vitrification

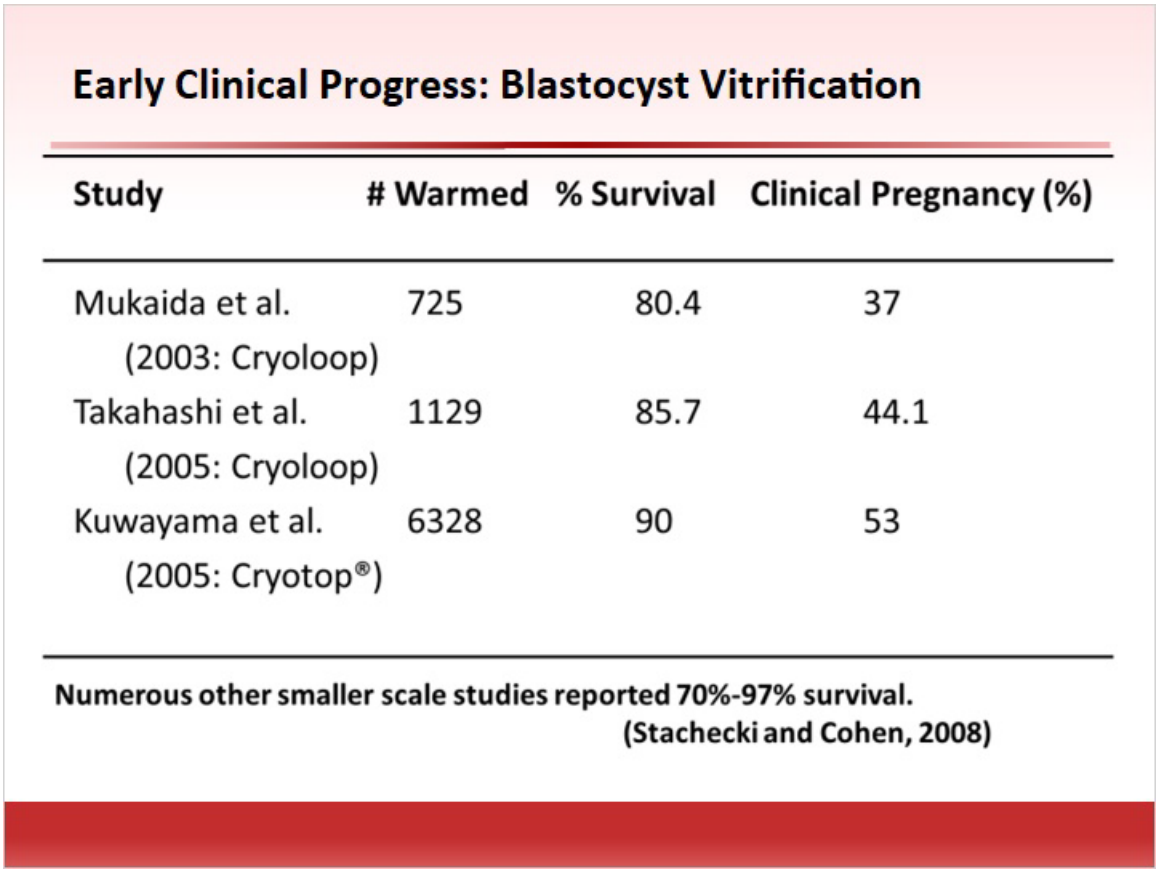


Notes:

The greatest success in terms of maintaining the viability of fresh embryos has been with the development and clinical application of blastocyst vitrification (Ebner et al., 2009). Post-warming, blastocysts tend to appear completely intact, with the occasional appearance of a necrotic outer layer. Nonincorporated cells and fragments commonly appear degenerated post-warming. It is uncommon to observe a completely lysed blastocyst, and care should be taken to culture these embryos as they may have experienced an initial transitional phase and appear quite normal a few hours later.

Today, complete survival rates routinely exceed 95%, and vitrified embryo transfer (VFET) live-birth outcomes can be equal to or higher than fresh embryo transfer success. This is thought to be due to impaired endometrial receptivity in fresh, controlled ovarian stimulation cycles (Shapiro et al., 2011).

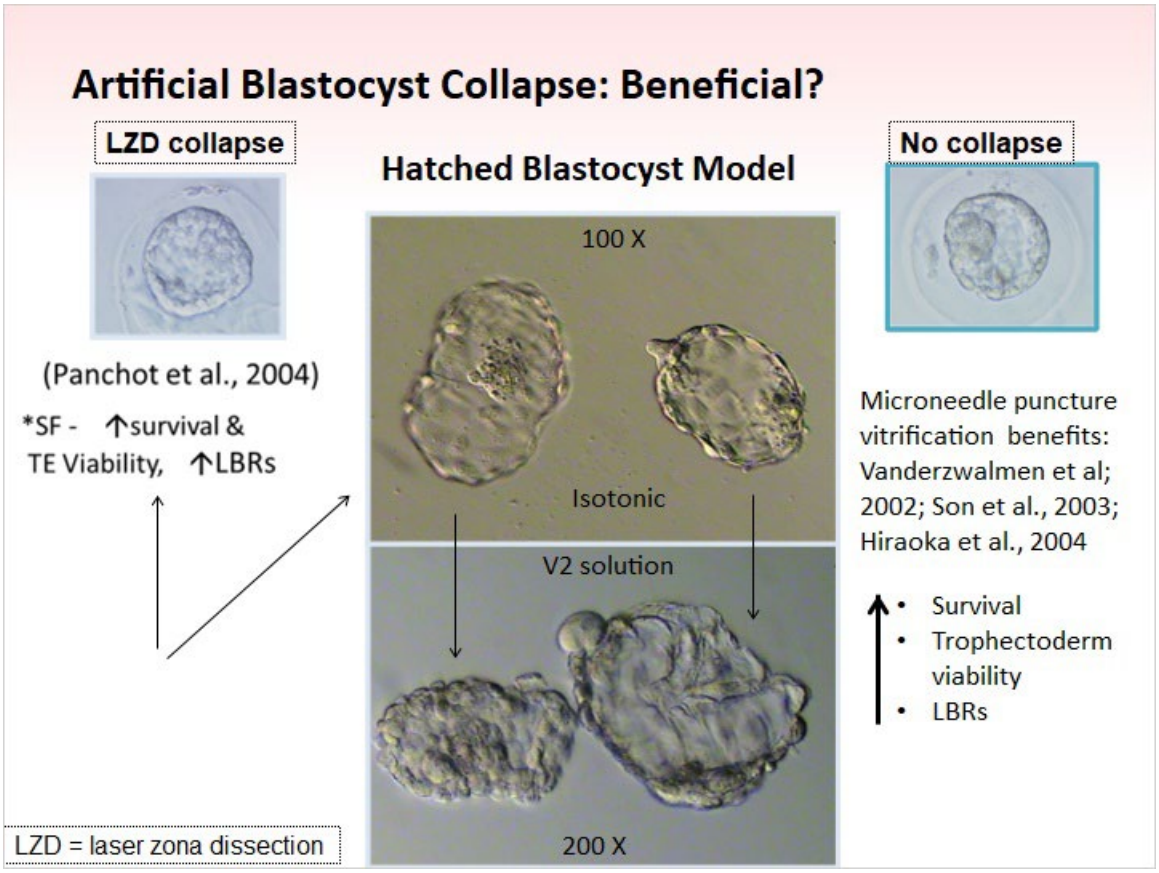
1.36 Early Clinical Progress: Blastocyst Vitrification



Notes:

Early efforts with blastocyst vitrification showed its reliability to sustain good embryo survival and viability. Reports have demonstrated 70% to 97% survival.

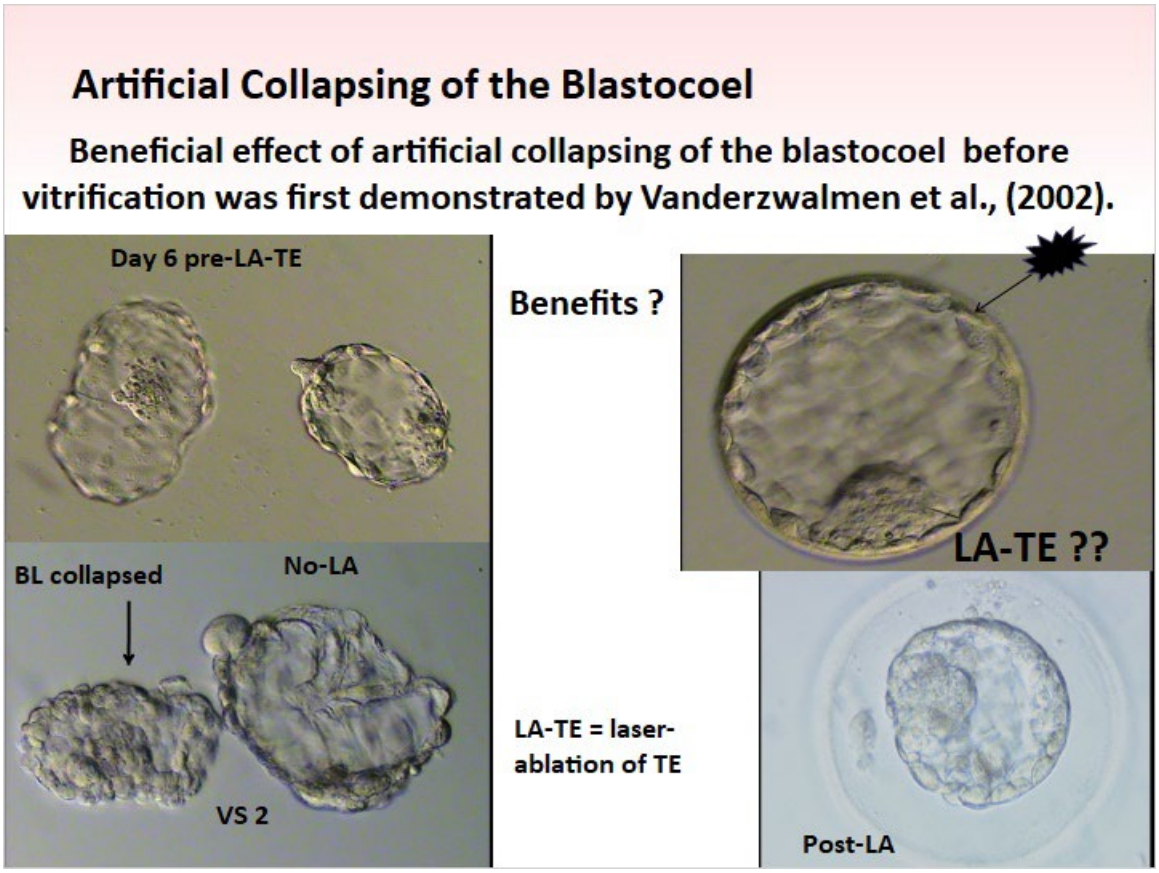
1.37 Artificial Blastocyst Collapse: Beneficial?



Notes:

Post-thaw viability rates utilizing slow-freeze protocols were being enhanced by simply collapsing the blastocoel cavity via either a laser ablation or microneedle puncturing of the trophectoderm cells prior to exposure to the cryoprotective agent. By improving the overall survival of trophectoderm cells, embryos had an improved chance of implanting and sustaining a full-term pregnancy. Similar improvements in post-warming survival of vitrified blastocysts have been seen with different vitrification devices using ethylene glycol/DMSO vitrification solutions.

1.38 Artificial Collapsing of the Blastocoel



Notes:

The artificial collapsing of blastocysts prior to vitrification has been demonstrated to be effective using a variety of methods: trophectoderm laser ablation, microneedle puncture/aspiration, and mechanical-reduced diameter pipetting (Vanderzwalmen et al., 2002; Panchot et al., 2004). A hatched blastocyst (left panel) and a fully expanded blastocyst (right panel) experience a significant reduction in volume when collapsed, and subsequent enhanced survival rates.

1.39 Blastocyst Vitrification using Cryoloop:

Blastocyst Vitrification using Cryoloop: Artificial Shrinkage of the Blastocoel Improved Survival and Pregnancy Outcomes
(Mukaida et al., 2006)

	# Vitrified	Survival Rate	Implantation Rate
Intact blastocyst	569	86%	21%
Pre-collapsed	502	97%*	47%*

Notes:

This early comparative trial found benefit of conversion from a cryoloop technique to the routine collapsing of the blastocoel before vitrification.

1.40 Clinical Results of S3 Vitrification

Clinical Results of S³ Vitrification

Non-DMSO; Simplified method using straws or mS-vitrification

Clinic	Thawed Intact		Pt. ETs	No. BLs	FHB	Preg/Transfer
A	201	163 (81.1%)	83	158	89 (56.3%)	60/83 (72.3%)
B	160	141 (88.1%)	77	131	43 (32.8%)	37/77 (48.0%)
C	41	35 (85.4%)	19	35	16 (45.7%)	12/19 (63.1%)
D	566	509 (89.9%)	209	509	N/A	116/209 (55.5%)
E	13	13 (100%)	8	13	5 (62.5%)	5/8 (62.5%)
F	125	118 (94.4%)	46	117	34 (29%)	30/46 (65.2%)

Stachecki et al., 2008 Updated by J. Stachecki for PG07, ASRM 2010

"Technical Signature": replication of published results with some devices is highly variable due to technical experience and execution

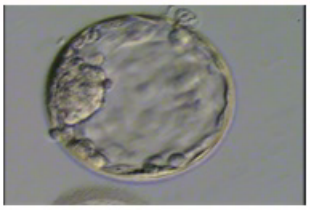
Notes:

Reports by 6 independent programs of use of an alternative vitrification solution, applied to macro-(straw) and micro-(Flexipet®) volume systems, showed that DMSO was not required to obtain consistently high clinical outcomes. Stachecki and colleagues (2008) introduced the concept of "technical signature," and that their vitrification system was a simple, safe, and secure way to vitrify blastocysts using closed or aseptic-closed devices.

1.41 Vitrified Embryo Transfer (VFET) over Fresh ET

Vitrified Embryo Transfer (VFET) over Fresh ET

- Controlled ovarian stimulation can induce changes in uterine environment that are less conducive to implantation and sustained pregnancy (Shapiro et al., 2011)
  - Slow-freeze/thawed 2PNs grown to blastocyst stage produced high pregnancy and implantation rates.



- Vitrification of all cycles becoming increasingly more routine
  - Highly efficient vitrification methods, experiencing little change in post-warming viability
  - Useful for blastocyst biopsy: results not available for fresh transfer

Notes:

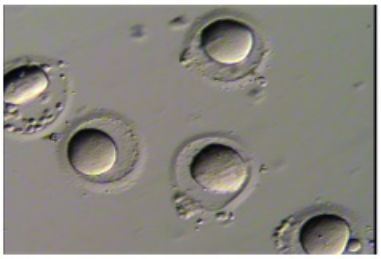
It has been reported for years, that controlled ovarian stimulation can induce changes in the uterine environment that are less conducive to implantation and sustained pregnancy (Shapiro et al., 2011). Slow-freeze/thawed 2PNs grown to blastocyst stage for transfer produced high pregnancy and implantation rates.

Highly efficient vitrification methods, experiencing little change in post-warming viability, have enabled vitrification of all cycles to become increasingly more routine. This may be useful in preimplantation genetic testing cycles when blastocyst biopsy has occurred and the test results are not available for fresh transfer.

1.42 Applications for Cryopreservation of Oocytes

Applications for Cryopreservation of Oocytes

- Government restrictions / legislation
- Fertility preservation
  - Medical
  - Social
- Emergency cycle management
  - Failure to obtain sperm
- Elective cycle management
  - Ethical/Religious reasons
  - Ovarian hyperstimulation syndrome (OHSS)
- Donor egg banking



Notes:

Tremendous improvements have been made in the cryopreservation of human oocytes in the last 20 years, moving to the application of vitrification in place of slow-freeze methods. Although improvements in outcomes of vitrified oocytes over fresh oocytes have not yet been shown, there are reports of health and well-being of the children born from oocyte vitrification (Noyes et al., 2009; Cobo et al., 2014). Oocyte vitrification is considered an effective technology for several clinical situations, as shown here. Live-birth rates following oocyte vitrification and warming from younger women (<35 years old) typically ranges from 35%-45% among IVF programs worldwide.

1.43 Oocyte Vitrification for Fertility Preservation (FP)

Oocyte Vitrification for Fertility Preservation (FP)
Medical vs. Social

	Oncological	Non oncological
Nº patients FP	361	907
Nº Patients using vitrified oocytes	11	35
Mean age at vitrification	31.9 ± 5.1	35.9 ± 4.2
Mean age at warming	36.1 ± 6.1	38.1 ± 2.8
Nº oocytes warmed	69 (6.2 ± 0.1)	250 (7.0 ± 3.5)
Survival rate	88.6	92.3
Nº embryos transferred	2 ± 0.1	2 ± 0.7
Nº patients with surplus embryos	5 (45.5)	22 (62.9)
CPR/patient	6 (54.5)	15 (42.8)
OPR/patient	5 (45.5)	11 (31.4)
Live birth	4	8
Ongoing pregnancies	1	3

Updated 5-years' experience of applying oocyte vitrification for fertility preservation at IVI courtesy of Dr. Ana Cobo

Garcia-Velasco et al., 2013

Notes:

Perhaps one of the most important applications for oocyte cryopreservation is to provide a "fertility preservation" option for women undergoing potential sterilizing medical treatments, or medical uncertainty during their remaining fertile years. As medical care continues to improve for women faced with life threatening diagnoses, oocyte vitrification is an option to preserve their future fertility if unmarried or unwilling to use donor sperm to create embryos for vitrification. The application of oocyte cryopreservation has also become an increasingly elected clinical procedure for women choosing to delay their childbearing years. Although efficacy of this technology in women <38 years old has been about half that seen with blastocyst vitrification, the database is relatively small worldwide. One concern surrounding the reclassification of oocyte cryopreservation technology as "nonexperimental" by the ASRM, is that this application may be misused in the clinical industry by promoting a business option without fully informing young women of the risk of poor or suboptimal outcomes. Based on current vitrification technology, one simply cannot ensure that all cases of vitrified eggs will be comparable with fresh eggs. There is still more to learn about the correlation between ovarian stimulation/cytoplasmic maturation and oocyte/embryo developmental competency. This is one area where investigation

is vital to optimizing future fertility preservation efforts.

1.44 Oocyte Vitrification for

Oocyte Vitrification for Emergency Cycle Management and Other Causes

	No semen sample the day of oocyte pickup	Gynecological causes
Nº of patients	18	74
Mean age	34.9 ± 3.6	37.9 ± 3.9
Nº of embryo transfers	18 (100)	68 (91.9)
Nº of vitrified oocytes	188 (9.56 ± 1.5)	899 (10.3 ± 4.1)
Survival rate	172 (91.8)	758 (84.3)
Mean number of ET	1.9 ± 0.5	1.9 ± 0.4
Implantation rate	41.7	37.1
Pregnancy rate	11 (61.1)	41 (60.3)
Clinical pregnancy rate	11 (61.1)	40 (58.0)
Ongoing pregnancy rate	11 (61.1)	35 (51.4)

Cobo et al., 2011

Notes:

Another application of oocyte vitrification would be for emergency IVF cycle management on the day of oocyte retrieval. There are cases when semen is not available for various reasons, such as the male partner's inability to collect the sample, no sperm are found with testicular sperm extraction, or unexpected travel without frozen sperm back-up. These represent situations where oocyte vitrification may be valuable to "rescue" the cycle. Other indications that favor oocyte preservation include gynecologic causes, such as inadequate endometrial thickness and endometrial bleeding the day of oocyte retrieval. The data shown here indicate that for both clinical situations, positive pregnancy outcomes can occur.

1.45 Oocyte Vitrification for Elective Cycle Management

Oocyte Vitrification for Elective Cycle Management

- Patients at risk of ovarian hyperstimulation syndrome (OHSS)
- Patients for whom embryo cryopreservation not an option due to religious or ethical reasons

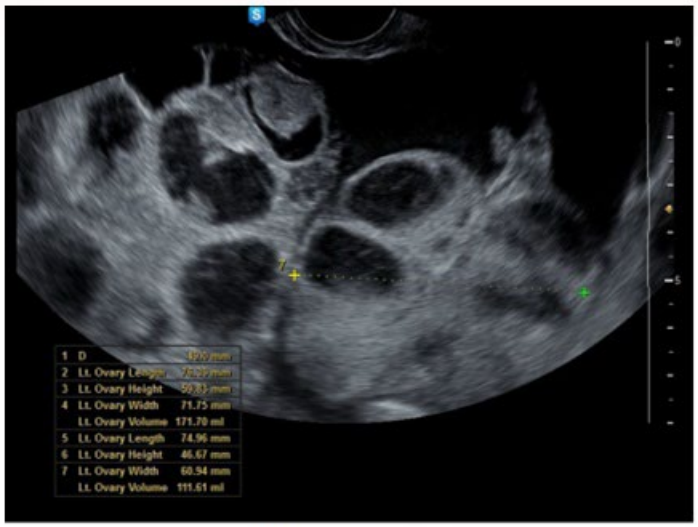
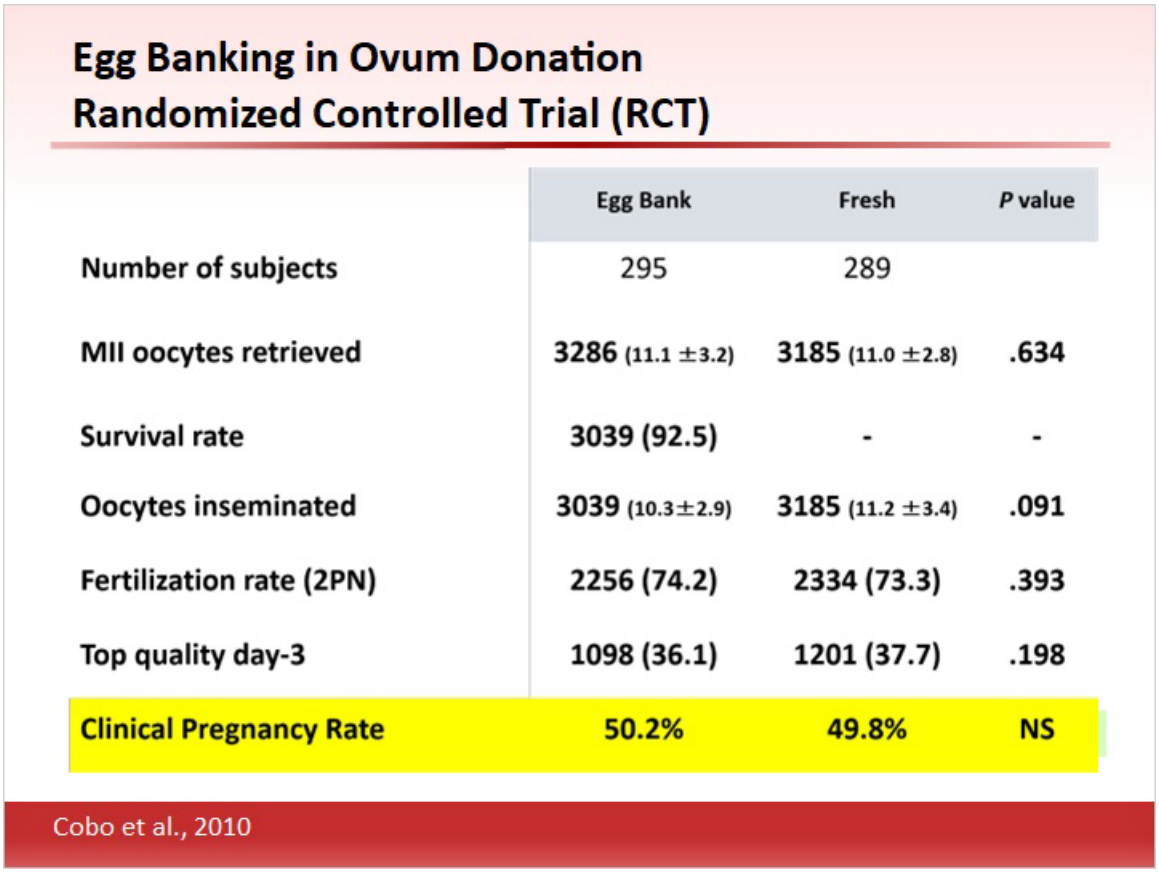


Image courtesy of Marcelle Cedars, MD

Notes:

Oocyte vitrification may also be an option in cases of patients at risk of ovarian hyperstimulation syndrome (OHSS). If embryo cryopreservation/vitrification is not a feasible option for a patient due to religious or ethical reasons then the availability of an efficient technique of oocyte cryopreservation is a valuable option, avoiding the storage of large number of embryos per patient.

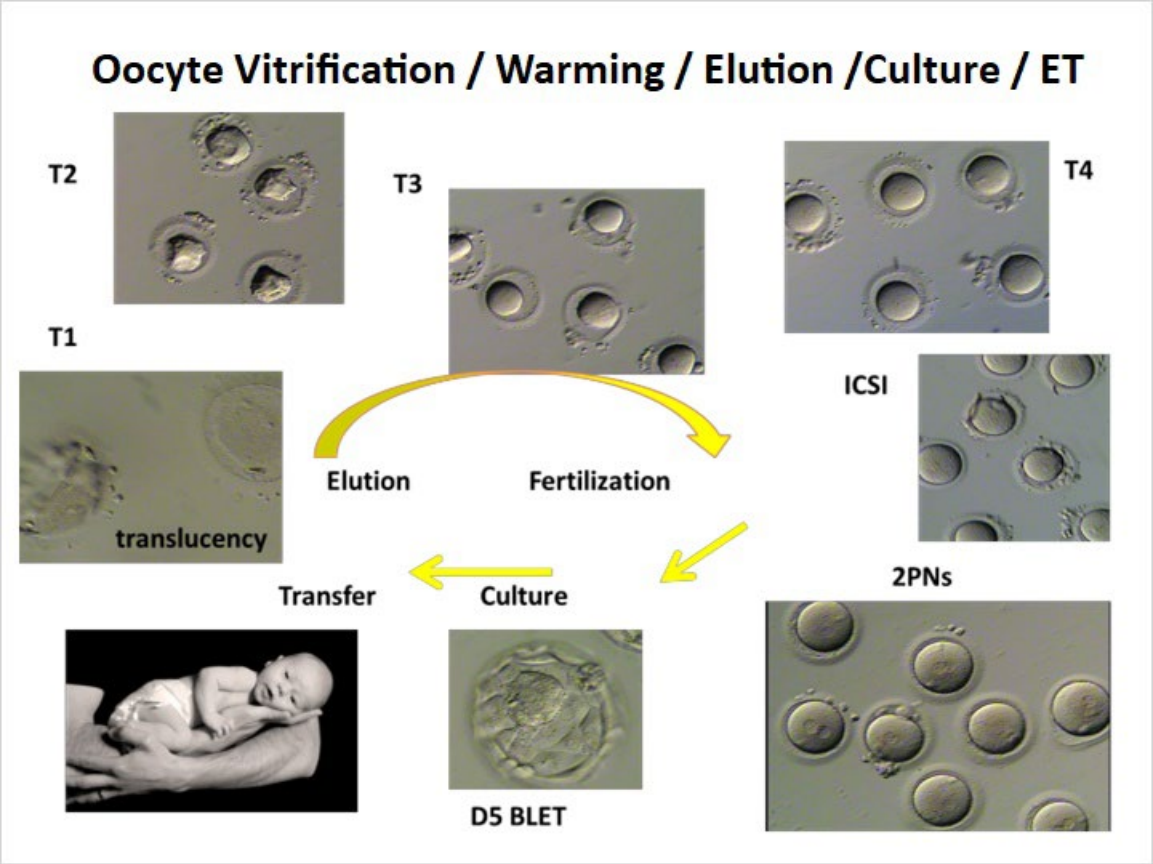
1.46 Egg Banking in Ovum Donation



Notes:

A randomized controlled trial examined the effectiveness of oocyte cryobanking in an ovum donation program and compared the outcomes of vitrified-banked oocytes with fresh oocytes. The study found that ongoing pregnancy rates for fresh oocytes were not superior to those for vitrified egg-banked oocytes.

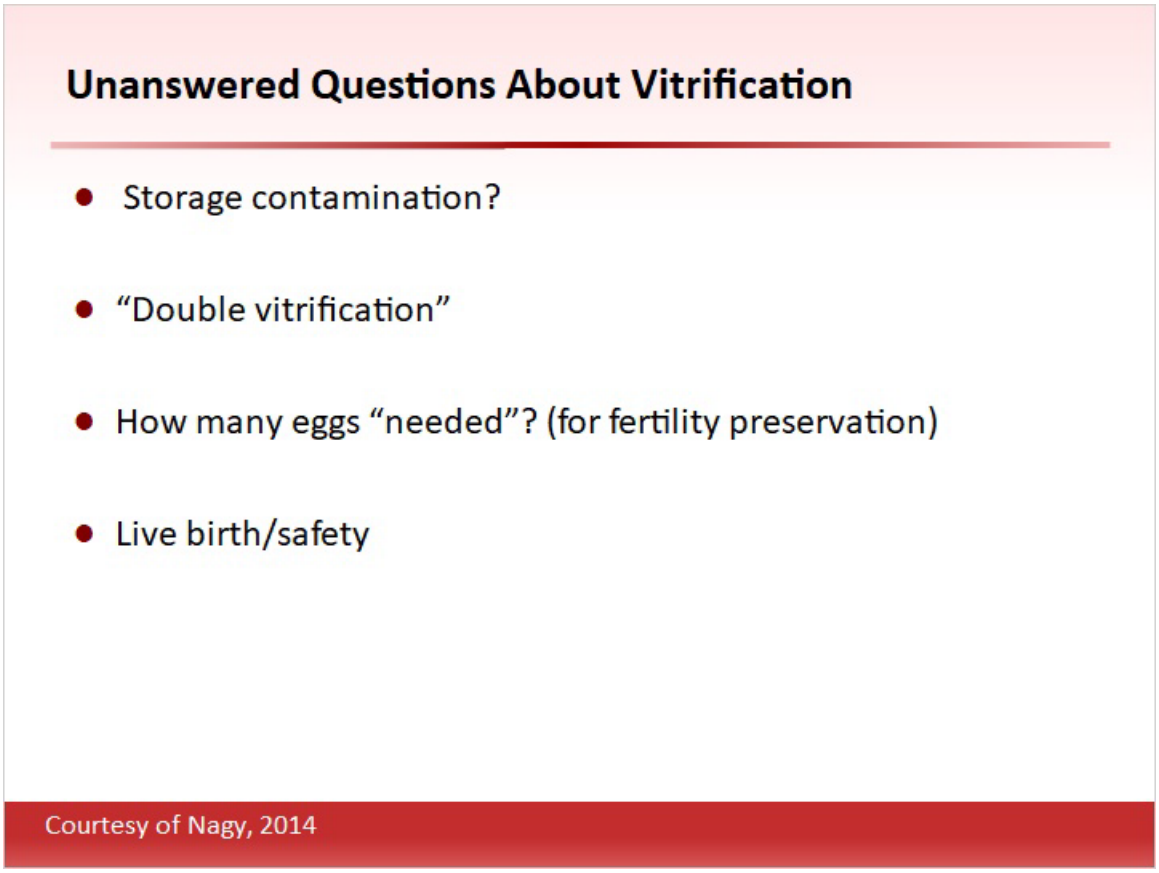
1.47 Oocyte Vitrification / Warming / Elution /Culture / ET



Notes:

Oocyte cryopreservation has proven to be a viable and effective treatment option. Yet, its overall efficacy has not been consistently demonstrated with the success rates achieved with blastocyst vitrification. While fresh donor egg live-birth rates in the United States are approximately 55%, and many top ART programs are able to routinely attain live-birth rates of 70%-80%, commercial egg banks have yet to demonstrate comparable outcomes. It is clear that there is more to learn about oocyte cryopreservation; specifically how egg quality and developmental competence is influenced by factors such as hormonal stimulation, cytoplasmic maturation, organelle functionality and membrane permeability. Furthermore, randomized controlled trials are needed to compare open and closed vitrification devices, cooling rates and warming rates, as well as unstable and metastable nonequilibration solutions, for optimizing the reliability and repeatability of oocyte vitrification.

1.48 Unanswered Questions About Vitrification



Notes:

There are several important questions related to vitrification that remain unanswered.

1)Is cross contamination between samples a real problem with LN_2 storage?
2)Can oocytes and embryos survive repeated/secondary vitrification?
3)Based on today's experience, what is the projected number of eggs needed for a woman to cryopreseve for future use (i.e., preservation of fertility), and retain a good chance of becoming pregnant?
4)What is the safety of vitrification technology to infant health and child development.

1.49 How Many Eggs? RCT: IVF Patients 30–39 years

How Many Eggs? RCT: IVF Patients 30–39 years

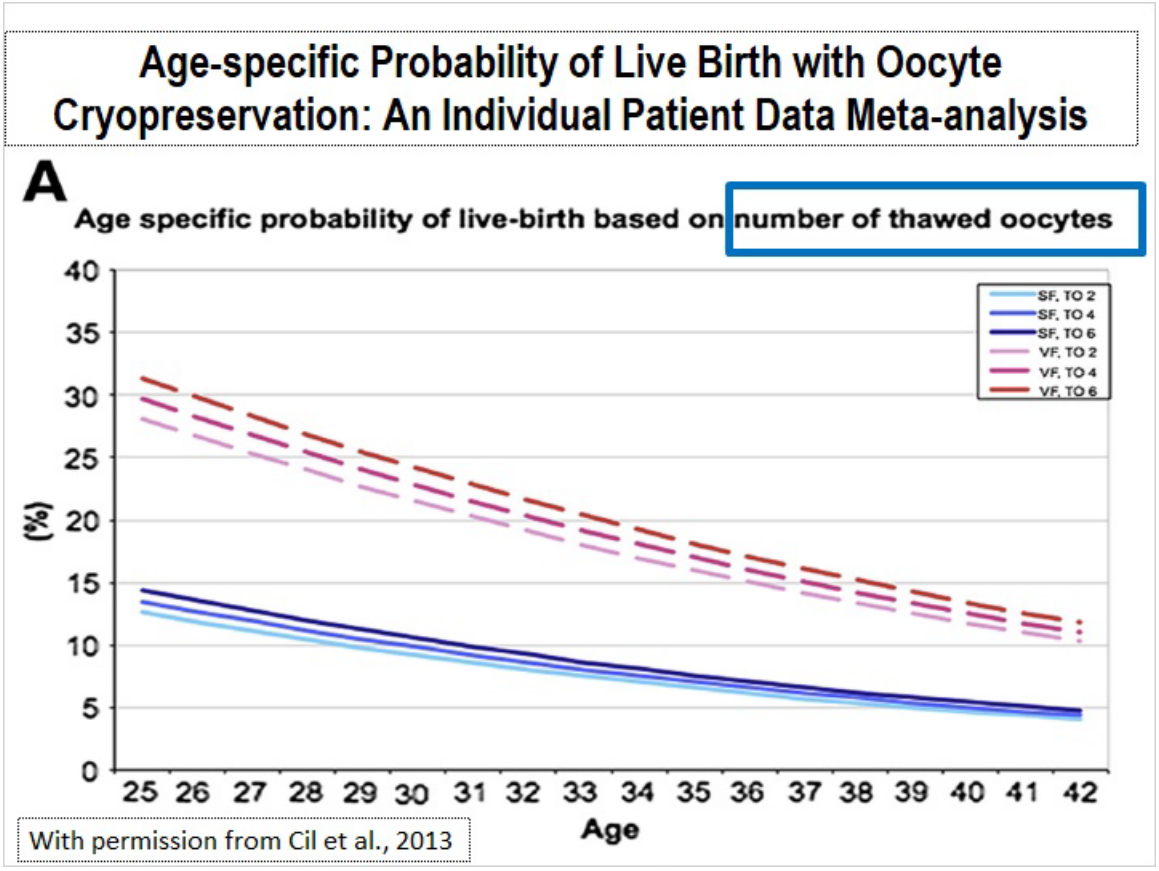
	Young 30–36 y (n=11)	Advanced 37–39 y (n=11)	*P*
Patient age (mean±SD)	32.9 ±1.9	37.9 ±0.8	<.01
Survival rate (%)	**82.5**	**76.4**	NS
Fertilization rate (%)	70.1	62.9	NS
Day 3 good Embryo (%)	55.6	40.4	<.05
Embryos transferred	24 (2.18)	29 (2.64)	NS
Clinical pregnancies (%)	**7/11 (63.6)**	**3/11 (27.3)**	NS
Implantations (%)	10/24 (41.7)	6/29 (20.7)	NS
Take-home babies (%)	6/11 (54.5)	2/11 (18.2)	NS
N° of live births	8	3	–
Oocyte to live birth (%)	**8/97 (8.2)**	**3/89 (3.3)**	NS

Chang et al., 2013

Notes:

Findings of a randomized controlled trial in which oocyte vitrification was applied to 22 normal cycling patients' eggs (11 per age group) revealed appreciable variation among patients with suboptimal survival and fertilization rates (donor egg index: ≥88% or 75%, respectively). Overall, pregnancy outcomes were good, being similar to or lower than expected fresh embryo transfer rates in each group respectively. Based on these representative data, the number of eggs needed to "freeze preserve" and use to attain similar success is predicted at approximately 12 eggs for women less than 36 years of age, or 33 eggs for women over 36 years of age.

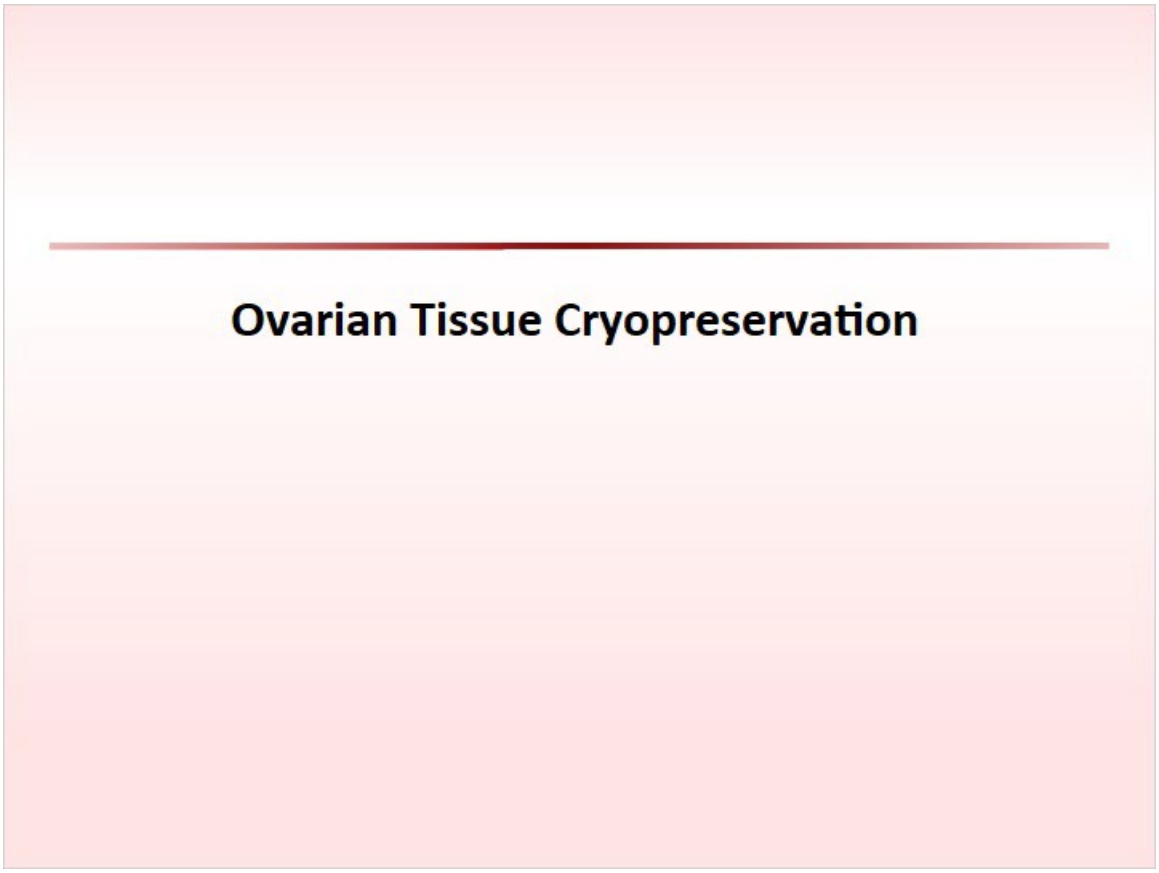
1.50 Untitled Slide



Notes:

A meta-analysis of oocyte cryopreservation effectiveness to-date confirms the expected effectiveness of vitrification vs. slow-freeze is nearly double. It would appear that greater than 6 eggs per warming would be necessary to achieve live-birth success comparable to fresh eggs/embryos.

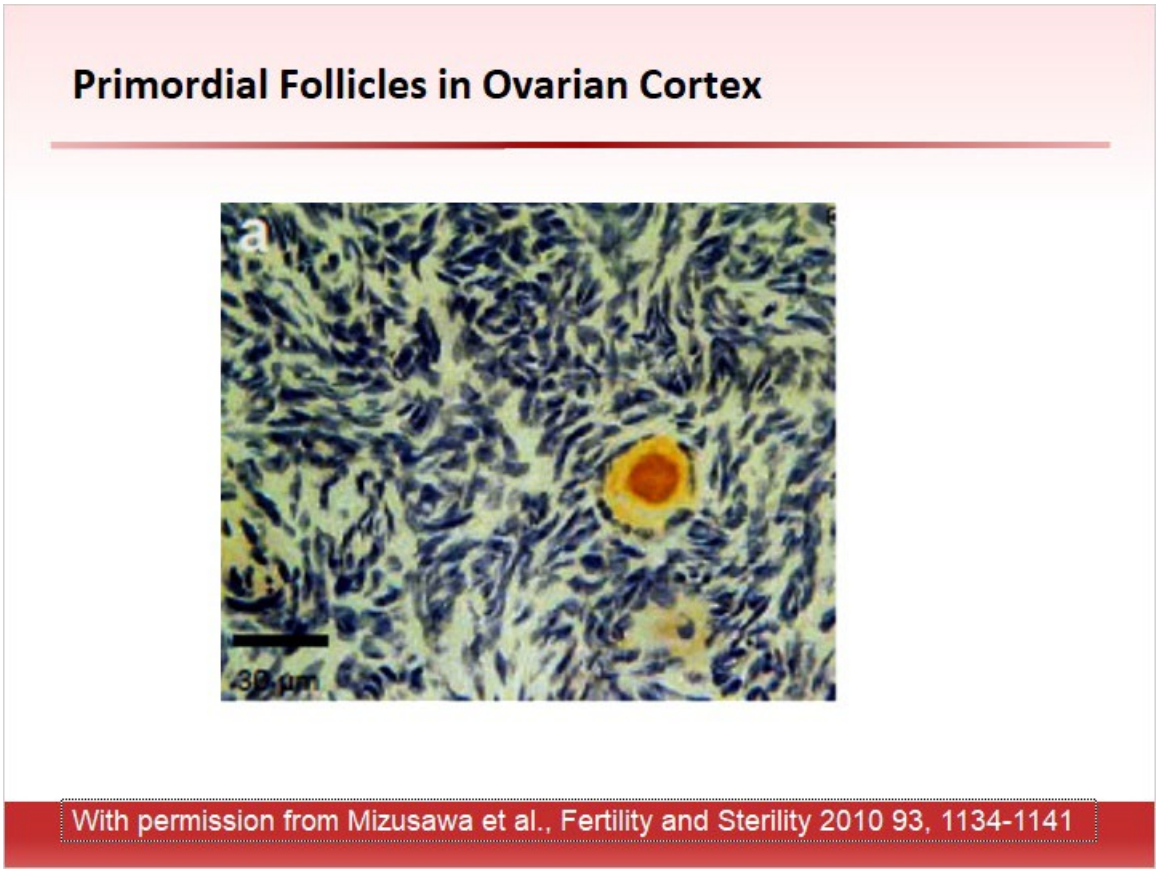
1.51 Untitled Slide



Notes:

Ovarian tissue cryopreservation is an option to preserve reproductive potential in patients who must urgently undergo aggressive chemotherapy and/or radiotherapy or who have other medical conditions requiring treatment that may threaten ovarian function and subsequent fertility. Two methods of cryopreservation have been applied to ovarian tissue: slow-freezing and vitrification. The classic standard for the cryopreservation of ovarian tissue has been slow-freezing. However, the vitrification method has gained popularity recently owing to good results obtained with vitrification of oocytes and embryos.

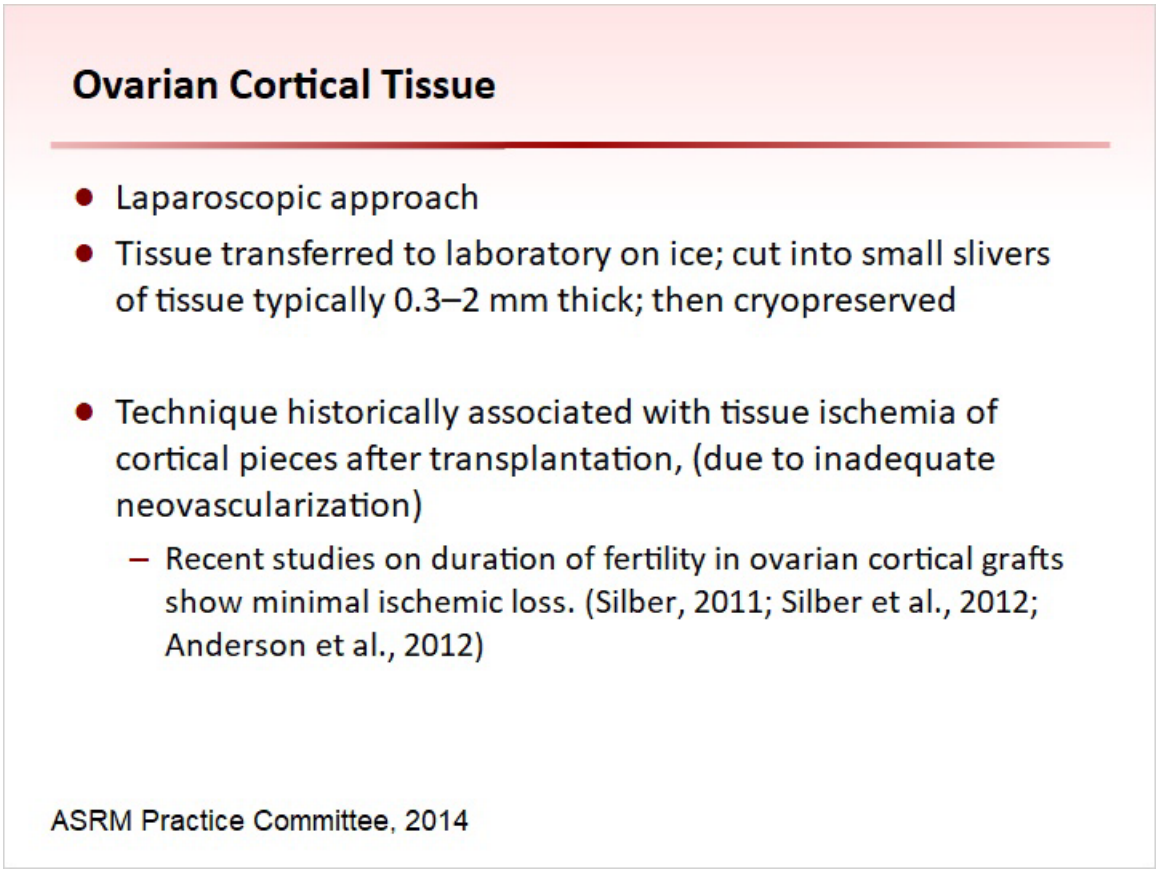
1.52 Primordial Follicles in Ovarian Cortex



Notes:

Most oocytes are located within primordial follicles in the ovarian cortex; therefore, obtaining a small volume of cortical tissue potentially enables cryopreservation of large numbers of oocytes.

1.53 Ovarian Cortical Tissue

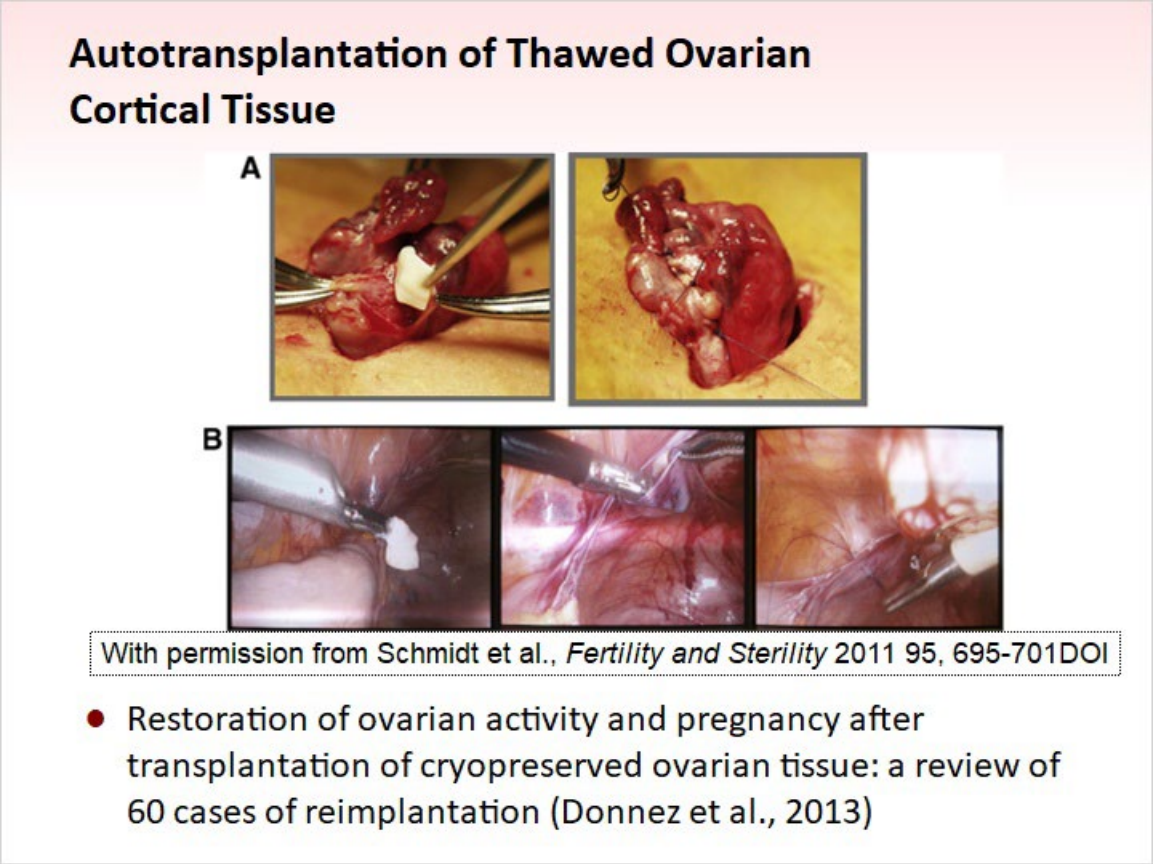


Notes:

The most common method to obtain ovarian tissue is by a laparoscopic approach, although tissue may be obtained by minilaparotomy or at the time of ovarian transposition surgery. Once the ovarian cortical tissue is obtained, it is transferred to the laboratory on ice and cut into small slivers of tissue that are typically 0.3–2 mm thick and then cryopreserved.

Although this technique has historically been associated with tissue ischemia of the cortical pieces after transplantation, owing to the inadequate process of neovascularization, recent studies on the duration of fertility in ovarian cortical grafts show minimal ischemic loss.

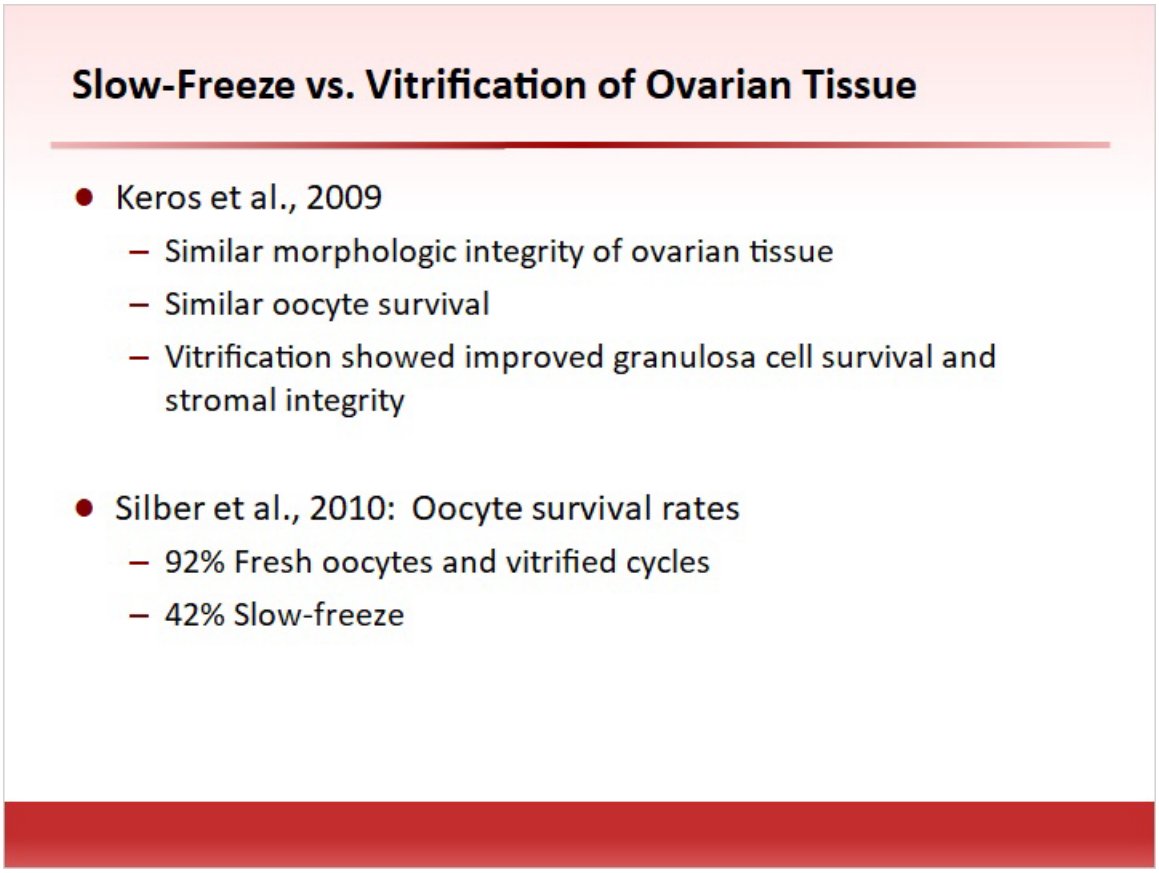
1.54 Autotransplantation of Thawed Ovarian



Notes:

Ovarian tissue may be transplanted into a pelvic (orthotopic) or extrapelvic (heterotopic) site. The process is shown here: In panel A, pieces of thawed ovarian cortex are transplanted in a subcortical pocket in the in situ ovary. In panel B, pieces of thawed ovarian cortex are transplanted in a subperitoneal pocket corresponding to the pelvic wall.

1.55 Slow-Freeze vs. Vitrification of Ovarian Tissue



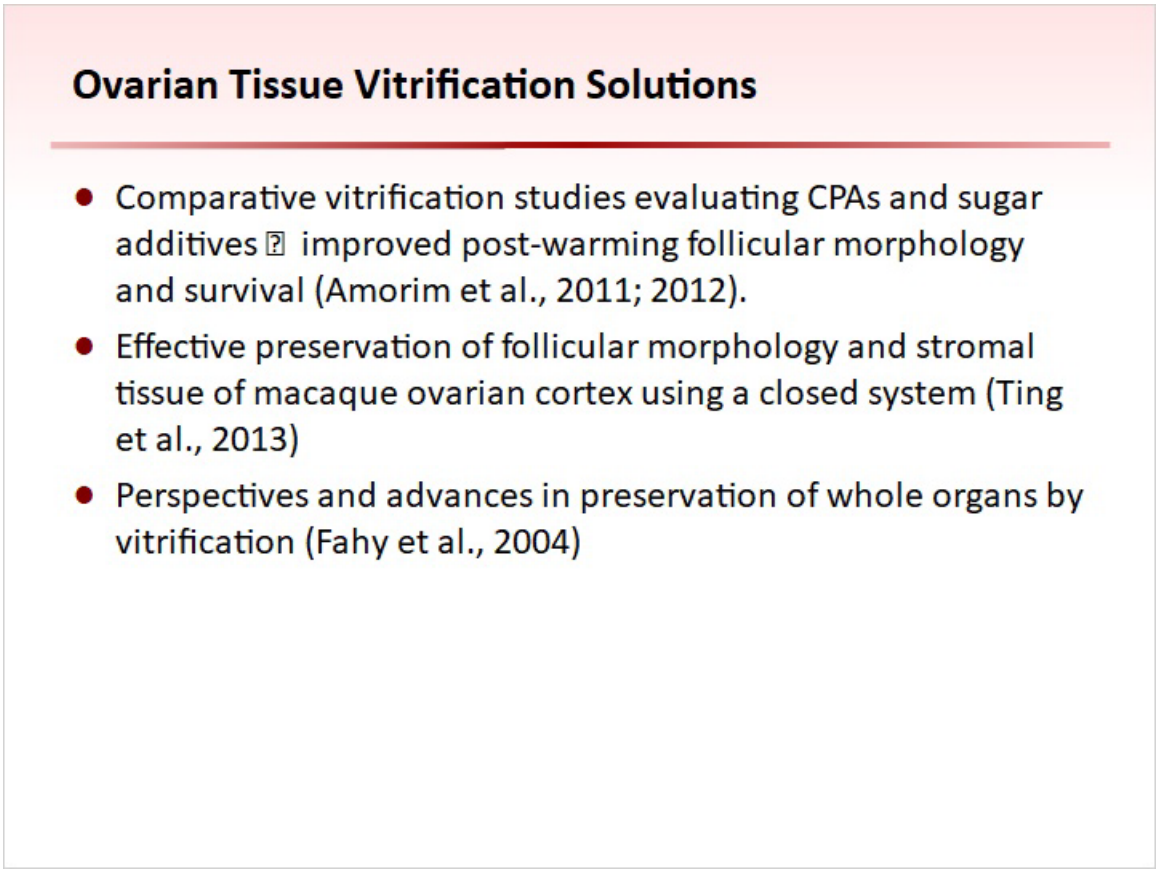
Notes:

In a systematic comparison of vitrification and slow freezing of ovarian tissue followed by tissue culture to assess subsequent oocyte viability, vitrification was demonstrated to have an outcome similar to slow freezing with preservation of the morphologic integrity of the ovarian tissue. Although the survival of oocytes was similar between the two methods, granulosa cell survival and the integrity of the stroma were improved with vitrification.

Another study evaluating the impact of slow-freezing and vitrification compared with fresh oocytes on oocyte survival rates demonstrated higher and similar viability of oocytes in the fresh and vitrified cycles (92%) compared with the slow-freeze cycles (42%), further favoring vitrification.

Although initial data suggest that vitrification of ovarian tissue may be the favored approach, outcome studies are needed before vitrification replaces slow freezing as the standard method of ovarian tissue cryopreservation.

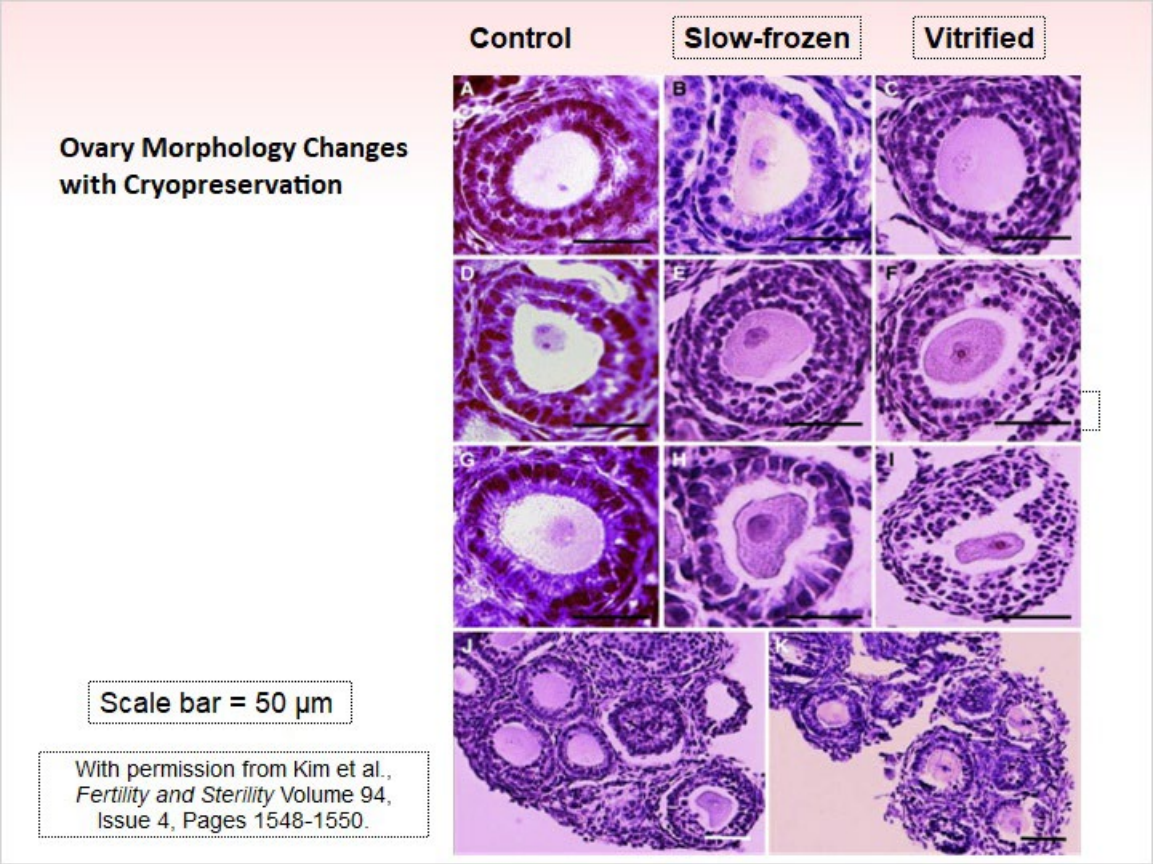
1.56 Ovarian Tissue Vitrification Solutions



Notes:

Comparative vitrification solution trials have been initiated to identify optimal solutions for ovarian tissue cryopreservation. Recent studies of metastable vitrification solutions in a closed device applied to a macaque model have provided promising results in its efficiency to maintain the structural viability of ovarian tissue. In addition, there have been promising developments in the cryopreservation by perfusion of whole organs (e.g., kidneys) with metastable vitrification solutions that could have direct application to ovaries.

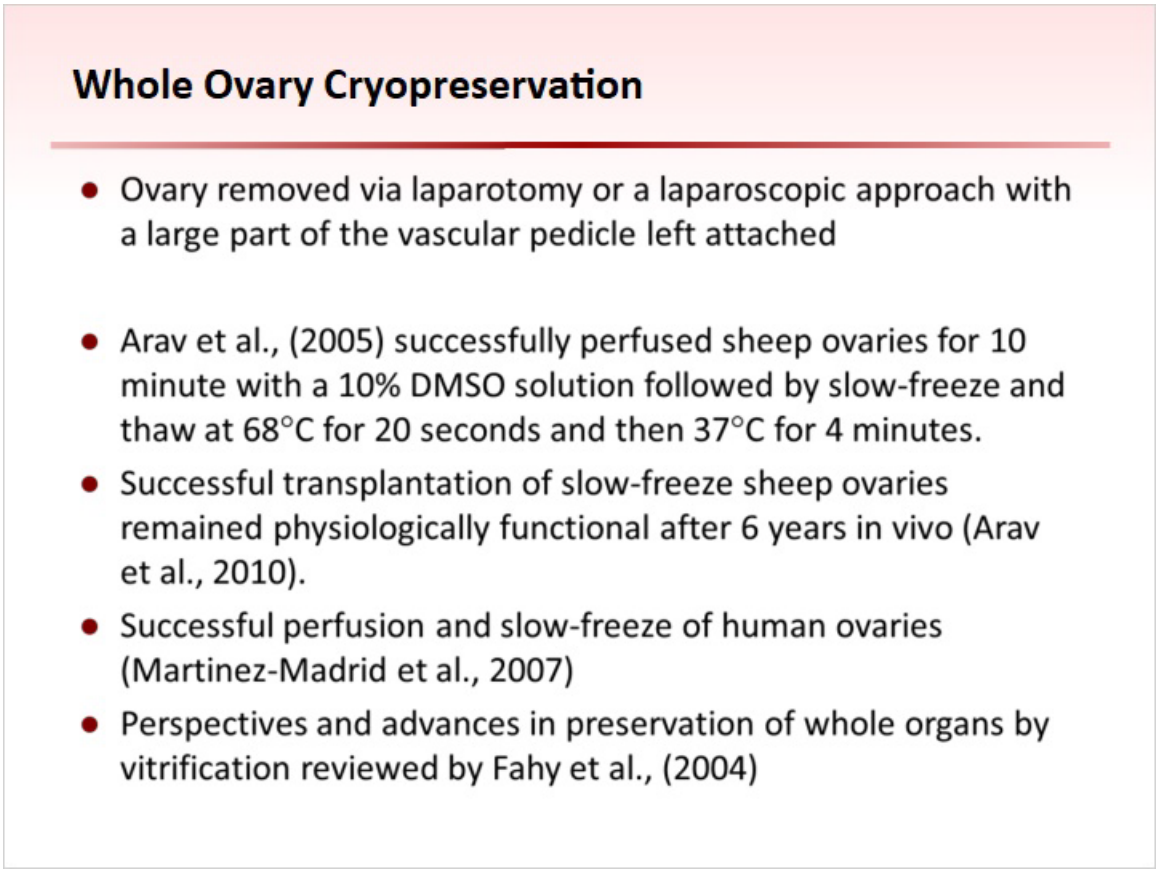
1.57 Ovary Morphology Changes with Cryopreservation



Notes:

Concerns have been raised regarding the safety of vitrification with induction of cellular toxicity and osmotic trauma with the high concentration of cryoprotectants (Fahy et al., 1990). This image illustrates the morphology changes in the ovaries cryopreserved with a slow-freeze or a vitrification protocol. Section of the paraffin-embedded intact ovaries (**A**, **D** and **G**), or ovaries cryopreserved with a slow-freezing (**B**, **E**, **H**, **J**) and a vitrification (**C**, **F**, **I**, **K**) method were stained with haematoxylin and eosin. Cryodamage such as shrunk (**E** and **F**) or deformed (**H** and **I**) cytoplasm of intrafollicular oocytes, vacuoles in ovarian stromal tissue (**K**), and dissociation of follicular cells from basement membrane of preantral follicles (**J** and **K**) were prominent in cryopreserved follicles, regardless of freezing methods.

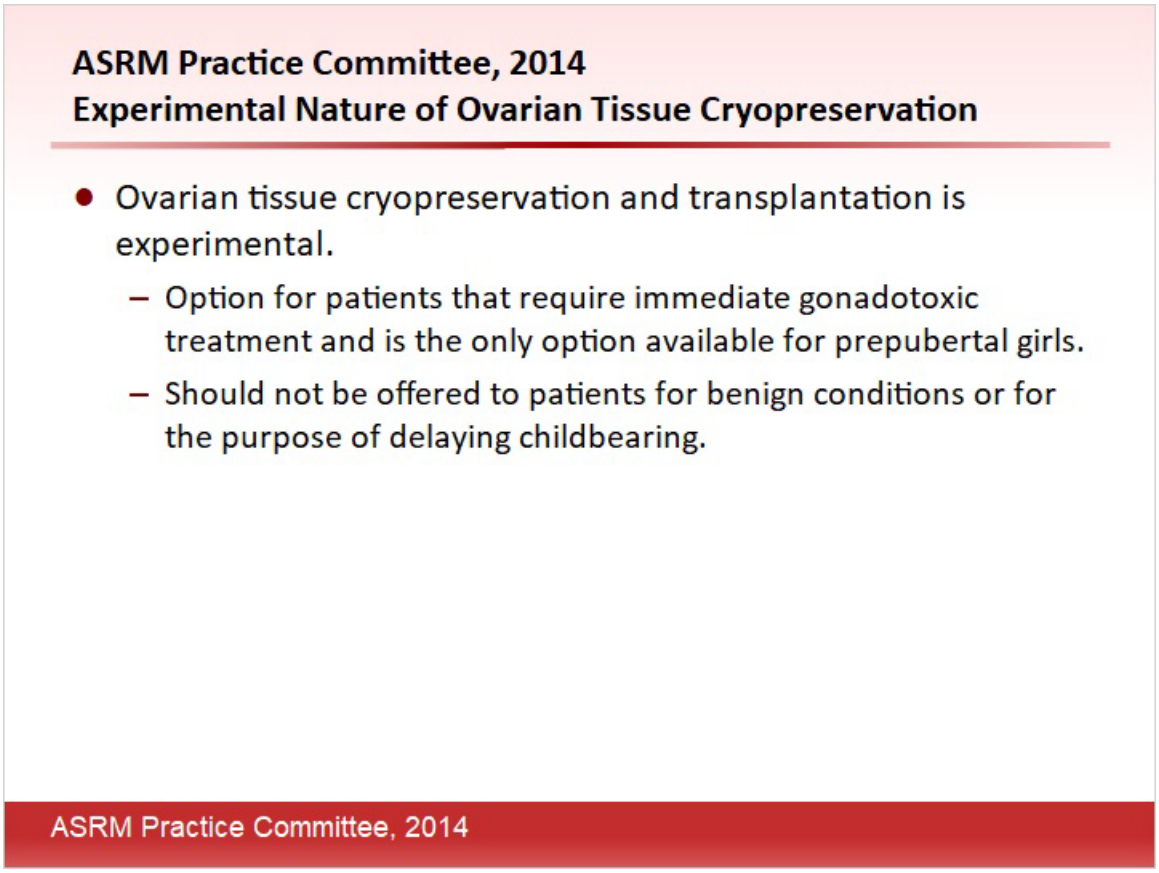
1.58 Whole Ovary Cryopreservation



Notes:

In patients in whom complete ovarian failure after treatment is anticipated, whole-ovary cryopreservation may be another option. For this indication, the ovary is removed by laparotomy or a laparoscopic approach with a large part of the vascular pedicle left attached. Inclusion of a large vascular ovarian pedicle enables the use of special perfusion equipment to introduce cryoprotectant to all cells within the ovary and remove the cryoprotectant at the time of thawing via the ovarian vessels. The limited applications for this procedure have revealed positive results. With advances in the vitrification of ovarian tissue, the future perfusion of whole ovaries with metastable vitrification solutions will likely yield promising outcomes in the years ahead.

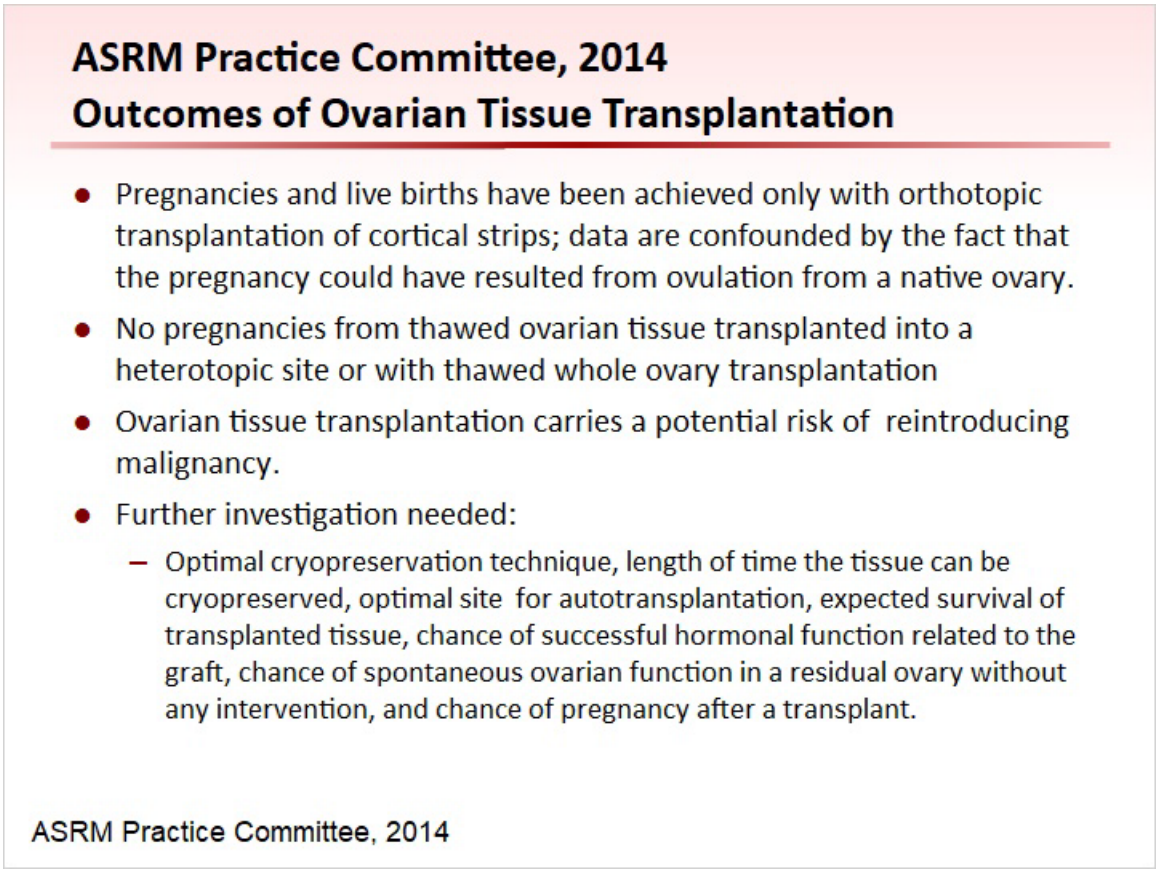
1.59 ASRM Practice Committee, 2014



Notes:

The American Society for Reproductive Medicine currently considers ovarian tissue cryopreservation to be an experimental technique for fertility preservation. This procedure is an option for patients who require immediate gonadotoxic treatment of aggressive malignancies when there is insufficient time to allow the woman to undergo ovulation induction, oocyte retrieval, and cryopreservation of oocytes and/or embryos. Ovarian tissue cryopreservation is the only option available for fertility preservation in young girls who are prepubertal or in women who have hormone-sensitive malignancies and should not be offered to patients for benign conditions or for the purpose of delaying childbearing.

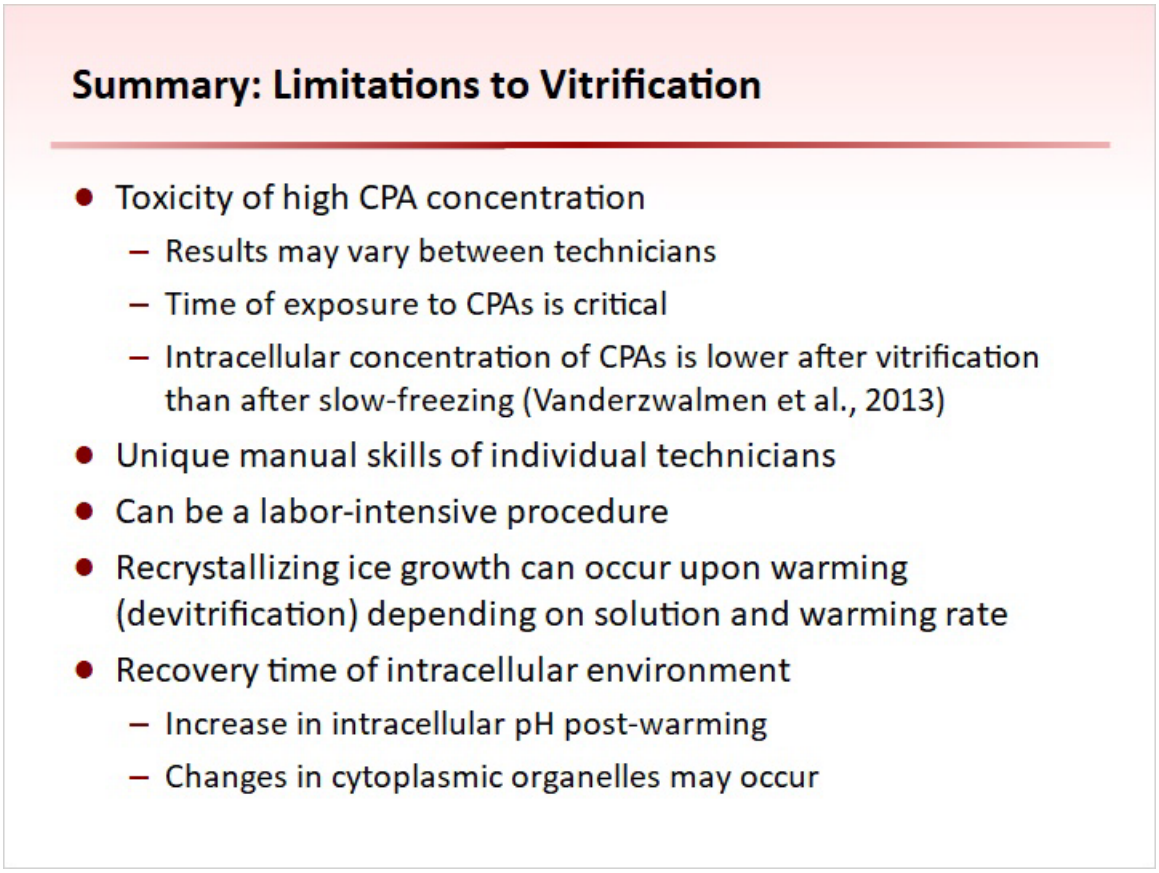
1.60 ASRM Practice Committee, 2014



Notes:

Pregnancies and live births have been achieved only with orthotopic transplantation of cortical strips; however, data are confounded by the fact that the pregnancy could have resulted from ovulation from a native ovary.
No pregnancies have been reported to date either from thawed ovarian tissue transplanted into a heterotopic site or as a result of thawed whole ovary transplantation.
Ovarian tissue transplantation carries a potential risk of reintroducing malignancy.
Issues that require further investigation include: optimal cryopreservation technique, length of time the tissue can be cryopreserved, optimal site for autotransplantation, expected survival of transplanted tissue, chance of successful hormonal function related to the graft, chance of spontaneous ovarian function in a residual ovary without any intervention, and chance of pregnancy after a transplant.

1.61 Summary: Limitations to Vitrification

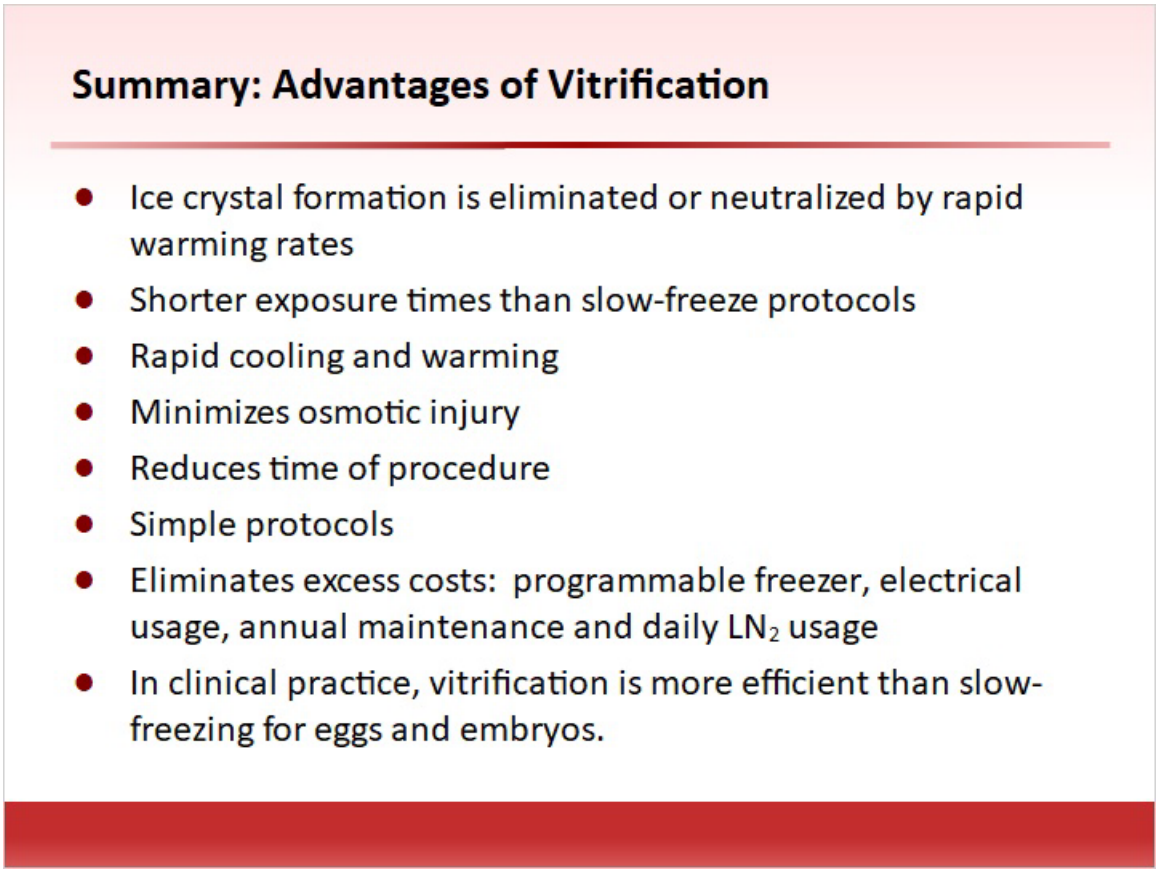


Notes:

In summary, there are a number of limitations to the vitrification procedure. The first has already been discussed: that the use of high concentrations of cryoprotectants can result in toxicity and osmotic injury. However, these toxic effects can be modulated by reducing the concentrations of cryoprotectant, shortening the time of exposure, and lowering the temperature during exposure.

In addition, results may vary from technician to technician depending on their skill at moving embryos through viscous solutions. And, if conditions are not strictly controlled, ice may still form in vitrification solutions upon warming. Furthermore, even with vitrification there may be changes in intracellular phenomena such as an increased intracellular pH post-thaw. Others have shown changes in cortical granule release and microtubule assembly. Therefore, successful vitrification still requires education and training.

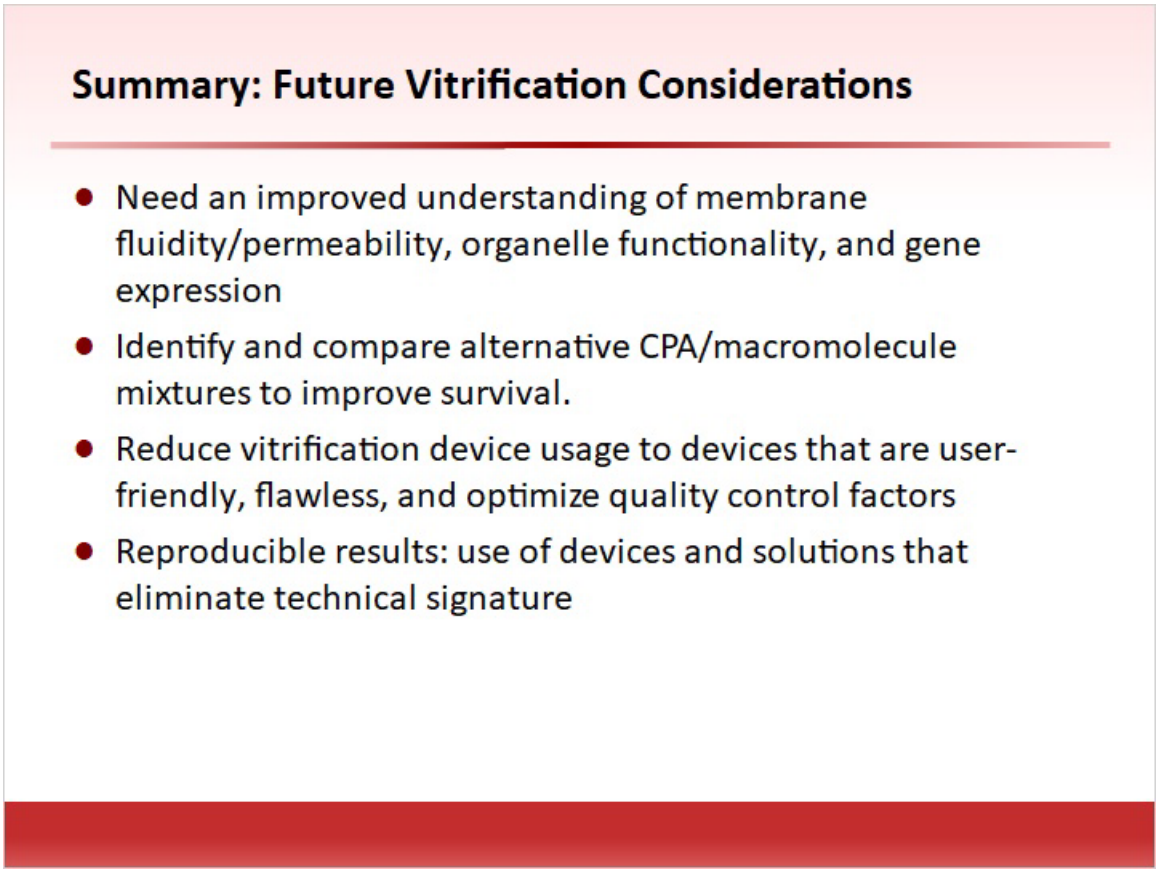
1.62 Summary: Advantages of Vitrification



Notes:

Nevertheless, the advantages of vitrification still appear to outweigh potential downsides and include the following: under well-controlled conditions, there should be little or no ice crystal formation to cause osmotic and physical disruption of the cells; vitrification requires shorter exposure times; the process can be done rapidly with minimal osmotic injury, which means that it takes less technician time to perform a procedure; the protocols are simple; and their use eliminates the cost of buying and/or maintaining a programmable freezer. Additionally, in clinical practice, vitrification is more efficient than slow-freezing for eggs and embryos.

1.63 Summary: Future Vitrification Considerations



Notes:

As with all procedures, there is always room for improvement in application and outcomes. In particular, a greater understanding of membrane biomechanics, organelle functionality/gene expression, cryoprotectant interactions, and improvements in reduced technical variation may provide important advancements in vitrification for the future. Ideally, vitrification devices will optimize quality control considerations and be user-friendly without inherent design flaws. The goal is to eliminate technical signature by reducing intra- and inter-laboratory variation by using improved devices and vitrification solutions.

1.64 Thank you!

Notes:

Thank you for participating in this educational activity.