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Chapter

# Female Fertility Preservation: Different Interventions and Procedures

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## Abstract

A human being is made up of two living cells: the egg and the sperm, which pass the torch of life to the next generation. After zygote, the fertilized egg undergoes a series of mitotic divisions. First division into two cells is called blastomeres, and then four cells to 64 cells are called the morula stage. Five days after fertilization, the embryo reaches the blastocyst stage. This blastocyst is attaching itself to the uterine wall for implantation. Implantation is complete when the blastocyst is fully embedded in the endometrium a few days later. Cryopreservation of ovarian tissue, oocytes, embryos, and blastocysts has become an integral part of improving the success of infertility treatment and fertility preservation. Various cryopreservation strategies have been proposed to enhance cell survival and preserve cellular function. It also increases the efficiency of assisted reproductive technology (ART) procedures, enables biodiversity conservation, and provides protection to a valuable biological material. However, successful cryopreservation requires the use of cryoprotectants. The chemical and physical effects of these reagents/processes cause extensive cryogenic damage to the plasma membrane, leading to changes in its normal function. In this chapter, we will discuss different interventions to preserve fertility, including cryopreservation methods and cryoprotectants used.

**Keywords:** zygotes, embryos, freezing, slow freezing, vitrification

## 1. Introduction

The unexpected discovery of the cryoprotective properties of glycerol revolutionized the field of cryopreservation and sparked a great deal of further research [1]. However, cryoprotectant toxicity, a fundamental barrier to realizing the full potential of artificial cryoprotection, generally remains a little-known phenomenon.

Successful cryopreservation of sensitive cells, tissues, and organs requires the use of cryoprotectants [2]. Cryoprotectants work by increasing the concentration of

solutes in cells. However, in order to be biologically viable, they must be easily permeable and non-toxic to cells. The toxicity of CPA depends on the inherent properties of the chemical itself. Therefore, the toxicity of cryoprotectants limits the concentration available, thereby limiting the cryoprotective efficiency of these agents [2–5].

Of course, longer CPA exposure time increases toxicity, which may be non-specific toxicity due to the interference of water molecules with cell membranes or specific toxicity due to CPA type and concentration [6, 7].

Gook identified the delicate balance between protection and toxicity associated with the use of glycerol and other cryoprotectants such as propylene glycol and ethylene glycol [8]. However, some cryo-damage is inevitable regardless of the use of cryoprotectants, as each cell type responds differently to cryoprotectants and cryopreservation.

There are two main categories of cryoprotectants: osmotic cryoprotectants and non-permeable cryoprotectants [9, 10].

### **1.1 Permeable cryoprotective agents (CPAs)**

Permeable cryoprotectants include glycerol, methanol, dimethyl sulfoxide (DMSO), dimethylacetaldehyde, ethylene glycol, and propylene glycol. They are low molecular weight chemicals that penetrate the plasma membrane and displace water in cells [11]. However, high concentrations of osmotic cryoprotectants can prevent ice formation during cryopreservation of cells, tissues, and organs at low temperatures. However, CPA becomes more and more toxic as the concentration increases [6].

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#### *1.1.1 Glycerol*

Glycerol is a non-electrolyte compound, so it can reduce the electrolyte concentration in the remaining unfrozen solution in and around the battery at any given temperature. It has been used for many years in cryobiology as a cryoprotectant for blood cells, animal sperm, and bacteria, which can be stored in liquid nitrogen at low temperatures [12].

In 1937, glycerol was used as a freezing medium for semen from bulls, rams, stallions, boars, and rabbits during the freezing stage ( $-21^{\circ}\text{C}$ ). About 10 years later, Polge et al. demonstrated the positive effect of glycerol on frozen poultry sperm [1]. However, glycerol is toxic to spermatozoa through protein denaturation, modification of actin interactions, and induction of plasma membrane fragility during cryopreservation [13, 14]. Good cryoprotective effects were obtained when the glycerol concentration was in the range of 0.5–2 M [15].

Therefore, mixtures of cryoprotectants have been shown to be less toxic and more effective than using a single CPA [6].

#### *1.1.2 Dimethyl sulfoxide (DMSO)*

DMSO and glycerol are probably the two most widely studied CPAs, although the relative success rates of each generally vary by species [16]. Since the early history of

cryopreservation, DMSO has been the cryoprotectant of choice for most animal cell systems.

Lovelock and Bishop were the first to document the ability of DMSO to attenuate freezing-induced cellular damage during slow cooling of bull semen [12]. DMSO also protects against certain aspects of biological damage and radiation damage suffered during cryopreservation. It is estimated that the hydrogen bond between DMSO and water is about 30% stronger than that between two water molecules [17].

DMSO crosses biofilms with great ease, with minimal evidence of damage, and has extensive solvation properties [18]. In 1988, Friedler et al. showed that DMSO was more effective than glycerol [19].

Nonetheless, its impact on cell biology and apparent toxicity to patients has been an ongoing topic of discussion, driving research into less cytotoxic CPAs. Cell membrane toxicity is the most common type of specific toxicity associated with DMSO.

Shu et al. also reviewed the effects of DMSO on a variety of organisms and biological systems [20]. However, DNA methylation and histone alterations have been reported to reduce survival and induce cell differentiation [21, 22].

### *1.1.3 Ethylene glycol (EG) and propylene glycol (PG)*

Ethylene glycol (EG) is approximately half as permeable to human egg cells as propylene glycol (1,2-propanediol) (PG) or dimethyl sulfoxide (DMSO), thus increasing membrane damage through osmotic stress. However, EG is the cryoprotectant of choice because it is less toxic than other drugs [23].

It is important to note that using a relatively high concentration of EG (15%) was prepared in an equimolar mixture with DMSO. This suggests that post-vitrified blastocyst transfer has no negative impact on perinatal outcomes compared to post-vitrification post-transfer [24].

Most embryos are more permeable to PG than to glycerol. PG has few systemic toxic effects and is used safely in many foods. PG has been used as an antidote for EG poisoning [25]. Nevertheless, PG (1,2-propanediol) is often toxic when used as a CPA agent. For example, 1,2-propanediol above 2.5 M has been shown to impair the developmental potential of mouse zygotes by lowering the intracellular pH [26].

## **1.2 Non-permeable cryoprotectants**

Impermeable cryoprotectants are a special class of high molecular weight, impermeable molecules. These include starches such as hydroxyethyl starch and polyethylene oxide, sugars such as trehalose and sucrose, and polyvinylpyrrolidone. They cannot enter cells and therefore remain extracellular during cryopreservation by exerting osmotic effects to support rapid cell dehydration, lower freezing temperature of the medium, and reduce extracellular ice formation [27, 28]. They are used to protect cells from rapid cooling [9–11].

Due to their low toxicity, they are commonly used as extracellular CPAs. Typically, these are not used alone, but together with permeable intracellular standard CPA [29]. However, glucose has specific toxicities such as binding to proteins [30] and causing glycation as a reducing sugar [31].

Many studies report that trehalose is superior to other sugars such as trehalose. In maintaining membrane stability, liposome stability is maintained during drying and preservation of biomaterials [32].

Sucrose is considered a cosmic [33]. Sucrose is used as an extracellular CPA for embryo and oocyte vitrification [34]. However, under acidic conditions, sucrose is more easily hydrolyzed to reducing sugar monosaccharides than the disaccharide trehalose [32].

## **2. Cryopreservation methods**

### **2.1 Controlled slow-rate freezing**

Slow freezing or slow programmable freezing technology was introduced in 1966 [35]. This cryopreservation technique was introduced in the 1980s. As the name suggests, it involves slow freezing of eggs or embryos. Treat the cells first with antifreeze (antifreeze) to protect them in the process. Then gradually cool; at a rate of 1–2°C per minute: from +24°C to –7°C, then sowing, 10 minutes after sowing the temperature drops to –30°C, and finally immersion to –196°C for storage of liquid nitrogen [36]. However, optimal cooling rates vary widely between cell and tissue types [37].

### **2.2 Vitrification**

The word vitrification comes from the Latin vitrum, meaning glass [38]. Vitrification has replaced traditional slow freezing as the primary method for gamete and embryo cryopreservation, while reproductive cryopreservation is slowly shifting research focus to vitrification, a cheaper, faster, and simpler technique [39–41].

Vitrification differs from slow freezing in that it avoids the formation of ice crystals in the intracellular and extracellular spaces [38]. So many laboratories around the world have completely replaced slow freezing with vitrification in order to improve cryogenic survival outcomes [42, 43].

Vitrification is an alternative freezing method based on the solidification of solutions at low temperatures, not by ice crystallization, but by a large increase in viscosity during cooling [44]. It is achieved by briefly exposing the embryos to high concentrations of cryoprotectant (~7–8 M), followed by direct immersion of the embryos in liquid nitrogen, resulting in ultra-rapid cooling at approximately 20,000°C/min. With this technique, a glassy amorphous state can be achieved, and the formation of intracellular and extracellular ice crystals is prevented [45].

This technique can be used to freeze sperm, oocytes, fertilized oocyte (zygotic) embryos, umbilical cord blood, and reproductive tissue in testes or ovaries [46].

## **3. Fertility preservation interventions**

Fertility preservation may be indicated for the following indications: Women diagnosed with cancer, women with a disease, surgery, or treatment that may affect future fertility (including lupus, endometriosis, and Turner syndrome) Fertility declines with age in women, transgender men, and women worried about aging.

### **3.1 Oocyte cryopreservation**

Oocytes are cells about 120 microns in diameter with a thick membrane called the zona pellucida. Egg cells are often referred to as the largest cells in the human body.



Surface and volume play important roles in the outcome of cryobiological processes. Therefore, freezing and thawing of unfertilized oocytes require extensive empirical and theoretical knowledge [45, 47].

Oocyte cryopreservation has become an important method for preserving female fertility in medical and non-medical indications [48, 49].

For women with age-related selective fertility, without a male partner, or without donor sperm, oocyte cryopreservation may offer another experimental option under stringent institutional review board (IRB) protocols, early data show promising results [50].

Unfortunately, oocyte cryopreservation is technically more complex than embryo cryopreservation, and unfertilized oocytes are more susceptible to damage during cryopreservation, so these procedures may have lower rates of unsuccessful pregnancies [51]. Cryopreservation of unfertilized oocytes is more technically challenging than embryo cryopreservation but has less ethical and legal implications.

Cryopreservation of human oocytes can be performed by conventional slow freezing or vitrification [46, 52]. Cryopreservation of immature oocytes in prophase I (follicle stage) has been proposed as an alternative to standard oocyte cryopreservation, as these oocytes are thought to be less susceptible to cryo-damage due to the absence of a spindle and different Membrane permeability [53].

ICSI is recommended for insemination of frozen and thawed oocytes because this method offers a reasonable chance of fertilization compared to in vitro fertilization [54].

Chen in 1986 reported the first pregnancy resulting from the slow freezing and rapid thawing of human eggs using DMSO (dimethyl sulfoxide) as a cryoprotectant [55].

Van Uem reported the second litter after cryopreservation of oocytes using a cryopreservation technique different from that described by Chen [56]. Several pregnancies have been reported after oocytes were cryopreserved—thawed and received intracytoplasmic sperm injection (ICSI) [57].

Kuleshova announced the birth of the first child from vitrified oocytes. The newborns were normal and healthy [58]. Other authors successfully used vitrification and found another 10 pregnancies [59].

The total number of children born after fertilization of frozen and thawed oocytes worldwide exceeds 1500 [51, 60]. Furthermore, no intellectual and/or developmental deficits have been found in children born from frozen oocytes [54, 61, 62].

Slow freezing is one of the methods of cryopreservation of oocytes. Compared with the fresh cycle, it has some limitations, such as low oocyte survival [63–65], increased risk of oocyte senescence [63, 66, 67], and reduced embryonic development [63].

Cao et al. conducted a randomized study to compare survival, fertilization, early embryonic development, and meiotic spindles in slowly frozen and vitrified and thawed human oocytes (n = 605) Assembly and Chromosome Arrangement. The vitrification group had significantly higher oocyte survival rate, fertilized egg and developing embryo division rate, and blastocyst development percentage than the slow freezing group (91.8%, 78.0%, 24.0%, 12.0% vs. 61.0%), 54.4%, 42.3%, and 33.1%, respectively). They also noted that vitrification was superior to slow freezing methods, resulting in improved oocyte survival, fertilization, and embryonic development in vitro [68].

Konc et al. used Polscope to determine the presence, position, and spindle dynamics/displacement in each oocyte. They examined frozen and thawed human oocytes

before and after thawing and for 3 h in culture and found that the spindle did not always return to its original position within the oocyte [69].

After thawing and culturing, they were able to see spindles in 84.3% of the oocytes. However, vitrified oocytes tend to reassemble their spindles more efficiently and faster than slowly cooled oocytes [70]. Cobo et al. found comparable spindle recovery after vitrification and slow freezing for 3 hours [62].

Cobo et al. in an oocyte donation program published the results of a randomized controlled trial of more than 3000 fresh oocytes and 3000 vitrified oocytes (92.5% survival rate). Randomized controlled trials demonstrated no adverse effects of vitrification on subsequent fertilization, development, or implantation [71]. Nagy et al. have also reported similar results in an oocyte donation program and Herrero et al. use the same vitrification protocol [72, 73].

### **3.2 Pronuclear stage (2PN) cryopreservation**

Until recently, our laboratory and others in Germany have focused on cryopreservation of embryos at the prokaryotic stage (PN). PN freezing was performed because of the reported clinical success rates and if embryo selection and thawing techniques improve over time, it ensures that patients have access to a larger cohort of potential embryos [74]. However, at the PN stage, there is evidence that cryopreserved embryos may suffer from damage to prokaryotic integrity and thus their developmental potential may be significantly impaired [75].

Veeck et al. to improve the overall pregnancy rate per search cycle [76], have described cryopreservation of excess prokaryotic stage embryos. They reported that if cryopreserved prokaryotic oocytes survive freezing, thawing has similar implantation and pregnancy potential compared to fresh conception. However, a limitation noted by these researchers is the low rate of embryo survival after thawing (68%) [76].

In another study of more than 300 single frozen embryo transfers of day 2 4-cell stage embryos and embryos that lost only one blastomere (25%), a similar transfer was performed on fully intact frozen embryos and an efficient operation, and fresh embryos were also obtained [77].

As a result, many centers have completely phased out the use of slow freezing and, after long-term adoption of traditional slow freezing, have been replaced by conventional vitrification procedures.

Schroeder et al. reported a pregnancy rate of 10.2% using slow cryopreservation of cryopreserved human fertilized eggs [78].

Sang Shan et al. compared slow freezing and vitrification methods in cryopreservation of 2PN zygotes and reported a 100% survival rate of 5881 vitrified zygotes using cryotop as a carrier [79].

Among 340 vitrified embryos, the zygotic PN stage after vitrification was reported to have a 100% survival rate, a high pregnancy rate (36.9%), and a low miscarriage rate (17.42%). In addition, vitrification of 2-PN fertilized eggs has a high pregnancy rate of 46.2% and a survival rate of 97% [80].

### **3.3 Embryo cryopreservation**

Embryo freezing and thawing are considered to have a higher survival rate than oocyte cryopreservation. The first successful embryo cryopreservation was achieved when the research team froze mouse embryos in polyvinylpyrrolidone

(PVP) [81] and the earliest pregnancy in frozen-thawed human embryos was reported in 1983 [82].

Rall and Fahy successfully vitrified embryos using high concentrations of cryoprotectant (CPA) and relatively low cooling and heating rates [38].

Embryo cryopreservation is a critical procedure for embryo transfer (ET) discontinuation due to the risk of ovarian hyperstimulation, endometrial bleeding, elevated serum progesterone levels on the day of ejection, or other unexpected events. There is still much debate about optimal tiers, protocols/procedures, and the use of cryoprotective additives (CPA).

Successful pregnancies and live births by thawing frozen human embryos were first reported in the 1980s. Ferraretti et al. showed that the pregnancy rate (PR) and live birth rate (LBR) of patients who subsequently received cryopreserved embryo thaw were like those of patients who received fresh transfer [83].

The average potential of cryopreserved embryos to become live is about 4%, and babies born from cryopreserved embryos do not exceed 8–10% of the total number of babies born with AR [84].

The results of a retrospective study of 11,768 cryopreserved human embryos that underwent at least one thaw cycle between 1986 and 2007 showed that the length of storage, whether by in vitro fertilization or oocytes, had a significant effect on clinical pregnancy, miscarriage, implantation, or survival. Yield did not significantly affect the donation cycle [85].

Compared to traditional slow-freezing methods, embryo vitrification is a recently introduced ultra-rapid cryopreservation method that prevents freezing within the suspension, transforming it into a glass-like solid, avoiding damage to cells or tissues [86, 87].

Embryo vitrification (VT) was first clinically introduced in Australia in 2006 and is now used for nearly three-quarters of the autologous thaw cycles for transferring blastocysts [88, 89].

Vitrification has been reported to significantly improve pregnancy, delivery, and implantation rates compared to slow freezing of cleavage-stage embryos and blastocysts [90].

Sifer et al. presented the results of a prospective observational study (58 cycles) where early cleavage stage good quality embryos were vitrified and warmed with the results of a retrospective series (189 cycles) where embryos were thawed after a slow freezing procedure (SF). They concluded that the post-thaw survival of vitrified embryos was significantly better than those of embryos resulting from slow freezing procedure. Then, a better clinical pregnancy rate (CPR) per thawed embryo cycle was observed following vitrification [91].

Debrock et al. compared the live birth rate (LBR) per embryo (day 3 cleavage stage embryos) after freezing and thawing by slow freezing or vitrification. They showed that the survival rate after vitrification was significantly higher than that after slow freezing, and the LBR per embryo was significantly higher after vitrification (16%) than after slow freezing (6%) [92].

Zhu et al. compared a retrospective cohort study of 5613 infertile patients with 7862 frozen and thawed day 3 slow frozen (SF) embryos and 3845 vitrified and heated embryos. Day 3 embryos. The proportion of high-quality embryos after thawing in SF was lower than in VT. In a single frozen embryo transfer (FET) cycle, pregnancy and implantation rates were similar between the two groups (35.0 vs. 40.8% and 34.6 vs. 35.9%, respectively). Also, for dual FET, pregnancy rates per cycle were similar between groups (58.8% vs. 58.4%). The implantation rate per embryo transfer was



significantly higher in SF than in VT (38.8% vs. 34.6%). However, SF protocols for cryopreservation of day 3 embryos should be considered [93].

Pooled data from 7 randomized controlled trials (RCTs) (3615 embryos) showed a significant increase in cryopreservation of embryos after vitrification compared to slow freezing ( $P < 0.001$ ) [94].

When embryos are placed in a freezing solution containing intracellular cryoprotectants (ethylene glycol, propylene glycol, glycerol, dimethyl sulfoxide (DMSO)), due to the extracellular concentration (osmolarity) of cryoprotectants from naive cells Gradient) is higher, intracellular water will leak from the cell. After reaching equilibrium, it gradually diffuses into the cell by cryoprotectant and shrinks until osmotic equilibrium is reached; the cell returns to its normal appearance [95, 96].

The main problem with using cryopreservation techniques is that embryos may be lost due to cryogenic injury. Possible risks of injury to cryopreserved and thawed embryos include exposure to biochemical intracellular ice formation (ICC), cytotoxicity of cryoprotectants due to hyperosmolarity, physical damage (zona pellucida), and deoxygenation during embryo handling ribonucleic acid (RNA) damage. During embryo storage [97].

The most important known mechanism of damage to the cells that occur during cooling to low sub-zero temperature includes chilling injury, ice crystal formation, and fracture damage. In controlled slow freezing, embryos are osmotically equilibrated by incubating in approximately 1–2 M permeable and impermeable CPA prior to freezing. This protects the embryo from the formation of intracellular ice crystals. The extracellular ice is then seeded to form, and the embryos are then cooled at a controlled rate to  $-30$  to  $-70^{\circ}\text{C}$  using a programmable slow-speed freezer at  $0.2$ – $2.0^{\circ}\text{C}/\text{minute}$  (min). Finally, embryos are immersed in liquid nitrogen (LN2) for short- or long-term storage [98, 99].

The only danger to cryopreserved cells is suspected to be DNA damage caused by background radiation. Human gametes can safely withstand 3–4 G radiation. Thus, human cells can safely survive for hundreds of years at typical terrestrial background radiation levels of  $0.1$  cGy/year. However, cosmic rays may be less harmful to embryos stored in high-quality cryogenic tanks than previously thought [100].

Sang Shan et al. vitrified cleavage stage embryos with EG + DMSO+ sucrose and showed a small but significant improvement in survival (98% vs. 91%), but no difference in pregnancy rates relative to slow cooling [79]. In a similar comparative study, slow cooling and vitrification were found to have no differences in survival and implantation rates [101].

Balaban et al. observed a higher survival rate (94.8% vs. 88.7%) and a higher rate of intact embryos (73.9% vs. 45.7%) in the vitrification group using the PG + EG + sucrose solution group compared to the slow solution group). Day 3 embryos were frozen in 1.5 MPG + 0.1 M sucrose [102].

### **3.4 Blastocyst cryopreservation**

Cryopreservation of blastocysts is a challenging task due to the size of blastocysts and presence of blastocysts. Since blastocysts contain a lot of water, the formation of ice crystals may be a major factor affecting blastocyst survival. Cohen et al. reported the first infant born after frozen/thawed blastocyst transfer [103].

Cryopreservation at the blastocyst stage has mainly been performed using slow methods with acceptable results [104–106]. It has been suggested that vitrification results in less apoptosis in blastocysts compared to slow freezing [107].

Outcomes of blastocyst-stage vitrification have improved significantly since 2001 [108, 109], with survival rates as high as 100% [110, 111] and 53% pregnancy rates reported by various investigators [79, 110–112].

Several studies have reported increased blastocyst survival when vesicle volume is artificially reduced using glass microneedles [113], 29-gauge needles [86], and hand-drawn Pasteur pipettes for micropipettes [113, 114].

Liebermann and Tucker reported a survival rate of 80.6%. Therefore, highly reproducible vitrification using the Cryotop method is superior to slow freezing. Furthermore, to date, no other technique has consistently achieved the excellent results obtained using this method [115].

Kuwayama et al. found in a comparative study that vitrified blastocysts had a slightly higher survival rate (90%) than slowly cooled blastocysts (84%). However, pregnancy and live birth rates per transfer were not significantly different [79].

In a study of more than 500 blastocysts per group, Liebermann and Tucker found significant differences in survival (96.5% vs. 92.1%), pregnancy per transfer (46.1% vs. 42.9%) and implantation rate (30.6% vs. 28.9%) between the vitrified and slow freezing groups [116].

Loutradi et al. found that blastocyst survival after vitrification was significantly higher than after slow freezing (odds ratio [OR]: 2.20, 95% confidence interval [CI]: 1.53–3.16) [117]. In addition, Hong et al. found a high pregnancy rate (70.5%) and implantation rate (40.6%) when using the new vitrification technique [118].

Recent studies have reported similar clinical outcomes between vitrified blastocyst transfer and fresh blastocyst transfer cycles when similar quality blastocysts were transferred [93, 119].

Cobo et al. vitrified 6019 embryos with cryogenic glass and showed that 97.6% of embryos survived on day 6, compared to 95.7% on day 5, 94.9% on day 2, and 94.9% on day 3, 94.2% at 6 days [120].

### **3.5 Ovarian tissue cryopreservation**

The ovary has hundreds of primordial follicles containing immature oocytes that are small, quiescent, less differentiated, and devoid of banded cells. Due to the lack of zona pellucida and cortical granules, this immature oocyte can tolerate cryopreservation [121].

Ovarian tissue cryopreservation (OTC) is an evolving technique, although limited outcome information is available. Ovarian tissue can be cryopreserved for later ovarian tissue transplantation in prepubertal patients or when immediate chemotherapy is required [122].

Ovarian tissue is collected laparoscopically and frozen and can later be thawed and reimplanted in situ (in the pelvis) or ectopic (into the subcutaneous tissue of the forearm or abdomen). The cryopreservation process of ovarian tissue involves freezing thin slices of ovarian cortex, which contain a rich reserve of primordial follicles. This method of investigating fertility preservation requires only ovarian cortical tissue [123].

The first frozen-thawed ovarian transplantation was reported in 2000, and since then, several successful pregnancies due to these procedures have been reported [124]. Studies have reported restoration of ovarian function using both approaches [125, 126].

The potential risk of cancer recurrence in preimplantation tissue not exposed to chemotherapy may limit its use in cancer patients, at least until in vitro maturation of immature oocytes becomes more standard [125, 127, 128].

The advantage of cryopreservation of ovarian tissue compared to mature oocytes is that primordial follicles in the ovarian cortex are more resistant to cryo-injury [129]. However, long-term studies have reported graft function for up to 11 years [127, 130].

Porcu et al. reported the first birth of healthy twins in a patient who underwent bilateral oophorectomy for ovarian cancer and was pregnant with her own cryopreserved oocytes [131]. Besides, 131 pregnancies and 75 live births (expected to exceed 200 by 2020) have been reported after slow freezing and transplantation, whereas only 4 deliveries have been described after vitrification [132].

In addition, many deliveries between identical twins using fresh ovarian transfer have been reported [133]. There are also reports of births from two sisters with HLA-compatible whole-fresh ovarian transplants [134].

There are two methods of OTC: slow freezing and vitrification. Early studies have shown that slowly frozen ovarian cortex preserves better than vitrified ovarian tissue [135]. Slow freezing has been the traditional technique for many years, despite reports of massive follicular pool loss and excessive stromal cell damage [136].

Xiao et al. reported that a new vitrification technique was comparable to slow freezing in preserving primordial follicles in human ovarian tissue. The proportion of morphologically prominent primordial follicles was significantly reduced by vitrification compared with slow freezing [93].

To date, only two live births following vitrification of ovarian tissue have been reported and all other live births were caused by slow freezing of the ovarian cortex [137, 138]. Twelve studies collected data on intact primordial follicles, and an overall pooled analysis showed no difference between vitrification and slow freezing for this endpoint [139].

However, it has recently been suggested that vitrification has beneficial effects on granulosa cells and ovarian stroma, providing equal or better results than slow cooling to protect ovarian tissue [139].

#### **4. Storage in LN2 containers after slow freezing and vitrification**

In the field of assisted reproductive technology, little is known about the risks of long-term storage of cryopreserved cells, because vitrification is the solidification of a liquid without forming a crystalline structure—a physically disorganized and therefore potentially unstable system. This raises the question, if this changes over time, does this significantly affect the cryosurvival and implantation potential of vitrified gametes and embryos?

Subsequently, the possible effects on neonatal health remain largely unknown. A study by Wirleiter et al. showed that storage of vitrified blastocysts under sterile conditions did not affect blastocyst viability. In addition, no significant differences were observed in survival rates after warming between the first year of storage and after 5–6 years of storage (83.0% vs. 83.1%); nor did the pregnancy rate decrease (40.0% vs. 38.5%). Furthermore, no increase in neonatal malformation rates was observed over time [140].

To date, there have been no reports of cross-contamination between germ cells and tissues stored in cryovials. Cobo et al. showed that viral sequences (HIV, HBV, and HCV) were not detected in liquid nitrogen samples from containers containing oocytes and embryos from chronically infected patients [120].

To date, neither open systems nor closed systems have resulted in disease transmission during vitrification. However, to ensure biosafety during cryopreservation, aseptic methods are recommended [43].

Germ cells and tissues must be cryopreserved and stored in accordance with European Organization Directive 2006/17/EC (European Union [EU] Directive 2006/17/EC) to prevent pathogen transmission or cross-contamination of samples. Patients must be screened for blood-borne viruses (BBV), such as HIV, Hepatitis B, and C, before processing and freezing gametes/embryos and storing germ cells and tissues for positive and negative patients, respectively. Periodic cleaning of storage containers is also considered good laboratory practice (GLP) for decontamination of viral and microbial agents [141].

## 5. Conclusion

Vitrification is now the method of choice for cryopreservation of oocytes due to better results than slow freezing, but more standardized applications are still needed. Transfers of fresh or cryopreserved embryos still performed statistically better than embryo transfers obtained from cryopreserved oocytes. Only a few centers with extensive experience in cryopreservation are comparable between frozen embryo transfer or oocyte cryopreservation embryo transfer.

## Conflict of interest

The authors declare no conflicts of interest.

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