Ovarian Tissue Vitrification: Modalities, Challenges and Potentials

Sarah Posillico1,4, Amr Kader1,2,3, Tommaso Falcone1,2 and Ashok Agarwal1,*

1Center for Reproductive Medicine, Glickman Urological and Kidney Institute, OB-GYN and Women’s Health Institute, Cleveland Clinic, Cleveland, Ohio, USA; 2Department of Obstetrics and Gynecology, West Virginia University, USA; 3Department of Obstetrics and Gynecology, University of Alexandria, Alexandria, Egypt; 4University of Vermont, Burlington, Vermont, USA

Abstract: Ovarian tissue cryopreservation is the key step towards the establishment of an ovarian tissue bank or the preservation of ovarian tissue for patients scheduled for gonadotoxic cancer therapies, aiming for fertility restoration later on. Conventional cryopreservation, or slow freezing, has been the mainstay of ovarian tissue cryopreservation. Vitrification has recently emerged as a new trend for biological specimen preservation. It has shown increasing success over slow freezing, especially with oocytes, which is mainly attributed to avoiding ice formation. Much research is underway to investigate the application of vitrification to ovarian tissue. Ovarian tissue vitrification may have specific challenges and requirements that differ from single cell or oocyte vitrification. The medical literature was searched for studies on ovarian tissue vitrification using the keywords: ovary, ovarian tissue, transplantation, vitrification, cryopreservation, and freezing. After authors’ agreement, relevant citations were analyzed. Thirty studies reported the ovarian tissue vitrification of 11 species, using different vitrification methods and different outcome measures. The vitrification of ovarian tissue is a promising alternative to slow freezing. However, proper ovarian tissue preparation and the specific method of vitrification are both key factors that determine the viability and functionality of preserved tissue in other applications, notably transplantation.

Keywords: Ovarian tissue, oocyte, cryopreservation, vitrification, transplantation, fertility preservation.

OVARIAN CRYOPRESERVATION AND TRANSPLANTATION

For the past 2 decades, ovarian tissue cryopreservation and transplantation have been under intense investigation. Currently, the most reliable method of fertility preservation is still embryo cryopreservation. However, cryopreservation and transplantation of ovarian tissue can lead to the restoration of both steroidogenic and gametogenic functions of the ovary, which in turn can restore quality of life in patients who are facing premature ovarian failure [1-6]. Ovarian cryopreservation also avoids the ethical problems relating to the need for a male gamete at the time of embryo preservation, thus allowing women without male partners to preserve their fertility [4, 7, 8]. Ovarian tissue can be obtained at any time during the menstrual cycle [8-10]. It can also be obtained from pre-pubescent girls, and it is a better option for women who are planning to receive gonadotoxic treatment and cannot wait for in vitro fertilization (IVF) [4, 7, 9, 11].

Once ovarian tissue cryopreservation is optimized for transplantation, various patient populations will be able to take advantage of this technology. These populations include cancer patients undergoing chemo- or radiotherapy and women who have a social interest in preserving fertility while delaying childbearing [7, 11-13]. This technology may also be used to preserve fertility in girls with mosaic Turner syndrome, who experience premature menopause, and therefore usually do not have the option of conceiving during the normal fertile years [14]. In addition, ovarian tissue cryopreservation and subsequent transplantation have even been acknowledged by some as a possible way to ensure the future preservation of endangered animals [3, 15]. The cryopreservation and transplantation of ovarian tissue is currently being investigated in various large mammals, most notably sheep and pigs [8, 16-19].

However, while some success for has been recorded with transplantation, the results thus far fall short of desired expectations [16]. One meta-analysis determined that fresh grafts were more likely to recover ovarian function than grafts that had been cryopreserved [12]. Although women with cryopreserved grafts did regain ovarian function, over 20% experienced recurrence of ovarian failure [12]. Graft failure is mainly caused by ischemic damage [4, 6, 12, 18-21].

Most preservation techniques have relied on the transplantation of ovarian tissue that had been slowly cryopreserved using conventional methods [16, 17, 22]. In 2003, Bedaiwy et al. reported that intact sheep ovaries survived cryopreservation and autotransplantation with vascular anastomosis in 3 out of 11 grafts [17]. Imhof et al. reported similar success with a long-term follow up of cryopreserved whole sheep ovaries grafted with microvascular anastomosis. Four out of 9 ewes resumed luteal function, with one achieving spontaneous pregnancy and birth after spontaneous intercourse. However, long term follicular survival was 1.7 -7.6%
after 18 months of transplantation [16]. In a recent report of their experience, Donnez et al. described the follow up of 5 patients who received orthotopic transplantation of cryopreserved ovaries [23]. They reported that orthotopic transplantation of frozen ovarian tissue restored endocrine function in all 5 of their patients and that the grafts were still functioning after a mean of approximately 3 years in three of the patients who had had chemotherapy-induced amenorrhea before transplantation [23].

Ovarian tissue can be cryopreserved in various forms, such as fragments, slices, semi-ovaries, or whole ovaries with a vascular pedicle for future vascular anastomosis. The smaller forms—fragments, slices, and isolated follicles—may have a higher probability of being viable after cryopreservation because the cryoprotectants can more fully permeate the tissue, and thus prevent cryodamage due to ice formation. Whole ovaries tend to be more difficult to preserve due to their dense tissue structure, intricate vascular system, and diversity of cell types, all of which makes proper protection with cryoprotectants challenging and results in poor heat transfer and uneven cooling rates [4, 24, 25].

OOCYTE CRYOBIOLOGY & OVARIAN TISSUE CRYOPRESERVATION

The oocyte has always been the most challenging specimen to cryopreserve due to its relative intolerance to cryopreservation [3]. This intolerance is attributed to a number of variables including the oocyte’s large cytoplasmic volume and complex cellular structure, which is especially vulnerable to cryoinjury caused by intracellular ice formation [4, 11, 26]. Due to the oocyte’s sensitivity, fertilization and birth rates between studies are highly variable, causing many to regard conventional oocyte cryopreservation as experimental [27].

Since vitrification aims to avoid ice formation, it should theoretically provide a better alternative to oocyte preservation than slow freezing. The oocyte needs an adequate media protocol and vitrification methodology to achieve proper biological preservation [28, 29]. In a meta-analysis, Oktay et al. reviewed the current state of mature oocyte vitrification [30]. They found that the low survival or fertilization rates that were initially achieved in early trials have dramatically improved in the last few years. These improvements reflect the maturation of this technology, the improvement of protocols, and the accumulation of experience among its practitioners [30]. More recent studies revealed that vitrification using the Cryotop method reduced oocyte cryo-damage and resulted in a significant number of healthy births [31]. Other clinical reports showed comparable oocyte maturation and fertilization rates between vitrified and fresh oocytes [7, 32]. Despite these promising results, pioneering efforts have yet to successfully determine the optimal vitrification protocol for ovarian tissue and whole ovary vitrification for vascular ovarian grafts.

Primordial follicles (PMF), which comprise about 90% of the follicular population of each ovary, are less cryosensitive than mature oocytes, embryos, and maturing follicles, primarily due to their morphology—less cytoplasm and cryosensitive cytoplasmic components, no zona pellucida, fewer granulosa cells—as well as the low metabolic rate associated with their dormancy [4, 9, 33]. Therefore, PMF consequently stand a better chance of surviving slow cryopreservation or vitrification than any growing stage follicles [9, 18]. However, little knowledge is available about the paracrine functions of the ovarian tissue cells and matrix, and many key roles required for inducing oocyte maturation remain unidentified, thereby preventing the achievement of a standard and efficient setting for prolonged in vitro maturation (IVM) and the use of PMF in a clinical setting [5, 15, 34]. Successful IVM of PMF can avoid the ischemic damage in ovarian tissue grafting techniques and avoids the risks of reintroducing malignancy through tissue transplantation [5]. Until IVM of PMF can be developed, the PMF must be stored in situ in their natural encasing: the ovarian tissue [5, 15].

VITRIFICATION

Although the conventional slow freezing method has been used successfully for a number of years, it has disadvantages; the most noticeable of which is intracellular ice formation and subsequent cell damage. On the other hand, vitrification is a rapid cooling cryopreservation technique that results in solidification without crystallization, thus avoiding cryodamage resulting from ice formation [11].

The first critical step in vitrification is the cooling rate, which must exceed the solution’s critical cooling rate (CCR). This is the slowest cooling rate that still allows a solution to turn into glass before ice crystals can organize [35]. Water has an extremely high CCR, which makes vitrification of pure water almost impossible in lab settings. Therefore, as much as possible, water needs to be replaced with cryoprotectants such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG), which have lower CCRs. By achieving the adequate cooling rate that exceeds the sample’s CCR at a given viscosity and dehydration level, cryodamage due to intra- and extracellular ice formation can be avoided, as the sample transforms immediately from an aqueous to a vitreous state [3, 19]. Accordingly, the thawing rate must also be rapid to prevent devitrification, which then results in ice formation. A prolonged exposure to subzero temperatures during a slow warming process may still allow damaging ice crystal organization [35]. The high rates of cooling and thawing necessary for vitrification are achieved with classic protocols in which a small volume of the specimen is loaded into a loading device along with the minimum necessary volume of cryoprotectant. The specimen is either directly exposed to liquid nitrogen or is loaded into a closed, thin-walled loading device to achieve maximum heat transfer rates [31].

MATERIALS AND METHODS

The literature was searched using the keywords ovary, ovarian tissue, oocyte, transplantation, vitrification, and cryopreservation, and relevant citations were identified. In addition, cross-referencing and general knowledge of the field were also used. After agreement between authors, only selected citations of relevance to the topic were further analyzed. Thirty studies describing ovarian tissue vitrification using 11 different animal species, including humans, along with 14 different loading devices and a multitude of different vitrification media protocols were identified and analyzed.
OVARian TISSue VITrification EXPERIENCE

To date, there have been various efforts investigating ovarian tissue vitrification in a variety of species using different tissue shapes and different vitrification protocols. Table 1 lists the different studies, species used, carrier method, and final vitrification media used.

One of the earliest of these experiments was performed by Sugimoto et al., who autotransplanted vitrified whole infantile rat ovaries using a vitrification solution containing 20.5% DMSO, 15.5% acetamide, 10% PROH, and 6% PEG, performing the vitrification in a glass test tube [2]. Cyclicity resumed in all but one of the rats, and histological examination of the transplants showed that there was no variation between the fresh and vitrified grafts. At 84 days post-transplantation, all the transplants contained corpora lutea as well as antral follicles, and although the antral follicle count was indeed higher in the fresh grafts, the results showed that rat ovaries could be vitrified in a glass test tube and survive rapid-cooling [2]. In a unique study, since it used ovaries from 5 different species, Kagabu and Umezo vitrified ovaries extracted from mice, Chinese hamsters, rabbits, Japanese monkeys, and rats and transplanted them into rat uteri [36]. The vitrification was carried out in freezing tubes using a 3 step protocol with a final solution containing 20% DMSO, 15.5% Acetamide, 10% PROH and 6% PEG. The study showed survival of PMF after vitrification and grafting of 16.9% in mice, 14.6% in monkey, 13.6% in Chinese hamsters, 11.2% in rats and 6.5% in rabbits as compared to the original counts [36].

In a study similar to Sugimoto’s trial, Salehnia vitrified whole mice ovaries using EG and acetamide for cryoprotectants and the cryovial as the loading device and autotransplanted the ovaries intraperitoneally [37]. Salehnia obtained results comparable to those of Sugimoto et al., in that normal primordial and primary follicles were found in the ovaries despite a decrease in overall follicular count [2, 37]. Also using a 1ml cryotube, Migishima et al. vitrified whole mouse ovaries using DAP213 [38]. After orthotopic transplantation, the vitrified-thawed grafts had normal folliculogenesis and corpora lutea formation, and the authors concluded that mouse ovaries could retain their fecundity after vitrification and transplantation, as demonstrated by the spontaneous birth of pups in both the fresh graft and vitrified graft groups [38]. It is important to note, however, that the frozen-thawed grafts contained significantly fewer follicles than the fresh grafts, and there were fewer pups born in and fewer oocytes collected from the frozen-thawed group [38]. Liu et al. also vitrified mouse ovaries in cryotubes using DAP213, and transplanted the ovaries after thawing [39]. They observed no difference between the fresh and vitrified transplanted ovaries in regards to the number of litters [39].

Using the cryotop as a loading device, Kagawa et al. vitrified ovarian tissue fragments in a 2 step vitrification protocol, with equilibration first in 7.5% DMSO, 7.5% EG and 20% SSS, followed by a final vitrification media containing 15% DMSO, 15% EG and 20% SSS [40]. Following vitrification and warming, the fragments were autotransplanted under the kidney capsules of adult mice. The transplant resulted in the development of preantral follicles and eventually led to the birth of 10 healthy pups after IVM and IVF [40].

In another experiment, ovarian tissue from mice, rabbits, and pigs were collected. The author evaluated the viability of the tissues post-vitrification by in vitro growth (IVG), IVM, and IVF/ET [34]. Hasegawa et al. equilibrated their samples in a solution of 7.5% DMSO and 20% SSS and transferred them to a vitrification solution containing 15% EG, 15% DMSO, 0.5 M sucrose, and 20% SSS [34]. The mouse ovaries and rabbit and pig ovarian tissues were loaded onto polyester sheets, plunged into liquid nitrogen and then stored in cryovials [34]. Not only did the primordial and maturing follicles retain good morphology after vitrification, but the mouse oocytes aspirated from the preantral follicles that had been vitrified in situ in ovarian tissue also developed into pups after IVG-IVM and IVF/ET [34]. The live pup birth rate was similar between the vitrified and fresh ovary groups, and there were no histological changes in the ovaries in either group [34]. Ishijima et al. vitrified canine ovaries using DAP213 and transplanted them into SCID mice [41]. Four weeks after the grafting procedure, proliferating cell nuclear antigen (PCNA) was detected in many granulosa cells of primary follicles, indicating resumption of activity [41].

One study reported successful vitrification of ovine hemi-ovaries. The hemi-ovaries were stripped of their medullas and cut into 1 mm × 2 cm × 1 cm pieces, and the pieces were vitrified in a 1-ml cryotube after being exposed to VS4 vitrification solution [42]. Orthotopic autografting and subsequent histological analysis were performed, and the results showed that ovarian endocrine function had resumed and fecundity had been restored [42]. Four healthy lambs were born, one of which died immediately after birth due to congenital malformations. One year after transplantation, histological analysis showed that primordial follicle count from the extracted grafts was very low, ranging from 6 to 58 follicles per transplant [42]. Regarding the fourth lamb, it was determined that its congenital malformations and subsequent death could not be immediately blamed on the vitrification process. However, the authors did indicate the need for more research into the correlation between vitrification and malformations [42].

Direct cover vitrification (DCV) is a method where liquid nitrogen is poured into a cryovial containing the sample. Chen et al. used this technique to vitrify ovarian tissue with encouraging results [1]. In their study, DCV was performed using an equilibration solution of 7.5% EG and 7.5% DMSO and a vitrification solution of 15% EG, 15% DMSO, and 0.5 M sucrose [1]. The tissue’s viability, morphology, and ultrastructure integrity were preserved, and fecundity resumed. Fresh ovarian tissue and tissue vitrified using DCV were found to have higher rates of morphologically normal primary and secondary follicles compared to tissue that had been preserved with traditional vitrification in a straw using Salehnia’s protocol [1, 37]. DCV was shown to be better than slow freezing and conventional vitrification, and its results were similar to those of the fresh control [1]. In conjunction with these results, Isachenko et al. vitrified human ovarian tissue via direct contact with liquid nitrogen and found that the stroma and follicles were adequately protected from

<table>
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<tr>
<th>Species</th>
<th>Vitrification Solution</th>
<th>Results</th>
<th>Authors</th>
</tr>
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<tbody>
<tr>
<td>Mouse</td>
<td>20.5% DMSO, 15.5% Acetamide, 10% PROH, and 6% PEG</td>
<td>Cyclicity resumed, histological examination showed no variation</td>
<td>Sugimoto et al.</td>
</tr>
<tr>
<td>Mouse</td>
<td>EG and acetamide for cryoprotectants and the cryovial as the loading device</td>
<td>Survival of PMF after vitrification and grafting</td>
<td>Kagabu and Umezo</td>
</tr>
<tr>
<td>Mouse</td>
<td>7.5% DMSO, 7.5% EG and 20% SSS</td>
<td>Development of preantral follicles</td>
<td>Liu et al.</td>
</tr>
<tr>
<td>Mouse</td>
<td>7.5% DMSO, 7.5% EG and 20% SSS</td>
<td>Birth of pups</td>
<td>Hasegawa et al.</td>
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</tbody>
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<tr>
<th>Species</th>
<th>Vitrification Solution</th>
<th>Results</th>
<th>Authors</th>
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<tbody>
<tr>
<td>Dog</td>
<td>DAP213</td>
<td>Survival of PMF after vitrification and grafting</td>
<td>Migishima et al.</td>
</tr>
<tr>
<td>Dog</td>
<td>DAP213</td>
<td>Normal folliculogenesis and corpora lutea formation</td>
<td>Liu et al.</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.5 M sucrose, 7.5% DMSO, and 15% EG</td>
<td>Survival of PMF after vitrification and grafting</td>
<td>Hasegawa et al.</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.5 M sucrose, 7.5% DMSO, and 15% EG</td>
<td>Survival of PMF after vitrification and grafting</td>
<td>Ishijima et al.</td>
</tr>
<tr>
<td>Ovine</td>
<td>0.5 M sucrose, 7.5% DMSO, and 15% EG</td>
<td>Survival of PMF after vitrification and grafting</td>
<td>Chen et al.</td>
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</tbody>
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<th>Species</th>
<th>Vitrification Solution</th>
<th>Results</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Direct cover vitrification (DCV)</td>
<td>Survival of PMF after vitrification and grafting</td>
<td>Isachenko et al.</td>
</tr>
</tbody>
</table>
Table 1. List of Reports on Ovarian Tissue Vitrification in Different Species, using Different Vitrification Protocols and Loading Devices

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Loading Device</th>
<th>Vitrification Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugimoto et al. (2000)</td>
<td>Rat</td>
<td>Glass test tube</td>
<td>20.5% DMSO, 15.5% acetamide, 10% PROH, 6% PEG</td>
</tr>
<tr>
<td>Kagabu and Umezu</td>
<td>Mouse, Chinese hamster, rabbit, Japanese monkey, rat</td>
<td>Cryotube</td>
<td>20% DMSO, 15.5% acetamide, 10% PROH, 6% PEG</td>
</tr>
<tr>
<td>Salehnia (2002)</td>
<td>Mouse</td>
<td>Cryovial</td>
<td>30% Ficoll, 0.5 M sucrose, 10.7% acetamide, 40% EG</td>
</tr>
<tr>
<td>Migishima et al. (2003)</td>
<td>Mouse</td>
<td>1 ml cryotube</td>
<td>DAP213</td>
</tr>
<tr>
<td>Isachenko et al. (2003)</td>
<td>Human</td>
<td>DCV (straws, copper grids)</td>
<td>40% EG, 0.35 M sucrose, 5% egg yolk</td>
</tr>
<tr>
<td>Rahimi et al. (2004)</td>
<td>Human</td>
<td>0.25 ml straws, copper EM grids</td>
<td>25% glycerol, 24% EG, 1% Supercool® X-100, egg yolk</td>
</tr>
<tr>
<td>Bordes et al. (2005)</td>
<td>Sheep</td>
<td>1 ml cryotube</td>
<td>VS4</td>
</tr>
<tr>
<td>Hasegawa et al. (2006)</td>
<td>Mouse, rabbit, pig</td>
<td>Cryovial</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose, 20% SSS</td>
</tr>
<tr>
<td>Chen et al. (2006)</td>
<td>Mouse</td>
<td>DCV (in cryovial)</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
</tr>
<tr>
<td>Ishijima et al. (2006)</td>
<td>Dog</td>
<td>Cryotube</td>
<td>DAP213</td>
</tr>
<tr>
<td>Hani et al. (2006)</td>
<td>Mouse</td>
<td>Cryotube</td>
<td>DAP213</td>
</tr>
<tr>
<td>Gandolfi et al. (2006)</td>
<td>Human, cow, pig</td>
<td>0.5 ml cryostraw</td>
<td>Method 1: 5.64 M EG, 5% PVP, 0.4 M trehalose, 20% FBS in TCM 199 Method 2: 3.58 M EG, 2.82 M DMSO, 20% FBS in PBS</td>
</tr>
<tr>
<td>Kagawa et al. (2007)</td>
<td>Mouse</td>
<td>Cryotop</td>
<td>15% EG, 15% DMSO</td>
</tr>
<tr>
<td>Santos et al. (2007)</td>
<td>Goat</td>
<td>SSV</td>
<td>20% EG, 0.5 M sucrose</td>
</tr>
<tr>
<td>Li et al. (2007)</td>
<td>Human</td>
<td>Carrier-less method</td>
<td>2 M DMSO, 2 M PROH, 0.2 M sucrose</td>
</tr>
<tr>
<td>Choi et al. (2007)</td>
<td>Mouse</td>
<td>EM grids</td>
<td>40% EG, 18% Ficoll, 0.5 M sucrose, 20% FBS</td>
</tr>
<tr>
<td>Isachenko et al. (2007)</td>
<td>Human</td>
<td>1.8 ml cryovial</td>
<td>Method 1: 20% DMSO, 20% EG, 10% SSS Method 2: VSI</td>
</tr>
<tr>
<td>Liu et al. (2008)</td>
<td>Mouse</td>
<td>Cryotube</td>
<td>DAP213</td>
</tr>
<tr>
<td>Huang et al. (2008)</td>
<td>Human</td>
<td>SSV</td>
<td>20% DMSO, 20% EG, 25 mg/ml HSA</td>
</tr>
<tr>
<td>Lin et al. (2008)</td>
<td>Mouse</td>
<td>SSV (metal cube surface)</td>
<td>6 M EG, 0.4 M trehalose, 10% FBS</td>
</tr>
<tr>
<td>Aerts et al. (2008)</td>
<td>Mouse</td>
<td>SSV (Cryologic method)</td>
<td>2.8 M DMSO, 3.6 M EG, 1 M sucrose</td>
</tr>
<tr>
<td>Kagawa et al. (2009)</td>
<td>Cow</td>
<td>Cryotissue</td>
<td>15% EG, 15% DMSO</td>
</tr>
<tr>
<td>Bagis et al. (2008)</td>
<td>Mouse</td>
<td>Cryotube</td>
<td>20% DMSO, 20% EG, 0.5 M sucrose</td>
</tr>
<tr>
<td>Kader et al. (2008)</td>
<td>Pig</td>
<td>Ohio-Cryo</td>
<td>15% EG, 15% DMSO, 0.5 M sucrose</td>
</tr>
<tr>
<td>Zhang et al. (2009)</td>
<td>Mouse</td>
<td>0.25 ml plastic straw</td>
<td>Group B: 20% EG, 20% DMSO, 60 μM Z-VAD-FMK, 0.4 M sucrose, 15% FBS in PBS Group C: 20% EG, 20% DMSO, 0.4 M sucrose, 15% FBS in PBS</td>
</tr>
<tr>
<td>Keros et al. (2009)</td>
<td>Human</td>
<td>0.5 ml cryostraws (hand-cut to be open)</td>
<td>VS1: 0.35 M DMSO, 0.38 M PROH, 0.38 EG VS2: 0.7 M DMSO, 0.75 M PROH, 0.75 M EG VS3: 1.4 M DMSO, 1.5 M PROH, 1.5 M EG, supplemented (10% w/v) with PVP (each solution was dissolved in HBSS with 10 mg/ml HSA)</td>
</tr>
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</table>
cryodamage but only when rapidly cooled in a vitrification solution composed of 40% EG, 0.35 M sucrose, and 10% egg yolk [10].

Santos et al. compared vitrification of caprine ovarian fragments using both conventional vitrification in a straw and solid surface vitrification (SSV), a method in which a metal surface floats on liquid nitrogen, thus being cooled to -196°C and able to vitrify samples that touch the surface [43]. The results obtained by Santos et al. showed that when four different vitrification solutions were tested, preantral follicles had the best survival rate when a cryoprotectant mixture of 20% EG and 0.5 M sucrose was used during conventional vitrification and when 35% EG and 0.5 M sucrose was used during SSV [43]. When the follicles were processed for in vitro culture, only the preantral follicles vitrified via SSV survived, whereas those frozen with conventional vitrification in a standard straw were less viable, which denotes a possible beneficial effect of the rapid cooling rate of SSV [43].

The results of a similar study by Huang et al., which used human ovarian tissue processed into 5 mm × 1 mm × 1 mm slices, indicated that SSV was just as successful as conventional slow freezing at preserving PMF [9]. In fact, SSV preserved 85.26% of PMF whereas slow freezing preserved 84.31%, although this difference was not statistically significant [9]. This study also demonstrated that dormant follicles were still viable after both SSV and conventional freezing, as follicles matured up to the secondary stage, and levels of estradiol and progesterone steadily increased in vitro [9]. Ovarian tissue was equilibrated in a vitrification solution composed of 20% DMSO, 20% EG, and 25 mg/ml HSA in sequential solutions of 25%, 50%, 75%, and finally 100% cryoprotectant concentrations [9].

Using a modified version of SSV called the “metal cube surface method”, Lin et al. vitrified murine whole ovaries, ovarian slices, and fragments [44]. The investigators examined the feasibility of vitrification in 6 μl droplets, instead of the 2 μl standard, to allow a larger volume of the ovarian tissue to be vitrified. After thawing, there was no difference in the survival rate. After IVM, cumulus-oocyte complexes (COC) and antral-like cavities were similar in the two volumes. Performing IVF on the mature collected oocytes resulted in similar 2-cell, 8-cell, and 16-cell stage embryos obtained in both groups [44]. However, COC and antral-like cavity formation were decreased when a 14-day-old mouse ovary was compared to a newborn ovary, denoting possible increased vulnerability to vitrification with age [44].

In the same context, Hani et al. have evaluated the vitrification of mice ovaries at 10 days, 4 weeks, 10 weeks, and 7 months of age [45]. The ovaries were vitrified using DAP213 in cryotubes and were transplanted in 4 or 10-week-old recipients. All grafts resulted in resumption of function with no change in litter size related to the age of the donor [45]. However, recipients of adult ovaries expressed a significant decrease in the number of pups delivered after the 2nd litter, which also may denote a possible negative effect of age on the outcome of the grafted vitrified tissue [45].

In another report on SSV, Aerts et al. studied the vitrification of murine ovarian tissue and its subsequent autotransplantation as compared to standard slow freezing [46]. Vitrified fragments were < 0.5mm³. The authors concluded that SSV using the Cryologic vitrification method was similar to standard slow freezing in regards to post-thaw survival [46]. However, following autotransplantation, proliferation of vitrified ovarian tissue was less than that of tissue subjected to standard slow freezing, denoting a possible deterioration of developmental potential following vitrification [46].

Kagawa et al. used a new open vitrification carrier called the Cryotissue to cryopreserve ovarian tissue from 4 cows [47]. Ovarian cortical slices were chopped into minute sections of 10 mm × 10 mm × 1 mm and equilibrated in an equilibration solution containing 7.5% EG and 7.5% DMSO with 20% SSS [47]. They were then transferred into a final vitrification solution of 15% EG and 15% DMSO, in accordance with the protocol they previously described in 2007 [40, 47]. The thin tissue slices were then placed on the Cryotissue, which is a thin metal strip with holes, and plunged into sterilized liquid nitrogen. The tissue survival rate was 88%, and all 4 cows resumed normal ovulatory function, which indicates that the Cryotissue method may be a viable and effective way to vitrify ovarian tissue [47].
Kader et al. also presented *in vitro* results on tissue viability and degeneration following vitrification of porcine ovarian tissue in a specialized closed vitrification device, the Ohio-Cryo [48]. Ovarian tissue was processed into tiny 0.125mm³ before being exposed to a vitrification media protocol with cryoprotectant concentrations similar to those previously described by Kagawa et al. and Hasegawa et al. [34, 40]. The Ohio-Cryo was used for both application of the protocol as well as the actual tissue vitrification [48]. Encouraging results showed no significant difference between the vitrified and fresh porcine ovarian tissue, and suggested some improvement over slow freezing [48, 49].

Additional experiments have focused on comparing various vitrification methods to conventional slow freezing. One study determined that both conventional cryopreservation and vitrification had similar efficacy rates, with efficacy being measured by cell viability and the amount of necrotic tissue in the experimental tissues, when compared to the fresh control [50].

In that study, ovarian tissue fragments were vitrified in either 0.25 ml insemination straws, which were plunged into liquid nitrogen or chilled metal powder, or on copper EM grids, which were also plunged directly into liquid nitrogen [50]. Huang et al. did not find a significant difference between slow freezing and vitrification of human ovarian tissue slices using SSV [9]. Another study compared a modified open method of vitrification with slow cryopreservation [51]. This carrier-less method involved the direct release of strips of ovarian tissue into a shallow container of liquid nitrogen. The samples were plated into cryovials after vitrification [51].

That study also used a two-step vitrification protocol using 2 M DMSO and 0.1 M sucrose, followed by 2 M DMSO, 2 M PROH, and 0.2 M sucrose [51]. Li et al. found that human ovarian tissue not only successfully survived this carrier-less vitrification method with normal follicle morphology, but also resumed normal endocrine function *in vitro*. While these results showed that the ovarian tissue could indeed survive the carrier-less method, there were no significant differences in either follicle formation or hormone secretion between conventional slow freezing and the modified vitrification method [51].

In 2008, Wang et al. compared slow cryopreservation of murine and human ovarian cortical fragments with needle-immersion vitrification (NIV) [52]. Vitrification was done by either directly dropping the sample into liquid nitrogen, following the procedure outlined by Li et al., with modifications, or by NIV [51, 52]. The NIV procedure employs acupuncture needles with 0.18 mm diameters and either 13 mm or 20 mm lengths, depending on the size of the specimen. After equilibration with the vitrification solutions, the needles are plunged directly into liquid nitrogen [52]. All cryopreservation methods resulted in morphologically comparable PMF, although there were significantly less PMF in cryopreserved tissue than in the fresh tissue control [52].

Vitrified ovarian fragments resulted in more abundant growing follicles compared to the other cryopreservation methods, suggesting that vitrification may result in more competent follicles post-thaw [52].

Following 5 days of *in vitro* culture, Choi et al. reported a decrease in the viability and an increase in apoptosis of both vitrified and slowly frozen murine ovarian tissue [53]. It is interesting to note, however, that there was less apoptosis in the vitrified ovarian tissue than in the slowly frozen tissue, although the difference was not statistically significant [53].

Only a few studies reported better results with slow freezing of ovarian tissue when compared to vitrification. Gandolfi et al. cut human, bovine, and porcine ovarian cortical tissue into 1-mm³ fragments and set out to compare the response of these tissues to slow and rapid freezing [54]. The rapid freezing protocols were divided into two methods. Method 1 involved equilibration in a solution of TCM 199, 20% FBS, and 0.64 M EG followed by transfer into a vitrification solution composed of 5.64 M EG, 5% PVP, 0.4 M trehalose, and 20% FBS in TCM 199 [54]. The tissue samples were then vitrified in 0.5 ml cryostraws with a small amount of vitrification solution. In method 2, tissue fragments were also vitrified in a 0.5 ml cryostraw, and the vitrification media in this procedure contained 3.58 M EG, 2.82 M DMSO, and 20% FBS in PBS [54]. The results showed that vitrification of the ovarian cortical fragments caused extensive damage to the PMF in both of the vitrification protocols they described, as indicated by poor follicle morphology [54].

The results also showed that the human ovarian tissues had better post-warming results with the second method, whereas the pig and bovine ovarian tissue were more efficiently preserved with the first vitrification procedure. When the two vitrification methods were compared with slow freezing, the data indicated that slow freezing was associated with the best results in all 3 of the tissue types [54]. Isachenko et al. determined the viability of cultured human ovarian tissue after both conventional and rapid freezing and obtained similar results [55].

Tissue fragments of almost 1 mm³ were vitrified with one of two methods: Procedure 1 used a vitrification solution containing 20% DMSO, 20% EG, and 10% SSS, and the tissue fragments were subsequently placed in a rotation shaker with 10 ml of holding media [55]. Forty ml of 50% EG and 10% SSS was added in a drop wise fashion to obtain a final EG concentration of 40%. Procedure 2 used VSI vitrification solution. The loading device was a 1.8 ml cryovial [55]. By observing follicle development and the *in vitro* production of hormones, the authors concluded that ovarian tissue was best preserved with slow freezing, which resulted in better preservation of all follicle stages [55].

More recent studies have demonstrated successful vitrification of both human and bovine ovarian tissue following various modifications of earlier vitrification protocols [56-58]. Investigating varying cooling rates, Isachenko et al. concluded that slow cooling tissue to -36°C, followed by directly plunging cryotubes into liquid nitrogen, which yielded a cooling rate of -220°C/min, preserves normal follicular structure and function to 98% in post-thawed *in vitro* cultured tissue, compared to fresh ovarian tissue [56]. Normal follicular morphology and function was demonstrated by hormonal activity (17-β estradiol and progesterone) and follicle viability assessment (by H&E histological examination) [56]. In subsequent studies, this same group determined that the optimal combination of cryopreservation media for ovarian tissue vitrification was 2.62 M DMSO, 2.6 M acetamide, 1.31 M PROH, and 0.0075 M PEG. The aforementioned molar concentrations of cryoprotectants,
combined with direct dropping of ovarian fragments into liquid nitrogen with a small amount of vitrification solution, yielded results that were very compatible with fresh ovarian tissue, as this vitrification protocol yielded the highest hormonal activity, the highest density of PMF, optimal follicular integrity, the highest percentage of normally developed follicles, and the best oocyte viability of all 6 experimental manipulations [56]. The authors concluded that by using the above-mentioned protocol, vitrification could yield results comparable to those of fresh tissue [56].

Isachenko et al. successfully obtained high oocyte viability and survival by vitrifying both human and bovine ovarian tissue using the Cryotissue metal grid and vitrification solutions containing 20% EG, 20% DMSO, and 0.5 mol/L sucrose for 15 minutes [57]. Grafted vitrified ovarian tissue resumed ovarian functions after transplant with no evidence of histological differences between fresh and vitrified tissue grafts [57]. The authors attributed success of vitrification to the thin tissue slicing, prolonged exposure to cryoprotectants, and rapid cooling by direct contact with liquid nitrogen [57]. They also claimed that better revascularization is another result of graft thinning [57].

Kagawa et al. compared the in-vitro follicular development and follicular function of fresh, vitrified, and slowly frozen ovarian tissue fragments [59]. The vitrification protocol in this study involved a vitrification solution of 2.62 M DMSO, 2.60 M acetamide, 1.31 M PROH, and 0.0075 M PEG, with ovarian tissue immersed in gradually increasing concentrations of solution [59]. The pieces of ovarian tissue, along with approximately 20 μl of 100% vitrification solution, were dropped directly into liquid nitrogen [59]. This vitrification protocol was chosen based on the results of a previous 2008 study by Isachenko, et al., which indicated that this protocol was the most successful vitrification protocol [56]. Ovarian tissue fragments that were conventionally frozen were put into 1.8 ml cryovials filled with a cryopreservation solution containing L-glutamine, 1.5 M DMSO, 0.1 M sucrose, and 10% SSS [59]. After immersion in an ice water bath, the cryovials were gradually cooled at rates of -0.1°C/min, then -0.3°C/min, and finally -10°C/min, after which, they were plunged into liquid nitrogen. It was determined, via histological examination of cultured tissue fragments, that 95% of follicles in the control group were normal, compared with 83% of follicles in the conventional freezing group, and only 80% of follicles from vitrified tissue [59]. The control group had the highest concentrations of both estradiol and progesterone (365 pg/ml and 3.82 ng/ml, respectively), while vitrified tissue had estradiol concentrations of 285 pg/ml and progesterone concentrations of 1.99 ng/ml [59]. Finally, the conventional freezing group had 300 pg/ml of estradiol and 1.95 ng/ml of progesterone [59]. Lastly, Isachenko, et al. determined that levels of glyceraldehyde 3-phosphate dehydrogenase gene expression, a housekeeping gene essential for many metabolic processes, were more reduced in vitrified tissue than in conventionally frozen tissue [59]. The cumulative results of this experiment indicate, as Isachenko, et al. concluded, that conventional slow freezing is more effective than vitrification at preserving the “developmental potential” of ovarian tissue [59].

To improve endogenous ovarian tissue cryotolerance to vitrification, Bagis et al. investigated the effect of antifreeze protein (AFP) type III using transgenic mice [60]. The AFP type III is a protein commonly found in marine organisms living in extremely cold conditions, and confers a natural tolerance to freezing [60]. Whole mouse ovaries were vitrified in a 1-ml cryotube using an equilibration solution of 10% EG and 10% DMSO and a vitrification solution of 20% DMSO and 20% EG [60]. Indeed, when expressed in the ovarian tissues of transgenic mice, AFP protected the tissue from cryodamage. When warmed and transplanted, the ovaries remained viable and pregnancy occurred [60].

Similarly, a recent study incorporated Z-VAD-FMK, a global caspase inhibitor, into the vitrification solution, in hopes of determining if cryopreservation could be more successful by inhibiting the apoptotic pathway induced by cryopreservation [24]. Mice were placed into either the fresh tissue control group (Group A) or into one of two experimental groups: Group B, caspase inhibitor included, and Group C, caspase inhibitor not included [24]. The vitrification solutions for Groups B and C were exactly the same, containing 20% EG, 20% DMSO, and 0.5 M sucrose in PBS with 15% FBS, except that Group B also had 60 μM of Z-VAD-FMK added, while Group C did not [24]. After 10 minutes in the vitrification solution, the ovarian strips were placed in 0.25 ml plastic straws with a minimum amount of vitrification solution and plunged into liquid nitrogen [24]. Zhang et al. reported that inclusion of the caspase inhibitor did indeed decrease apoptosis of the ovarian tissue and increase the functionality of the ovarian tissue grafts [24].

In another recent study using human ovarian tissue, Keros et al. compared vitrification and controlled-rate freezing and used light and transmission electron microscopy to evaluate oocyte and stromal morphology [61]. Strips of ovarian cortex to be vitrified were moved sequentially through 3 different solutions with increasing cryoprotectant concentrations (see Table 1 for components of VS1, VS2, and VS3 solutions) [61]. The investigators tested two incubation times by exposing the tissue specimens to each solution for 5 or 10 minutes. Finally, the ovarian strips were put into a 0.5 ml cryostaw, which had been hand-cut into an open device, plunged into liquid nitrogen, and stored in a cryotube [61]. The results of this experiment showed comparable follicle morphology between both protocols, but that the ovarian stroma was significantly better preserved with vitrification [61].

Yet another comparison study was performed in 2009, but this compared DCV with conventional vitrification (CV) using human ovarian tissue [20]. Ovarian tissue was placed into the control group, the CV group, or one of 3 DCV groups. Each DCV group had varying concentrations of cryoprotectants in both the equilibration and vitrification solutions (see Table 1 for compositions of each vitrification solution), and the CV group was exposed to the same solutions as the DCV3 group, except that the CV group also had 0.5 M sucrose added [20]. In the CV group, the ovarian cortical strips were released directly into a shallow well of liquid nitrogen, while ovarian pieces in the DCV group were put into a 1.8 ml cryovial, into which liquid nitrogen was poured [20]. Zhou et al. noted that the number of apoptotic
cells was greatly increased in the vitrified tissue compared to the fresh tissue, but of the experimental groups, the DCV2 group had the least damage and most morphologically normal follicles, as well as the highest follicular density after grafting into nude mice [20]. Because the lowest number of ultrastructurally abnormal oocytes was found in the DCV2 group, the authors determined that this protocol was optimal [20].

The ultrastructural deformities observed by Zhou et al. were similar to those observed by Kim et al. in a study that compared vitrified and slowly cryopreserved whole mouse ovaries [20, 62]. Kim et al. vitrified mouse ovaries using a solution of 15% EG, 15% DMSO, 0.5 M sucrose, and 20% FBS and an EM grid as the loading device [62]. The authors found that there were various deformities induced by both cryopreservation protocols and determined that neither method was superior [62]. Some of the many ultrastructural changes observed in both this experiment and in that of Zhou et al. are as follows: swollen, deformed, and vacuolized mitochondria; damaged zona pellucidas; damaged granulosa and thecal cell basement membranes; and many intracellular vacuoles [20, 62].

Moniruzzaman et al. demonstrated that PMF could retain their developmental potential after being vitrified, thawed, and transplanted into SCID mice, even though their developmental rate was slower than that observed in fresh tissue [63]. After incubation in a vitrification solution of TCM-199 with 15% EG, 15% DMSO, 20% FBS, and 0, 0.25, or 0.5 M sucrose, porcine ovarian cortical strips were placed on a Cryotop sheet and plunged into liquid nitrogen [63]. After storage and thawing, the cortical strips were either xenografted under the kidney capsules of male SCID mice or histologically examined [63]. Histological examination of vitrified-warmed cortical strips showed that tissue vitrified without sucrose had fewer PMF, but that the amount of abnormal oocytes increased with increasing concentrations of sucrose [63]. PMF in xenografted tissue developed to the secondary stage, while PMF in fresh tissue developed to the antral phase [63].

Finally, Silber et al. evaluated the long-term function of human ovarian cortical transplants [64]. Ovarian cortical transplants were either slowly frozen or vitrified using the Cryotissue method and a vitrification solution of 20% EG, 20% DMSO, and 0.5 M sucrose [64]. The authors found that there was no significant difference in oocyte survival between fresh (91.9% surviving oocytes) and vitrified (89.1% surviving oocytes) tissue, while the slowly frozen ovarian tissue only had 42% surviving oocytes [64]. The stromal cells, which exhibited cryodamage in the slowly frozen tissue, were essentially intact after vitrification, and this is important because these supporting cells may be essential to normal germ cell development and survival [65, 66].

PRE-REQUISITES FOR TISSUE VITRIFICATION

In order for vitrification to occur successfully, a number of prerequisites must be fulfilled, some of which are enumerated in Table 2.

OVARIAN VITRIFICATION PROTOCOLS

Since vitrification occurs by rapidly lowering temperature and increasing viscosity, the specimen should contain a high concentration of cryoprotectants, as well as be maximally dehydrated [35]. The degree of dehydration and exposure to highly concentrated cryoprotectants depends on the protocol used to prepare the specimen for vitrification. Therefore, it would be ideal for each specimen to have its own protocol through which it would be possible to achieve a certain level of dehydration and high viscosity, while at the same time, retaining its viability and vitality. The harms of unduly exposing a specimen to increasing dehydration and highly concentrated toxic cryoprotectants while it is still in a vegetative form would far outweigh the supposed benefit of achieving proper vitrification and devitrification. For a successful vitrification protocol to be designed, it is empirical to know the maximum tolerance of the specimen to dehydration, as well as the maximum intracellular concentration of permeable cryoprotectants that the specimen can stand without jeopardizing viability. The delivery of this protocol would then require the determination of the CCR of this highly viscous specimen so that the cooling rate required to achieve vitrification could be reached or exceeded [11]. This rate would also be dependant on the volume of the specimen to be vitrified. The probability of vitrification would therefore follow the equation [35]:

\[
\text{Probability of vitrification} = \frac{\text{Cooling and warming rates} \times \text{Viscosity}}{\text{Volume}}
\]

The cooling speed achieved during most vitrification protocols is usually above -300°C/min, with some experiments even obtaining estimated freezing rates ranging from -1500°C/min to -30,000°C/min, when using a small volume of highly concentrated solution of cryoprotectants [51, 67].

<table>
<thead>
<tr>
<th>Table 2. Pre-requisites for Tissue Vitrification</th>
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<tr>
<td>1. Delivery of high intracellular concentration of cryoprotectants</td>
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<td>2. Achieving adequate level of dehydration.</td>
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<tr>
<td>3. Controlling the contact time with vitrification solutions, according to tissue type and applied concentration of cryoprotectants, to avoid toxicity</td>
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<tr>
<td>4. Even delivery of the required protocol to the entire specimen.</td>
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<tr>
<td>5. Achieving rapid cooling and warming rates/high heat transfer (via small sample, small volume of surrounding cryoprotectant, direct contact with liquid nitrogen, or indirect contact in specialized carriers or devices.)</td>
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<tr>
<td>6. Even chilling/warming of the specimen.</td>
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<tr>
<td>7. A sample volume that can vitrify at a given viscosity and cooling rate</td>
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In another study, the mean ovarian tissue cooling rate was estimated using differential scanning calorimetry to be -342.9 ± 49.9°C/min, which was above the estimated CCR of the vitrification solution used in the study [19]. It is also necessary not only to have a rapid cooling rate, but also a rapid warming rate, in order to prevent recrystallization as the specimen is thawed [56]. Different methods and loading devices would have different cooling rates, and the estimated cooling rate of a certain method should always reach or surpass the actual CCR of a certain tissue after it has been prepared for vitrification.

The most common cryoprotectants used in ovarian vitrification are dimethyl sulfoxide (DMSO) and ethylene glycol (EG), although some vitrification solutions contain other cryoprotectants, such as polyethylene glycol (PEG), propylene glycol PROH, and acetamide [2]. Disaccharides, such as sucrose and trehalose are commonly included in vitrification solutions as they help dehydrate the sample by increasing the extracellular osmolarity. The debate on the use of disaccharides in the vitrification solution depends on the tolerance of the tissue to the dehydration level caused by the disaccharide, and if the actual added benefit of its high viscosity can be reached by increasing the concentrations of permeating cryoprotectants. The concentrations of cryoprotectants used in vitrification media are much higher than those used in solutions for slow freezing, and as a result, there is a significantly increased risk of toxicity [11]. In addition, these high cryoprotectant concentrations increase the risk of osmotic shock to the cells, as they cannot cope with rapid changes in their tonicity [11]. To minimize toxic effects from prolonged exposure to high concentration of cryoprotectants, many protocols expose the ovarian tissue to the cryoprotectants at 4°C [9, 42]. The protocol that has been most commonly applied so far for ovarian tissue vitrification is a two-step protocol consisting of:

1- Equilibration step: 7.5% DMSO + 7.5% EG + 20% Serum Substitute Supplement (SSS) or similar product (V/V)

2- Vitrification step: 15% DMSO + 15% EG + 20% SSS + 0.5 M sucrose.

However, duration of exposure as well as the size of ovarian tissue pieces vitrified and devices used did vary [1, 34, 40, 47, 49]. Another common vitrification protocol for ovarian tissue is the DAP213 protocol that is most commonly performed as a two-step procedure following an initial equilibration in 1 M DMSO [38, 39, 41, 45].

One important key element for the successful vitrification of ovarian tissue is the even permeation of cryoprotectants throughout the sample. For small samples such as tissue slices or fragments, complete permeation with cryoprotectants is achieved by impregnation and equilibration, and because adequate permeation occurs quickly, toxicity to the sample is reduced. The larger the sample, however, the more difficult it is to reach an even permeation. Whole ovaries require perfusion via the ovarian artery or vascular pedicle for an even delivery of cryoprotectants, but this is not without the challenges of toxicity and vessel damage. As a result, there is a greater probability of achieving successful vitrification with smaller fragments [31].

LOADING DEVICES AND METHODS FOR VITRIFICATION OF OVARIAN TISSUE

The maximal cooling rate can be achieved by immediately and constantly exposing the specimen to the liquid nitrogen temperature of -196°C or even less (such as with liquid nitrogen slush). Vitrification methods can be broadly categorized into two distinct types according to specimen exposure to liquid nitrogen: open devices (direct contact with liquid nitrogen) and closed devices (no direct contact with liquid nitrogen).

In open devices that allow direct contact with liquid nitrogen, the boiling liquid nitrogen can form bubbles around the specimen and impede the cooling rate. However, this can be partially overcome by preventing gas bubbles from accumulating by continually stirring the specimen, if possible. Solid surface vitrification (SSV) seems to offer the benefit of continuous and constant exposure to a -196°C environment while avoiding the bubbling phenomenon.

Researchers have used different direct methods to vitrify ovarian tissue, such as copper grids, direct cover vitrification (DCV), the carrier-less method, the Cryotop, and the Cryotissue. In the case of the copper grid, the sample is placed on top of a copper grid, which has holes of 42 mm², and is plunged into liquid nitrogen, at which point the liquid nitrogen passes through the holes and the sample is vitrified [10]. In DCV, liquid nitrogen is poured directly on top of the sample, which is contained in a cryovial [1]. The carrier-less method is a method of vitrification in which a tissue sample is dropped into a shallow container of liquid nitrogen where it is directly exposed without the need for a loading device [51]. The Cryotop is a direct method of vitrification in which the sample to be vitrified is placed on a thin, laminated film strip that is plunged into liquid nitrogen, and then encased and sealed in a special straw for storage in liquid nitrogen [40]. This device has been successfully used for vitrification of oocytes and embryos [68, 69]. Despite its success in vitrifying fragmented mice ovaries, it seems impractical to use it for vitrification of larger samples, such as whole human ovaries, even if they are fragmented [40]. In addition, the newest open method, the Cryotissue, utilizes a metal strip full of holes through which the liquid nitrogen travels. Also, the tissue slices used with this device are very thin, thus allowing full permeation of cryoprotectants and complete vitrification without ice formation [47]. Four groups of investigators separately assessed the effectiveness of different direct contact methods of vitrification, with studies using whole mouse oocytes (DCV method), mouse ovarian tissue fragments (Cryotop method), human tissue (grids and carrier-less methods), and cow ovarian tissue (Cryotissue). All studies reported one or more of the following: histologically normal follicles, restoration of endocrine function and follicle growth, and sufficient protection from cryodamage [1, 10, 40, 51]. Two other studies involving autotransplantation of vitrified-warmed mouse oocytes resulted in pregnancy and live birth, as well as follicle growth and live birth after IVF-ET [1, 40]. Resolution of cyclicity was also achieved in cows after transplantation of vitrified ovarian cortical tissue [47].

However, while these studies have encouraging results, they either did not evaluate or did not report data regarding contamination of ovarian tissue as a result of direct contact.
with liquid nitrogen. As viruses tend to be relatively cryoin-
sensitive, they can retain their virulence or possibly increase
their virulence after contact with liquid nitrogen [70, 71]. In
addition, UV light is usually used to sterilize the liquid ni-
trogen, but it is not 100% effective [70, 71]. Therefore, it is
imperative that a carrier is created that will both protect the
specimen from direct contact with liquid nitrogen, while at
the same time maximizing the cooling/warming rates [36].

Other researchers have attempted to vitrify ovarian tissue
by indirect contact with liquid nitrogen. The indirect or
closed vitrification devices that were used for ovarian tissue
vitrification are: the cryotubes, vials, straws, and bags,
SSV (now known as the Cryologic method), and the Ohio-
Cryo. The cryotubes, vials, straws, and bags are all regular
containers that were traditionally used for specimen slow
freezing. Though these devices have been adapted to tolerate
the liquid nitrogen temperature and maintain the specimen
they are containing at this temperature, none of them were
specially developed to deliver the high cooling and warming
rates that are required to surpass the CCR of the contained
specimen or to prevent devitrification at low temperatures,
respectively. Some are capable of holding large samples,
such as whole ewe ovaries, but they cannot provide any
specific advantages to improve the vitrification of a large
specimen, such as the whole ovary, the inherent nature of
which impairs optimal heat conduction in the tissue. The
metal cube method, also known as the Cryologic vitrification
method, is an evolution of SSV [46]. In this method, a metal
cube is partially immersed in liquid nitrogen, with just its top
surface remaining above the liquid nitrogen [46]. The metal
will acquire and remain at the liquid nitrogen temperature.
The specimen to be vitrified is then allowed to touch
the surface of the metal cube, where it vitrifies, and it is
then carried in a container for final storage. This method
results in a high cooling rate of around -20,000°C/min to
-40,000°C/min [44].

Our group has recently developed the Ohio-Cryo method
in an attempt to address some problem areas in ovarian tissue
evitrification. The device holds fragmented tissue without
allowing direct contact with liquid nitrogen, and almost all of
the processed ovarian cortical tissue of a large mammal can
be contained in 2 devices. Also, it allows rapid replacement
to vitrification media, which permits proper control of con-
tact time with the highly concentrated cryoprotectants and
avoids the need for a centrifuge [48, 49]. Then, a sample
surrounded by a minimal volume of vitrification media is
plunged into liquid nitrogen for vitrification and storage, all
inside the Ohio-Cryo. The tissues are pressed flat against the
bottom of the container in order to ensure maximum cooling
or warming rates when going into liquid nitrogen or into a
water bath [48, 49].

**WHOLE OVARY VITRIFICATION**

Whole ovary cryopreservation and transplantation with
microvascular anastomosis of the ovarian pedicle has been
advised as an approach to not only increase graft longevity,
and thus long-term restoration of fertility, but also to
minimize ischemic damage to the grafted tissue [6, 21, 64].
Ischemic damage is responsible for the massive follicle
loss observed in non-vascular ovarian cortical grafts, and in
principle, whole ovarian cryopreservation should avoid
ischemia, since it allows immediate revascularization [6, 18-
21, 72-74].

In fact, Zhou et al. observed that the follicular density of
vitrified-warmed human ovarian cortical pieces was similar
to that of fresh tissue, but that the follicular density dramati-
cally decreased after transplantation, indicating that the
ischemia from transplantation was more detrimental to fol-
licular viability than the process of vitrification [20]. Regard-
less, Kim mentions that whole ovary transplantation does not
automatically negate the possibility of ischemic damage and
subsequent follicular loss in a graft, for there is still the pos-
sibility of thromboses forming in the ovarian vascular system
after transplantation [73].

Different studies have showed restoration of ovarian
function following transplantation of slowly cryopreserved
vascular whole ovaries. Most of the studies have shown sig-
nificant loss of PMF or compromised graft longevity, al-
though few have shown persistent ovarian functions 2 years
after grafting [16, 64, 75].

Though slow freezing of ovarian tissue in a whole ovary
has been shown not to induce significant cell damage,
changes in ovarian tissue viability post-transplantation sug-
gest damage to the vasculature of the whole ovary. This has
been indicated by experimental data from Bedaiwy et al.,
Revel et al., Imam et al., and Arav et al., who each de-
scribed one or more of the following complications: multiple
adhesions, venous and capillary thromboses, arterial tearing,
capillary fibrosis, and atrophy of transplanted ovaries [16,
17, 21, 64, 75]. Recently, Arav et al. reported the continued
function of frozen-thawed whole ovaries 6 years after trans-
plantation into ewes [21]. The whole ovaries were slowly
frozen using directional freezing and were transplanted into
ewes by microvascular anastomosis [21]. This protocol has
resulted in long-term restoration of ovarian functions, as
indicated by normal histology, normal folliculogenesis (with
follicles in all developmental stages), intact and functional
vasculature, and in one of the three sheep, cyclic endocrine
function as measured by progesterone concentrations [21].
However, one ovary was atrophied, and the sheep were
stimulated with exogenous FSH, since the presence of adhe-
sions prevented normal conception [21]. Though this method
has not been fully investigated, these data could be attributed
to better preservation of the ovarian vasculature using this
unidirectional freezing technique.

Because vitrification, by definition, avoids ice formation,
it may be a potential answer to the problem of intravascular
ice development, which is at least partly responsible for the
aforementioned vascular injuries that occur after transplanta-
tion [6, 76]. Although microscopic tissue analysis of whole
ovaries, as employed in studies by Martinez-Madrid et al.
and Bedaiwy et al., indicate that frozen-thawed whole hu-
omans ovaries exhibit normal follicular viability, normal his-
tology and morphology, and normal intact vasculature, and
thus no significant injury from the process of slow freezing,
the presence of the aforementioned vascular abnormalities
and pathologies after transplantation and anastomosis in
various other experiments suggest the presence of submicro-
scopic injuries due to intravascular ice formation [6, 16, 17,
21, 64, 75, 77]. These submicroscopic changes would be any
change too small or subtle to be observed using traditional microscopy or staining, but which affect the normal functioning of the cells and/or tissues.

Sachs et al. suggested the possibility of submicroscopic changes in the vasculature after slow cryopreservation, as demonstrated by frozen veins that were morphologically similar to fresh veins, but displayed a high rate of thrombus formation and endothelial tissue loss after transplantation [78]. Further support comes from a study on the character and distribution of ice in slowly frozen ovarian tissue, in which the authors stated that microscopy and staining, key methods for determining cryodamage in previous studies, are inadequate for determining the “extent, character, or location of ice formed in cryopreserved tissue” [79]. Finally, data from cryosurgical testing in rats suggests that intravascular ice development, especially direct damage to the endothelium, can cause vessel wall tears, thrombus formation, and fluid leakage from vessels post-thaw, thus leading to necrosis [17, 80, 81]. Therefore, the formation of intravascular ice remains a rich and important area for investigation, especially for the cryopreservation of whole ovaries.

The vitrification of whole ovaries other than those of rodents has been far less investigated than whole ovary cryopreservation by slow freezing. Technically, vitrification of a whole human or large mammal ovary is difficult to achieve. The mammalian ovary has a dense stroma, low surface area to volume ratio, highly diverse cell types, and a delicate vascular system [4, 8, 11, 24]. These characteristics would inevitably retard both the permeation of cryoprotectants and the rates of cooling and warming, therefore minimizing the chances of proper vitrification and increasing the chances of ice formation and/or fractures, especially in the medulla [8, 25].

Vitrification of ovarian tissue not only depends on the size and shape of the sample to be vitrified, but also on the intrinsic histologic and thermodynamic tissue characteristics. These characteristics will vary with species, age, and tissue processing. Many studies have reported high success rates vitrifying mice ovaries, which are smaller and less fibrous than ovaries from larger mammals, and therefore would have higher heat transfer rates. Also, cryoprotectants can permeate whole mice ovaries more efficiently because they are more porous than ovaries from large mammals such as cows, pigs, sheep, and humans [5].

Using differential scanning calorimetry while attempting to vitrify a whole sheep ovary in a cryobag, Baudot et al. verified that the cooling rate was higher in the cortical surface than in the medulla, as a consequence of high tissue density and low thermal diffusion, thus leading to ice formation in areas where the CCR for vitrification was not achieved [8]. Importantly, slow cooling and warming may result in vessel fractures, further minimizing the chances for successful recovery of the vascular graft’s viability. Histological evaluation of small PMF after tissue rewarming indicated 61% follicular membrane integrity and only 48% normal follicles, which is in stark contrast to 86% normal follicle development observed in vitrified ovarian pieces in another study [8, 56]. However, the authors stated that 48% normal PMF indicated that the protocol was not toxic for the tissue [8]. Baudot et al. concluded that insufficient and non-homogenous permeation of cryoprotectants also plays an empirical role in inadequate whole ovarian vitrification [8].

In a study of transplantation of vitrified whole ewe ovaries with vascular anastomosis of the ovarian pedicle, the investigators reported vessel fractures and almost no graft survival after transplantation [4]. The vitrification solution was infused via the ovarian artery, and ovaries cryopreserved with their vascular pedicle were compared with fresh ovaries that were orthotopically autotransplanted without cryopreservation [4]. The vitrified ovaries were warmed in two steps, as previous data showed increased pedicle fractures after single step thawing, which the authors attributed to the stress from rapid thawing [4, 18, 19]. The 5 sheep in the control group had variable responses to transplantation, including resumed endocrine function in three sheep, two spontaneous pregnancies, no ovarian function in one sheep, and the death of one ewe [4]. Of the 5 sheep that underwent transplantation of a vitrified-warmed ovary, only one actually resumed endocrine function, and of the four ewes that did not, two had thrombosis in the ovarian pedicle, whereas another presented with arterial thrombosis and normal venous return denoting possible collateral development [4]. This experiment demonstrated that grafting vitrified whole ovaries with microvascular anastomosis of the ovarian pedicle did not reduce ischemic injury, as indicated by the massive follicular loss in all the study animals [4]. In addition, the vascular thrombosis detected in 3 out of 4 vascular pedicles suggested the possible impracticality of the technique using the present technology [4]. Interestingly, both Courbiere et al. and Baudot et al. used VS4 vitrification solution, and Courbiere et al. suggested that this media may be inadequate for whole ovary vitrification [4, 8].

Any attempts for whole ovary vitrification of a large mammal ovary would need to address the following challenges:

1. The low surface area to volume ratio of a whole ovary, which will impede the cooling and heating rates [11].
2. The cooling or heating would be centripetal, with the cooling rate decreasing as the specimen gets larger. At a certain size limit, the central areas of the ovary would fail to cool faster than the CCR required to achieve vitrification, and would thus be at a greater risk of severe damage [4, 8, 19].
3. The difficulty in optimizing a vitrification protocol for a complex tissue such as the ovary [11, 51]. Even when cryoprotectant levels and dehydration requirements are determined for the ovarian tissue at a certain thickness, it would be difficult to optimize a method of even cryoprotectant delivery to the entire tissue within a given time. Incomplete cryoprotectant penetration throughout the entire ovary can also increase the required CCR in certain areas of the ovary above the achieved rate, thus resulting in ice formation [11].

a. Exposure to the vitrification media can be done by permeation, but this will be affected by the tissue thickness, resulting in either increased toxicity in surface areas or decreased protection in other areas, like the core.
b. The other modality is to perfuse the whole ovary through the vascular pedicle if possible. So far, it has not been determined if this method may have an impact on the micro- or macrovasculature. However, it has been a common observation that either thromboses form in the main vessels, or there is still a massive loss of PMF in surviving grafts, which implies damage or impaired function at the microvascular level [4, 12, 17-19]. Moreover, improper vessel vitrification or warming may lead to vessel fractures or lacerations [4, 18, 19].

The delivery of highly viscous solutions like those required for vitrification, resulting in high concentrations of toxic cryoprotectants and high levels of dehydration, as well as the relatively rapid dehydration and rehydration are all additional challenges when designing a whole ovarian tissue protocol [8, 19].

The most successful ovarian tissue vitrification experiences have been with mice ovaries, or ovarian tissue processed into thin slices or fragments of different sizes [1, 12, 34, 40, 47, 49, 50, 54]. The ovarian tissue size and shape should therefore be taken into consideration when designing a vitrification protocol for ovarian tissue. Any change in these variables would necessitate re-evaluation of the vitrification procedure and may render the results less reproducible.

Finally, because whole ovarian cryopreservation is especially intended for those patients that will undergo chemotherapy, whole ovary preservation and transplantation should only be considered when it is highly unlikely that cancers or other pathologies could be reintroduced to the patient [4, 72].

CONCLUSION

Vitrification continues to be a promising technique for the preservation of ovarian tissue. There have been significant advances in the past 8 years with many accumulating reports suggesting various techniques for ovarian vitrification. The results of most of these studies are promising and suggest a possible superiority of the technique over traditional cryopreservation. The superiority of vitrification is already anticipated by the current experience and success demonstrated with oocytes. The vitrification of human ovarian tissue fragments has been shown to be successful, although not always superior to conventional slow freezing [51, 55, 82]. Even with reports showing no significant improvements in the outcome of ovarian tissue vitrification compared with slow freezing, vitrification remains more convenient and less expensive to perform.

However, there is still room for improvement, specifically in addressing four main problem areas:

1. We must be able to deliver the best possible vitrification protocols to complex tissues like the ovary, while at the same time avoiding toxicity and preventing uneven vitrification or devitrification, which may result in patchy areas of decreased vitality or tissue and vessel breaks.

2. There is a need to improve closed systems that can help achieve proper vitrification of the ovarian tissue while avoiding liquid nitrogen contamination.

3. Any future ovarian vitrification development should take into account the large volume of the sample needed if whole human ovaries are to be considered for vitrification, whether intact or processed.

4. The tissue processing may be an integral part of the vitrification process to create samples that can be more successfully vitrified with high post-warming survival.

As vitrification protocols continue to improve and as new carrier devices are created to address different specific needs or challenging problems, this technique will continue to evolve and progress. Further improvements in the outcome of transplantation of vitrified ovarian tissue are further anticipated, with improvements regarding other aspects of the procedure, namely the revascularization.

EXPERT COMMENTARY

The purpose of this article was to evaluate and review the current primary literature regarding ovarian tissue vitrification. Many studies have differing results regarding which cryopreservation method, conventional or vitrification, is superior or more successful. Although the slow freezing of ovarian cortical fragments is currently used in clinical settings, vitrification offers a less expensive and ice-free alternative for preserving ovarian tissue and is becoming more mainstream for oocyte and embryo preservation.

FIVE-YEAR VIEW

Vitrification has the potential to decrease costs to women seeking fertility preservation, while at the same time increasing viability of tissue post-thaw, as it avoids cryoinjury secondary to ice formation. However, there are some obstacles currently preventing the clinical use of vitrification for preserving ovarian tissue. These obstacles include the lack of a standardized and universal vitrification procedure, the difficulty of maximizing heat transfer when using closed devices to avoid liquid nitrogen contamination, and finally, the dense and complex structure of the ovary itself. While whole ovary vitrification and subsequent transplantation offer a solution to some of the problems inherent in cortical tissue preservation, such as shortened graft life and decreased follicular density and viability due to ischemic damage, whole ovaries present their own unique obstacles, including uneven permeation of cryoprotectants, uneven heat transfer across the entire specimen, possible vascular damage, and possible transfer of cancers and other pathologies. When tissue vitrification methods are consistently successful and can be implemented in the clinical setting, they will allow women who may experience premature ovarian failure, whether due to gonadotoxic chemotherapy or conditions such as mosaic Turner syndrome, as well as women who wish to preserve their fertility for personal reasons, a more efficient and cost-effective choice.

Currently, studies are underway that aim to minimize oocyte loss from transplanted tissue, to determine the optimal vitrification protocol, and to improve whole ovary vitrification. Fertility preservation via vitrification is a possibility not only for humans, but also to ensure the continued existence of endangered animals. As the technology evolves and research continues, the aforementioned challenges to clinical
applications of ovarian tissue and whole ovary vitrification may be overcome. Until one protocol is demonstrated to be consistently superior, slow cryopreservation and vitrification will most likely coexist in the clinical setting.

REFERENCES


Ovarian Tissue Vitrification: Modalities, Challenges and Potentials


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