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REVIEW

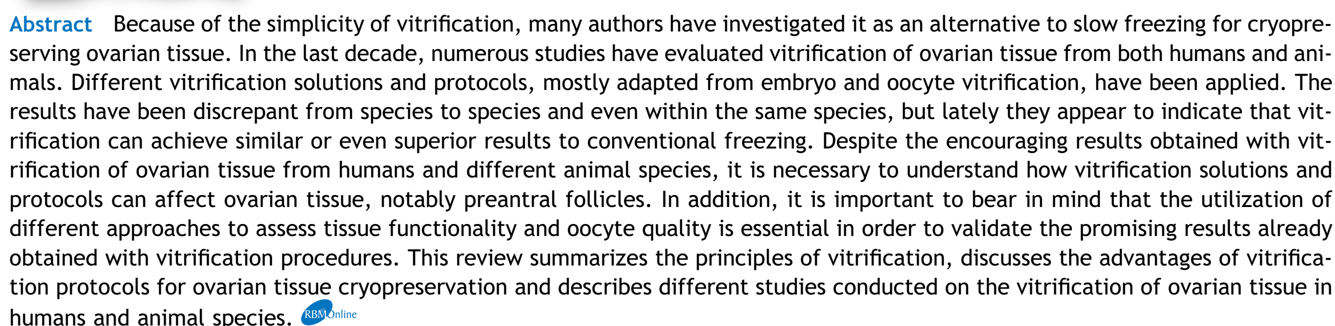
Vitrification as an alternative means of cryopreserving ovarian tissue

Christiani A Amorim, Mara Curaba, Anne Van Langendonckt, Marie-Madeleine Dolmans, Jacques Donnez *

Université Catholique de Louvain, Institut de Recherche Expérimentale et Clinique, Department of Gynecology, Avenue Emmanuel Mounier 52, bte 5247, 1200 Brussels, Belgium

* Corresponding author. *E-mail address:* jacques.donnez@uclouvain.be (J Donnez).

Christiani Andrade Amorim has a bachelor's degree in Veterinary Medicine and since her master and PhD thesis she has been working on preantral follicles. Initially, she focused on domestic animals, developing projects to safeguard native breeds from Brazil and Italy through the establishment of cryopreservation procedures for isolated follicles and ovarian tissue. In 2007, she joined the research team of Professor Jacques Donnez and since then she has been working with human preantral follicles. Her major research interests have focused on human follicle isolation, cryopreservation, in-vitro culture and xenotransplantation.



Abstract Because of the simplicity of vitrification, many authors have investigated it as an alternative to slow freezing for cryopreserving ovarian tissue. In the last decade, numerous studies have evaluated vitrification of ovarian tissue from both humans and animals. Different vitrification solutions and protocols, mostly adapted from embryo and oocyte vitrification, have been applied. The results have been discrepant from species to species and even within the same species, but lately they appear to indicate that vitrification can achieve similar or even superior results to conventional freezing. Despite the encouraging results obtained with vitrification of ovarian tissue from humans and different animal species, it is necessary to understand how vitrification solutions and protocols can affect ovarian tissue, notably preantral follicles. In addition, it is important to bear in mind that the utilization of different approaches to assess tissue functionality and oocyte quality is essential in order to validate the promising results already obtained with vitrification procedures. This review summarizes the principles of vitrification, discusses the advantages of vitrification protocols for ovarian tissue cryopreservation and describes different studies conducted on the vitrification of ovarian tissue in humans and animal species. RBMOnline

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KEYWORDS: cryoprotectants, fertility preservation, ovarian tissue, slow freezing, vitrification

Introduction

The concept of vitrification was first described in the early part of the last century. In order to achieve successful vitrification, scientists found that high cooling rates, as well as high concentrations of cryoprotectants (CPA), were required. As this appeared to be an insurmountable problem, the focus shifted to a more stepwise approach and the development of slow-freezing technology, which has dominated cryobiology until now (Kelly et al., 2003). Recently, however, vitrification has made a comeback and

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has quickly become the most significant development in cryobiology to date (Arav et al., 2002; Kelly et al., 2003).

Following this trend, in recent years, researchers working with cryopreservation of preantral follicles around the world have been seduced by the concept of vitrification. The idea of a simple and fast protocol that is easy to master and does not require special equipment is very appealing. Indeed, the procedure can be performed in any laboratory, even in the operating room simultaneously with patient surgery, or out in the field, soon after the death of a wild animal. The idea becomes even more attractive when added to the successful results obtained with embryos (Desai et al., 2007; Hiraoka et al., 2007; Vieira et al., 2008) and oocytes (Chang et al., 2008; Cobo et al., 2008; Somfai et al., 2007).

During the last decade, many studies have been published on the vitrification of preantral follicles from humans (Isachenko et al., 2002, 2003, 2006, 2008, 2009b; Kagawa et al., 2009; Keros et al., 2009; Li et al., 2007) and animals (dogs: Ishijima et al., 2006; hamsters: Kagabu and Umezu, 2000; goats: Santos et al., 2007; monkeys: Yeoman et al., 2005; mice: Haidari et al., 2007; Nagano et al., 2007; rats: Sugimoto et al., 1996, 2000; sheep: Courbiere et al., 2006; rabbits: Kagabu and Umezu, 2000; pigs: Gandolfi et al., 2006; marsupials: Czarny et al., 2009). However, the results have been discrepant from species to species and even within the same species, and therefore conclusions are not easily drawn. This is probably due to the many variables involved in vitrification protocols, such as ovarian tissue size, composition and exposure time of vitrification solutions and carrier systems, and the absence of studies on the impact of vitrification on ovarian tissue and its follicles. Such studies would need to elucidate how high concentrations of CPA and fast cooling rates can affect preantral follicles and the surrounding ovarian tissue.

The present study is an impartial review that aims to shed new light on the advantages of vitrification for ovarian tissue cryopreservation and to report studies that have used vitrification to cryopreserve preantral follicles from human and different animal species.

Conventional freezing versus vitrification

Cryopreservation conserves cells, tissues or organs by decreasing the temperature in order to suspend metabolic reactions in cells. Although this allows storage for an unlimited amount of time without impairing cell or tissue viability, the decrease and subsequent increase in temperature during cooling and warming often result in cryoinjury (Karlsson and Toner, 1996; Mullen and Critser, 2007), especially in the intermediate temperature zone between ~0°C and −15°C (Mazur, 1963). During cooling and warming, cells are exposed to different forces (thermal, chemical and mechanical), which may interfere with their normal function (Karlsson and Toner, 1996). These forces vary according to the cryopreservation procedure applied, which generally falls into one of two categories: freezing or vitrification. **Table 1** summarizes the main differences between the two approaches.

Freezing approach

As mentioned before, while vitrification requires high concentrations of CPA, protocols using lower CPA concentrations have been developed for traditional freezing methods. Both cryopreservation approaches involve initial equilibration of the cell suspension or tissue in the CPA solution and dehydration with conventional slow freezing, much of the equilibration and most of the dehydration only occurs once ice forms outside cells, and it is this ice that concentrates the extracellular solution and dehydrates the cells (Agca, 2000; Mazur, 1996; Polge et al., 1949). The rate of cooling dictates the rate and extent of water movement and ice nucleation (Karlsson and Toner, 1996), with relatively fast cooling achieving a lower degree of dehydration or concentration of solutes than slow rates. Faster rates are therefore best suited to cells that dehydrate rapidly or are sensitive to high solute concentrations, while slow rates are conventionally applied to oocytes. The ice that forms during slow freezing has the potential to cause physical

Table 1 Comparison of vitrification and slow freezing for cryopreservation of ovarian tissue.

Characteristics	*Vitrification*	*Slow-freezing*
Direct contact with liquid nitrogen	Yes	No
Ice formation	No	Yes
Time	Fast (minutes)[a]	Slow (hours)
CPA equilibration	Yes	Yes
CPA concentration	High (over 40%)	Low (10–15%)
Sample size (human)	Up to 5 × 1 × 1 mm[b]	Up to 2 × 4 × 12 mm[c]
Cooling rates (°C/min)	15,000–30,000	0.15–0.30
Cost	Protocol-dependent (usually inexpensive)	Equipment-dependent (usually expensive)
Special equipment	No	Yes
Technical expertise	Risky	Simple
Routinely applied for cryopreservation of human ovarian tissue	No	Yes

Adapted from Moore and Bonilla (2006).
[a]Considering just one cryocycle.[b]Li et al. (2007) and Huang et al. (2008).[c]Donnez et al. (2004).

and mechanical injury, as ice occupies a greater volume than water and the dimensions of individual ice crystals change during cooling and warming. Ovarian tissue contains very diverse cell types that differ in their optimal CPA, equilibration and cooling requirements. Slow freezing has nonetheless proved to be relatively effective for ovarian tissue in many species.

A number of highly regarded review papers describe how freezing can affect ovarian tissue and compare the results obtained with this technique (Amorim et al., 2003; Bromer and Patrizio, 2009; Demirci et al., 2003; Donnez and Dolmans, 2009; Donnez et al., 2006, 2009; Fabbri, 2006; Gook and Edgar, 2007; Hovatta, 2005; Nawroth et al., 2005; Santos et al., 2010; von Wolff et al., 2009). Although slow freezing is a simple and relatively effective way of cryopreserving ovarian tissue, the cooling component of the procedure is time consuming and requires expensive equipment in clinical practice. There is, therefore, considerable impetus to find a more rapid alternative such as vitrification.

Vitrification approach

In order to avoid extra- and intracellular ice formation and solution effects, cells may be cryopreserved using vitrification protocols. In contrast to conventional freezing methods, with vitrification, the entire solution is thought to remain unchanged during cooling and warming, both chemically and structurally, as no ice crystals are formed (Fahy, 1986; Liebermann et al., 2002). According to Fahy (1986), in the vitrification process, the viscosity of intra- and extracellular solutions progressively increases, which decreases the diffusivity of water, and molecules become immobilized. At this stage, the sample is no longer a liquid, but rather has the properties of a solid. In this procedure, the solution is rapidly cooled, forming a glassy, vitrified state, not by ice crystallization (as in slow freezing), but by an extreme increase in viscosity during cooling (Fahy et al., 1984) (**Figure 1**). Moreover, by preventing ice formation, vitrification methods also avoid solution effects (Pegg, 2005). Vitrification can be achieved by augmenting the cooling rate and CPA concentration, which results in total elimination of intra- and extracellular ice formation. According to Liebermann et al. (2002), an optimal cooling rate allows a high efflux of water for further extracellular vitrification. However, this drastic approach may be biologically problematic and technically challenging (Rall, 1987) due to the toxicity of high concentrations of CPA, and the risk of damage caused by the difficulty of achieving and controlling the required cooling and warming rates (Yavin and Arav, 2007).

Several disadvantages of vitrification protocols are all basically linked to high concentrations of CPA. Therefore, to prevent toxicity and excessive CPA permeation, exposure to vitrification solutions should be carefully managed (Glenister and Rall, 2001). However, efficient control of CPA equilibration, taking into account CPA concentrations and exposure time and temperature, is achievable only with cryopreservation of a small number of ovarian fragments. This is a very important detail to bear in mind since, so far, vitrification protocols have essentially been implemented for either very thin (up to 1 mm thick) or very small (usually around 1 mm^3) pieces of ovarian tissue, and a large amount of ovarian cortex, indicated for later fertility preservation, can be divided into dozens of such tissue cubes/slices.

Variables involved in vitrification methods

According to Liebermann et al. (2002), many factors influence the likelihood of a successful vitrification outcome, such as the type and concentration of CPA, temperature of exposure to vitrification solution, stepwise addition of vitrification solution, sample size, carrier system, quality of samples and technical expertise.

Type and concentration of CPA

The main CPA used to slow freeze ovarian tissue and isolated preantral follicles can also promote glass formation, although they do not all have the same capacity to form glass (Fahy et al., 2004a,b). To avoid freezing and achieve vitrification of large volumes it is common to use high concentrations (e.g. 40%) of one or more CPA, (Fuller and Paynter, 2004) and to add compounds that aid dehydration (e.g. sugars) and formation of a solid state (e.g. polymers) (Kuleshova et al., 1999, 2001; Wowk, 2005; Wowk et al., 2000). These solutes are not chemicals normally found in living organisms, at least not in high concentrations (Fuller, 2004), and they may be toxic (Pegg, 2005). The nature of

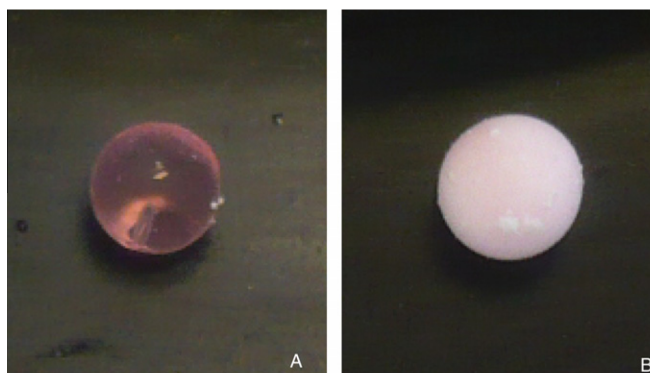

Figure 1 Vitrified droplets after contact with liquid nitrogen containing (**A**) high concentrations of ethylene glycol and dimethylsulphoxide and (**B**) a low concentration of dimethylsulphoxide solution.

chemical toxicities of CPA is complex given the range of different molecular structures (Fuller, 2004), and CPA concentrations above 30% w/w may lead to severe toxic or osmotic damage (Fahy et al., 1984). In order to reduce CPA toxicity without affecting cryoprotective capacity, relatively low concentrations of different CPA can be combined to obtain a vitrifiable concentration of total solutes (Wusteman et al., 2004) and decrease the specific toxicity of individual CPA (Vajta and Nagy, 2006). Another advantage of this approach is that the permeability of these CPA mixtures is higher than that of individual CPA (Vicente and Garcia-Ximenez, 1994), so in such combinations, at least one CPA should be permeable, even if the others are not (Vajta and Nagy, 2006) (**Figure 2**).

Permeable CPA

To vitrify ovarian tissue derived from humans and animals, permeable CPA, such as ethylene glycol (EG), dimethylsulphoxide (DMSO), glycerol (GLY), propylene glycol (PROH), acetamide and formamide, have been used. Although EG is a poor glass former (Mullen and Critser, 2007), it appears to have a low toxic effect and shows rapid diffusion into cells (Bautista and Kanagawa, 1998; Kuleshova et al., 1999; Orief et al., 2005), as well as high compatibility with other CPA (Bautista and Kanagawa, 1998). EG is the most commonly used CPA for human (Gandolfi et al., 2006; Huang et al., 2008; Isachenko et al., 2002, 2003, 2006, 2008; Rahimi et al., 2003, 2004; Wang et al., 2008) and murine (Amorim et al., 2011; Chen et al., 2006; Haidari et al., 2006, 2007, 2008; Hasegawa et al., 2004, 2006; Hemadi et al., 2009; Salehnia, 2002; Salehnia et al., 2002; Segino et al., 2003; Xiao et al., 2010; Zhang et al., 2009, 2010) ovarian tissue and is often combined with other CPA, polymers and proteins. While only a few studies on human ovarian tissue (Huang et al., 2008; Wang et al., 2008) have achieved results comparable to those obtained with slow-freezing procedures, all vitrification experiments in mice have been very successful, some even yielding live offspring (dela Peña et al., 2002; Hasegawa et al., 2006; Bagis et al., 2007; Kagawa et al., 2007; Wang et al., 2009b). EG has also been found to effectively vitrify ovarian tissue from monkeys (Hashimoto et al., 2010; Yeoman et al., 2005), rabbits (Hasegawa et al., 2006) and pigs (Hasegawa et al., 2006). EG alone has also been used for slow freezing of ovarian tissue and isolated preantral follicles (Andersen et al., 2008; Amorim et al., 2003, 2006; Candy et al., 1997; Oktay et al., 1997; Newton et al., 1996; Schmidt et al., 2003, 2005; Shaw et al., 1996). The inclusion of EG in vitrification solutions for ovarian tissue was probably based on successful results of oocyte and embryo vitrification (Ali and Shelton, 1985; Kuleshova et al., 1999; Mukaida et al., 2001; Yokota et al., 2000; Yoon et al., 2000).

For vitrification of ovarian tissue from other animal species, such as sheep (Baudot et al., 2007; Bordes et al., 2005; Courbiere et al., 2005, 2006, 2009b; Lornage et al., 2006), rats (Sugimoto et al., 1996, 2000; Kagabu and Umezu, 2000), hamsters (Kagabu and Umezu, 2000), rabbits (Hasegawa et al., 2006; Kagabu and Umezu, 2000), pigs (Gandolfi et al., 2006; Moniruzzaman et al., 2009) and dogs (Ishijima et al., 2006), DMSO, an effective glass-forming agent (Ali and Shelton, 2007; Fahy et al., 2004a), is usually combined with PROH, another strong glass-former (Ali and Shelton, 2007; Fahy et al., 2000), or weak glass-formers such as EG, acetamide and formamide (Ali and Shelton, 2007; Fahy et al., 2004a).

Non-permeable CPA

Serum proteins, sugars, polymers and other non-permeable CPA are important in the composition of vitrification solutions because they influence viscosity and promote glass formation, thereby decreasing toxicity, since their addition allows lower concentrations of permeable CPA to be used without compromising vitrification properties (Bautista and Kanagawa, 1998; Liebermann et al., 2002; Pegg, 2005). At low temperatures, these macromolecules show high viscosity, which prevents water molecules from bonding with growing ice crystals (Sutton, 1991, 1992). In addition, they control the rate of swelling before and after warming and assist in the formation of stable glass (Bautista and Kanagawa, 1998).

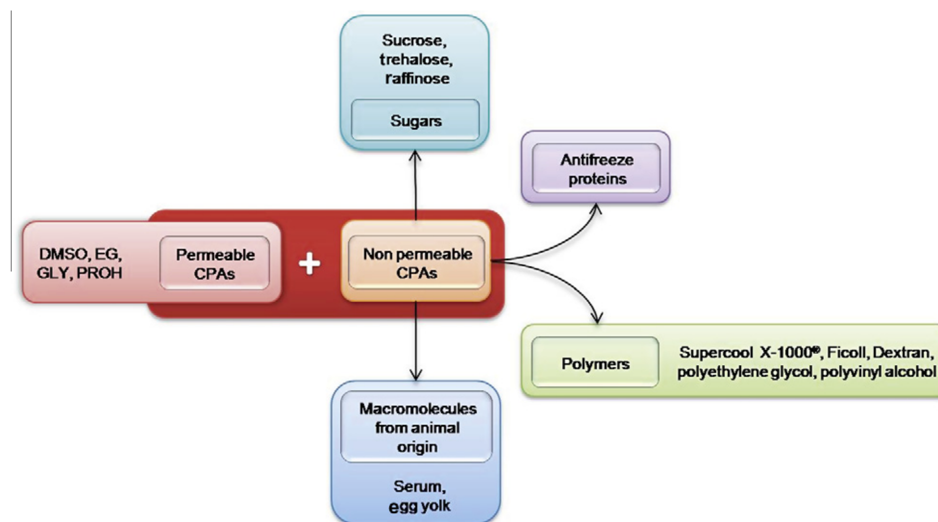

Figure 2 Different types of permeable and non-permeable cryoprotectants that can be used in vitrification solutions. CPA = cryoprotectant; DMSO = dimethylsulphoxide; EG = ethylene glycol; GLY = glycerol; PROH = propanediol.

There are some studies on the use of macromolecules of animal origin from sera (Amorim et al., 2011; Chen et al., 2006; Gandolfi et al., 2006; Lin et al., 2008) or egg yolk (Isachenko et al., 2002) to decrease the possibility of cryoinjury during vitrification of human and animal ovarian tissue. Such agents are less toxic than permeable CPA. Sera of different origin, serum albumin or recombinant albumin, may promote vitrification and protect cells against cryoinjury (Chen and Yang, 2007). However, since these additives have variable composition, they can change the characteristics of the vitrification solutions (Adamson et al., 1993). Furthermore, they may also be a potential source of infectious agents. It is therefore more desirable to develop solutions without additives of animal origin (Shaw et al., 1997). With this in mind, some authors (Hasegawa et al., 2006; Hashimoto et al., 2010; Isachenko et al., 2006, 2008, 2009b; Kagawa et al., 2007, 2009) use a synthetic serum substitute to replace animal serum in the formula of their vitrification solution.

Sucrose, trehalose, raffinose and other sugars have been shown to influence the properties of a solution, contributing to the vitrification outcome (Kuleshova et al., 1999; Sutton, 1991). Some studies (Kuleshova et al., 1999; Wusteman et al., 2003) have demonstrated that, by adding sugars, some of the CPA can be removed without affecting the vitrification properties of the solution. It has been suggested that sugars are capable of preserving membrane integrity at low water activities (Hotamisligil et al., 1996), and can reduce CPA toxicity as they line the outer membrane by formation of hydrogen bonds with phospholipids from the membrane (Anchordoguy et al., 1987). Moreover, adding sugar assists dehydration, decreasing the risk of intracellular crystallization (Courbiere et al., 2009a), and allows shorter exposure times, thereby reducing the toxic effects of permeable CPA present in the vitrification solution. Sugars also protect cells from intense osmotic shock during intracellular removal of the CPA, avoiding fast rehydration (Courbiere et al., 2009a). However, it is important to bear in mind that, like permeable CPA, sugars can also cause osmotic damage when used in high concentrations (Isachenko et al., 2007). Most studies on vitrification of ovarian tissue from humans (Amorim et al., 2011; Rahimi et al., 2003; Wang et al., 2008; Xiao et al., 2010; Zhang et al., 1995) and mice (Abedelahi et al., 2009; Chen et al., 2006; Segino et al., 2003; Wang et al., 2008, 2009a,b) have added sugar to their solutions. The percentage of intact human preantral follicles found after vitrification with solutions containing sucrose was similar to the results of slow freezing (Li et al., 2007; Wang et al., 2008). However, evaluating the dynamic growth and hormonal activity of human ovarian tissue after vitrification, Isachenko et al. (2002) suggested that sugars may have a negative effect on the vitrification outcome. Both sucrose (Bao et al., 2010; Czarny et al., 2009; Deng et al., 2009; Hashimoto et al., 2010; Moniruzzaman et al., 2009) and trehalose (Gandolfi et al., 2006) have been used for other animal species.

Polymers such as synthetic ice blockers could provide a cheaper and more stable alternative to antifreeze proteins (Wusteman et al., 2004). Polyvinyl alcohol (PVA), a synthetic polymer used to substitute serum in vitrification (Naitana et al., 1997) and freezing solutions (Sommerfeld and Niemann, 1999), can have extraordinary ice-inhibition properties (Wowk, 2005; Wowk et al., 2000), enhancing the vitrification capacity of solutions (Wang et al., 2009a). In the study by Wowk et al. (2000), PVA was tested in the form of a product called SuperCool X-1000, and even small concentrations of PVA were sufficient to inhibit ice formation in vitrification solutions. Despite these promising results, PVA was used in only one study on vitrification of ovarian tissue. Isachenko et al. (2002) tested different vitrification solutions supplemented with SuperCool X-1000 to vitrify human ovarian tissue. They reported that long exposure to this synthetic ice blocker could dramatically reduce the viability of ovarian tissue after warming, possibly due to the toxicity of this product. In two further studies, Isachenko's group compared a vitrification solution containing SuperCool X-1000 with a freezing solution and showed that, after xenografting of human ovarian tissue, the extent of necrosis did not differ between treatments (Rahimi et al., 2004), while the number of apoptotic cells was higher in vitrified tissue (Rahimi et al., 2009). Long-chain polymers can increase solution viscosity and replace some permeable CPA, which in turn reduces the toxicity of vitrification solutions (Courbiere et al., 2009a; Kuleshova et al., 2001). According to Acker (2007), the effectiveness of polymers may also be due to their impact on the physicochemical properties of solutions and their efficiency in terms of biomolecular stabilization. The addition of polymers like Ficoll, dextran, polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP) can allow lower CPA concentrations to be used without compromising the vitrification properties of solutions (Fuller, 2004; Liebermann et al., 2002; Pegg, 2005). Polymers have a very specific role in glass forming (Fuller, 2004) and may act through different interactions, exhibiting effects other than simple colligative and/or space-filling properties (Shaw et al., 1997). These CPA appear to have increasingly high viscosities at low temperatures, preventing water molecules from joining growing ice crystals (Fuller, 2004; Sutton, 1991, 1992). In addition, pure PVP shows high solubility and low toxicity (Hashimoto et al., 2010). Polymers are not often used in vitrification solutions for human ovarian tissue and, although Ficoll is less effective than PVP (Shaw et al., 1997), it has been more frequently utilized (Isachenko et al., 2002, 2003; Rahimi et al., 2003) than PVP (Gandolfi et al., 2006). Shaw et al. (1997) reported that the contribution of Ficoll to the vitrification characteristics of an EG solution was slightly greater than that of an inert substance that would occupy a similar proportion in the solution. PVP has been shown to contribute as effectively as EG to the properties of vitrification solutions (Shaw et al., 1997) and to increase the cryoprotective properties of EG solutions (Leibo and Oda, 1993). However, the unsatisfactory results obtained with vitrification of human ovarian tissue using an EG-based solution containing Ficoll or PVP do not allow conclusions to be drawn about the impact of these macromolecules on the vitrification of human preantral follicles. PEG was also used in two different vitrification solutions tested by Isachenko et al. (2008) but, since their results were as good as with one of the PEG-free solutions, it is not possible to attribute the positive findings to this macromolecule. While Ficoll is the macromolecule of choice to vitrify murine preantral follicles (Abedelahi et al., 2009; Hemadi et al., 2009; Salehnia, 2002; Salehnia et al., 2002; Segino et al., 2003), PEG is the

only macromolecule used in vitrification solutions for ovarian tissue from sheep (Bordes et al., 2005; Courbiere et al., 2005), hamsters, rabbits (Kagabu and Umezu, 2000) and rats (Sugimoto et al., 1996, 2000), and PVP is used for cows, pigs (Gandolfi et al., 2006) and monkeys (Hashimoto et al., 2010).

Temperature during exposure to vitrification solution

Another alternative to decrease the toxicity of CPA is to reduce the temperature during exposure of ovarian tissue to vitrification solutions. Since temperature affects CPA permeation, a lower temperature would duly increase the time of exposure which, in turn, increases the likelihood of osmotic injury (Newton et al., 1998). This was observed by Wusteman et al. (2003) when they equilibrated ECV304 cells with vitrification solution at low temperatures. The authors reported a significant degree of toxic injury, which was probably due to the longer CPA exposure required at lower temperatures. Therefore, it would probably be less harmful to ovarian tissue to raise the temperature of the vitrification solution to allow faster penetration of CPA and reduce the exposure period, than lowering the temperature and increasing the duration of contact of the tissue with the solution (Pegg, 2005).

Although the choice of temperature for CPA equilibration plays a vital role in the vitrification protocol because of the high concentration of CPA in the solution, studies on vitrification of ovarian tissue (human and animal) do not appear to follow any specific trend. To vitrify human ovarian tissue, for example, CPA equilibration was performed at 0–4 °C in most protocols (Huang et al., 2008; Rahimi et al., 2003). Isachenko et al. (2002, 2008) compared several vitrification solutions equilibrated at different temperatures and found them to differ. However, since these solutions had different compositions, it is not possible to determine if this was due to the equilibrium temperature.

Zhang et al. (2009) reported variation in follicular density in the same vitrification solution exposed to CPA at different temperatures and for different periods of time. These authors showed that exposure of murine ovarian tissue to CPA at room temperature positively affected follicular survival compared with exposure at 4°C. Some authors also described protocols with stepwise addition of vitrification solution, where the temperature varied according to CPA concentration: the higher the CPA concentration, the lower the temperature (Isachenko et al., 2008; Keros et al., 2009; Rahimi et al., 2010), and vice versa (Isachenko et al., 2002). They were probably aiming to decrease the toxic effect of high CPA concentrations and/or the risk of osmotic injury.

Stepwise addition

Due to the high concentrations of CPA required in vitrification procedures, stepwise exposure to the vitrification solution is advised in order to decrease osmotic and toxicity effects (Ishimori et al., 1992; Liebermann et al., 2002). In human ovarian tissue vitrification, only Isachenko et al. (2003) and Gandolfi et al. (2006) compared single-step exposure of vitrification solution with stepwise addition. Although they obtained better results with single-step addition, it is not possible to determine whether this option would be more suitable for human ovarian tissue, because they used a different vitrification solution for both methods. Rahimi et al. (2003) also added the solution in a single step. Their aim was to investigate the effect of different vitrification protocols on the survival of stromal cells and the production of reactive oxygen species in ovarian tissue. One cannot, therefore, know the impact of the addition of these single-step solutions on preantral follicles. In mice, while most studies (dela Peña et al., 2002; Hasegawa et al., 2004, 2006; Wang et al., 2009a,b; Zhang et al., 2009, 2010) have applied stepwise addition of the vitrification solution, a few (Abedelahi et al., 2009; Mazoochi et al., 2008; Salehnia, 2002; Salehnia et al., 2002) have reported single-step exposure and demonstrated that, ultrastructurally, ovarian tissue did not show any signs of damage or necrosis (Abedelahi et al., 2009; Salehnia et al., 2002) and that preantral follicles were morphologically normal after vitrification (Mazoochi et al., 2008; Salehnia, 2002), although none of them were aiming to obtain offspring, unlike some studies using multistep addition (Hani et al., 2006; Hasegawa et al., 2006; Kagawa et al., 2007; Migishima et al., 2006; Wang et al., 2009b). For vitrification of sheep (Courbiere et al., 2005), pig and cow (Gandolfi et al., 2006) ovarian tissue, single-step addition of vitrification solutions was implemented, but the results were disappointing. Thus, the role of stepwise exposure needs to be investigated in order to confirm if CPA toxicity can be overcome by this procedure.

Sample size

The size of ovarian tissue pieces also plays an important role in the success of the vitrification procedure. For instance, large ovarian fragments would require longer equilibration to allow penetration of the vitrification solution to the inner parts of the tissue. In turn, cells on the surface would be overexposed to CPA, which may result in injuries caused by the toxicity of high CPA concentrations. It is therefore very likely that cell survival would be higher in smaller ovarian fragments.

Use of small fragments would allow faster cooling rates, which are necessary to decrease the likelihood of chilling injury during the critical temperature zone that ranges from 15 to −5°C (Liebermann et al., 2002). When samples are plunged into liquid nitrogen, there is extensive evaporation on their surface, which creates a vapour coat around them. This isolates the samples from the liquid nitrogen and prevents them from sinking, decreasing the cooling rate (Liebermann et al., 2002; Vajta, 2007). In order to minimize the duration of vapour coat formation and increase the cooling rate, the size of tissue fragments can be reduced.

Probably taking into account the importance of sample size, all studies on vitrification of ovarian tissue from humans and animals have used small fragments (≤1 cm). The only exception were studies in sheep by Salle's group, which aimed to vitrify half (Bordes et al., 2005) and whole ovaries (Baudot et al., 2007; Courbiere et al., 2005, 2006, 2009b; Lornage et al., 2006). The results were very promising after vitrification of half ovaries, with the birth of four

lambs (Bordes et al., 2005) and recovery of ovarian endocrine function (Courbiere et al., 2009b). However, when whole ovaries were vitrified, the authors reported ice formation (indicating that the ovarian tissue did not completely vitrify) (Baudot et al., 2007), a lower percentage of viable follicles (Lornage et al., 2006), poor follicular survival rates after vitrification and grafting and a high incidence of pedicle thrombosis (Courbiere et al., 2009b).

Unlike small ovarian fragments, where simple soaking of samples in vitrification solution is enough to provide protection, whole ovaries require a more complex vitrification procedure, using special perfusion equipment for introduction and removal of the vitrification solution. Unfortunately, this time-consuming and delicate process does not guarantee vitrification in the entire organ. In addition, larger tissues are very likely to be subjected to non-uniform cooling, which can cause fractures, with devastating consequences for whole organs. Indeed, Courbiere et al. (2005) observed pedicle fracture in whole ovaries after vitrification. Such mechanical destruction of the tissue may preclude organ transplantation. According to Williams (1989), another problem caused by fractures is that they offer an interface for nucleation that could provoke devitrification. Finally, it can also be difficult to warm vitrified organs rapidly enough to avoid devitrification (Taylor et al., 2004). Therefore, vitrification of whole ovaries or large pieces of tissue (half ovaries) clearly remains a considerable challenge.

Carrier systems

To reduce the volume of vitrification solution as much as possible, different carriers have been applied in ovarian tissue vitrification, such as plastic straws (Amorim et al., 2011; Isachenko et al., 2002; Nagano et al., 2007; Rahimi et al., 2009), copper grids (Choi et al., 2007a,b; Isachenko et al., 2003), cryovials (Hemadi et al., 2009; Isachenko et al., 2006), handcut straws (Keros et al., 2009), metal strips (Kagawa et al., 2009), cryotops (Bao et al., 2010; Kagawa et al., 2007; Moniruzzaman et al., 2009; Trapphoff et al., 2010), glass tubes (Sugimoto et al., 1996, 2000), needles (Wang et al., 2008; Xiao et al., 2010) and cryosupport (Hashimoto et al., 2010). However, most carriers have large working dimensions and thick walls and are made of non-conductive materials (e.g., plastic), which may negatively affect the cooling rate. Some authors have therefore applied alternative carrierless systems, such as solid-surface vitrification (SSV; Amorim et al., 2011; Huang et al., 2008; Santos et al., 2007; Wang et al., 2009b) and minimum drop size (MDS; Amorim et al., 2011; Isachenko et al., 2008; Rahimi et al., 2010; Wang et al., 2008; Yeoman et al., 2005). Santos et al. (2007) compared a carrier approach (plastic straw) with a carrierless system (SSV) to vitrify caprine ovarian tissue and reported that follicular viability was higher after SSV. Similarly, Amorim et al. (2011) reported that a carrierless system (MDS) offered better results than plastic straws to vitrify human ovarian tissue. Wang et al. (2008) obtained different results, however. They showed the percentage of morphologically normal primordial follicles to be similar when human ovarian tissue was vitrified using needles or MDS, but ultrastructurally, preantral follicles and ovarian stroma were better preserved when needles were used. The authors suggested that the amount of vitrification solution around the tissue in the carrierless system might have decreased the cooling rate due to the formation of a nitrogen vapour coat. One can also surmise that the superior results obtained with the needle procedure were due to a lower concentration of CPA in the vitrification solution. However, needles also have some advantages that could explain the results. Before plunging into liquid nitrogen, the vitrification solution is removed from the ovarian tissue using absorbent gauze, minimizing its thermal mass and hence reducing vapour coat formation. Furthermore, use of a support to keep the tissue immersed in the liquid nitrogen prevents it from floating, which would also reduce formation of a vapour coat and increase the cooling rate.

One important concern for protocols both with and without carriers is the direct contact of ovarian tissue with liquid nitrogen, which could potentially allow cross-contamination of pathogenic organisms between these two elements, since the tissue may be contaminated with pathogenic organisms that are consequently preserved. One should therefore bear in mind that, despite the promising results obtained with different vitrification protocols, most carrier and carrierless systems will never be indicated for clinical applications (Isachenko et al., 2009a, 2010). In addition, there is a potential risk of international propagation of pathogenic agents that could have serious consequences in clinical and agricultural settings (Grout and Morris, 2009). An alternative is the SSV method developed by Dinnyés et al. (2000), since the ovarian tissue does not come into direct contact with liquid nitrogen, but is encased in a metal cube partially submerged therein. Based on the SSV system, Lindemans et al. (2004) developed the cryological vitrification method (CVM), which was tested by Aerts et al. (2008) to vitrify mouse ovarian tissue. CVM avoids contact between ovarian tissue and liquid nitrogen during and after vitrification, since the tissue is first vitrified in the metal box and then inserted into a straw with an in-built sealant before plunging into liquid nitrogen.

Warming rate

Vitrification of ovarian tissue does not guarantee the viability of preantral follicles. Another crucial step involves warming. During warming, the specimen passes through temperatures at which ice nucleation and ice growth might occur in marginally vitrified solutions or regions of tissue where the amount of penetrating CPA has not reached a sufficiently high concentration to fully vitrify. In these solutions or tissue regions, samples will devitrify (form ice crystals) (Pegg, 2005) rather than staying completely ice-free during warming. Ice nucleation and ice crystal growth and remodelling in these marginally vitrified solutions or regions can be reduced by increasing the warming rate or decreasing sample size. This was confirmed by Courbiere et al. (2006), who observed ice formation during fast warming of whole ovine ovaries after vitrification. These authors also reported the risk of fractures between liquid nitrogen (−196°C) and vitreous transition (−125.2°C) temperatures due to glass fragility. According to Fahy et al. (2004b), warming is a serious concern for vitrification of whole organs, since it is difficult to perform this step rapidly enough to avoid devitrification. As with the cooling rate, the

efficacy of the warming rate depends on different parameters, like the carrier system chosen and the methods applied to bring the tissue to temperatures above 20°C.

The literature describes different protocols to warm vitrified ovarian tissue. The most commonly implemented include tissue submersion in warming solution previously equilibrated at 37°C (Hani et al., 2006; Kagawa et al., 2007, 2009; Keros et al., 2009; Wang et al., 2008) and warming in a water bath at 37°C (Bordes et al., 2005; Gandolfi et al., 2006; Kagabu and Umezu, 2000). More extreme methods, such as use of boiling water (Isachenko et al., 2006) or agitation in ice (Sugimoto et al., 2000), have also been proposed. However, the authors do not give reasons for choosing a specific warming procedure or discuss its influence on follicular survival.

Technical expertise

In addition to the variables listed above, vitrification protocols are influenced by the technical expertise and skills of the person carrying out the procedure. As mentioned before, when working with such high concentrations of CPA, time and temperature are critical parameters for follicular survival, as are cooling and warming rates. However, exposure of ovarian tissue to vitrification solution and cooling/warming procedures are difficult to maintain under perfect control, so differences in results can be expected even using the same protocol.

Vitrification of human ovarian tissue

Apart from the pioneering work carried out by Zhang et al. (1995) at the end of the last century, vitrification of human ovarian tissue has only been intensively studied in the last 10 years. Isachenko's team in Germany has been the most productive in this field (Isachenko et al., 2002, 2003, 2006, 2008, 2009b; Rahimi et al., 2003, 2004, 2009, 2010), investigating various vitrification solutions and carrier systems, using a number of methods to evaluate the impact of different vitrification protocols on ovarian tissue. After numerous attempts and variable outcomes, they reported, in one of their last papers (Isachenko et al., 2009b), that slow freezing is more promising than vitrification for human ovarian tissue. Similar results were obtained by Gandolfi et al. (2006), showing vitrification to cause extensive damage to preantral follicles. However, most studies support vitrification as the cryopreservation method of choice for human ovarian tissue. Many authors (Huang et al., 2008; Keros et al., 2009; Li et al., 2007; Wang et al., 2008) have demonstrated that vitrification provides similar results to conventional freezing, with the additional advantage of preserving the ultrastructure of stromal tissue (Keros et al., 2009; Wang et al., 2008; Xiao et al., 2010) that is usually affected by freezing. Outstanding results were achieved by Kagawa et al. (2009), who reported that preantral follicle morphology and viability were not affected by vitrification and their percentages were similar to those in fresh ovarian tissue. However, it is important to interpret such results with caution. The analysis by Kagawa et al. (2009) was conducted as soon as the tissue was warmed and therefore follicles did not have time to express possible damage caused by vitrification. It would be prudent to have an incubation period, as proposed by Hovatta et al. (1996) and Keros et al. (2009), before fixing or preparing the tissue for further analysis, because it would probably allow detection of changes in organelles after cryopreservation. Indeed, when different carrier systems to vitrify human ovarian tissue were analysed, a significant difference in follicular morphology was observed after incubation compared with fresh ovarian tissue (Amorim et al., 2011). Very recently, Zhou et al. (2010) demonstrated the advantages of using direct cover vitrification to cryopreserve human ovarian tissue. This technique was developed by Chen et al. (2006) to vitrify murine ovaries and has the benefit of requiring a lower concentration of CPA than other vitrification solutions. When compared with the minimum droplet-size method, direct cover vitrification showed a higher percentage of morphologically and ultrastructurally normal follicles after 24 days of xenografting. In addition, the incidence of apoptotic cells was lower with the latter treatment (Zhou et al., 2010). **Table 2** summarizes studies published on vitrification of human ovarian tissue. As previously pointed out, with so many different vitrification solutions and methods, it is difficult to establish the best protocol to vitrify human ovarian tissue. However, it is interesting to observe that most protocols yielding successful results use solutions containing a mixture of DMSO and EG (Huang et al., 2008; Kagawa et al., 2009; Keros et al., 2009; Wang et al., 2008; Xiao et al., 2010; Zhou et al., 2010).

Vitrification of animal ovarian tissue

Vitrification procedures have mainly been studied in murine ovarian tissue. Mouse ovaries have a sponge-like structure rich in pores (Candy et al., 1997; Harp et al., 1994), which facilitates CPA permeation, decreasing the CPA exposure period, and increases cooling and warming rates. Probably due to this characteristic, vitrification methods are usually very successful in murine ovarian tissue (**Table 3**). Different authors have reported live births (Bagis et al., 2007; Hani et al., 2006; Kagawa et al., 2007; Migishima et al., 2003, 2006; Wang et al., 2009b) and fertilization of mature oocytes originating from vitrified preantral follicles (Hasegawa et al., 2004; Segino et al., 2003). Studies have revealed that, after vitrification, preantral follicles maintain the same survival rates, morphology and ultrastructure as follicles from fresh ovarian tissue (Haidari et al., 2006, 2007, 2008; Mazoochi et al., 2008; Salehnia et al., 2002). Some studies have also shown vitrification of isolated preantral follicles to be successful, yielding live births (dela Peña et al., 2002) and superior results compared with vitrification of ovarian tissue fragments (Choi et al., 2007b; Lin et al., 2008). Trapphoff et al. (2010) recently demonstrated that vitrification of preantral follicles isolated from mouse ovaries does not affect oocyte development or increase the risk of abnormal imprinting.

All studies on vitrification of sheep ovarian tissue (Baudot et al., 2007; Bordes et al., 2005; Courbiere et al., 2006, 2009b) have been carried out by Salle's team in France, who have extensive experience in cryopreservation and transplantation of ovine ovaries (Demirci et al., 2001, 2002; Massardier et al., 2010; Salle et al., 1998, 1999, 2002). After obtaining live births with vitrification of half ovaries (Bordes

Table 2 Ovarian tissue size, vitrification solution and procedure, evaluation after warming and main results of cryopreservation of ovarian tissue in humans.

Ovarian tissue	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
0.5–1 mm^3	4.2 mol/l DMSO + 0.35 mol/l sucrose + 15 mg/ml BSA	25 µl vitrification solution in straws directly plunged into LN_2	IVC (5–25 days); histological analysis	Vitrification did not affect in-vitro growth of primordial follicles	Zhang et al. (1995)
0.5 × 1 × 4 mm	(1) 40% EG + 0.35 mol/l sucrose + 10% egg yolk → 40% EG + 0.35 mol/l sucrose + 10% egg yolk + 1% Supercool X-1000 (2) 10% EG + 20% DMSO → 20% EG + 20% DMSO → 40% EG + 0.35 mol/l sucrose + 10% egg yolk → 40% EG + 0.35 mol/l sucrose + 10% egg yolk + 1% Supercool X-1000 (3) 40% EG + 18% Ficoll 70 + 0.35 mol/l sucrose → 40% EG + 18% Ficoll 70 + 0.35 mol/l sucrose + 1% Supercool X-1000 (4) 10% GLY + 20% EG → 40% EG + 18% Ficoll 70 + 0.35 mol/l sucrose → 40% EG + 18% Ficoll 70 + 0.35 mol/l sucrose + 1% Supercool X-1000 (5) 10% GLY + 20% EG → 25% GLY + 25% EG + 1% Supercool X-1000 (6) 10% GLY + 20% EG → 25% GLY + 25% EG → 25% GLY + 25% EG + 1% Supercool X-1000	Straws directly plunged into LN_2	IVC (21 days); hormone assay; histological analysis	No follicles were found after IVC	Isachenko et al. (2002)
0.8 mm^3	(1) 40% EG + 0.35 mol/l sucrose + 10% egg yolk extract (2) 40% EG + 18% Ficoll 70 + 0.35 mol/l sucrose (3) 10% EG + 20% DMSO → 20% EG + 20% DMSO	(1) Straws directly plunged into LN_2 (2) Inserted into metallic powder previously cooled in LN_2 (3) Ovarian tissue transferred onto copper grids prior to plunging into LN_2	IVC (21 days); hormone assay	Best results were obtained with vitrification solution (1) with the use of straws or grid for plunging into LN_2	Isachenko et al. (2003)
1 ± 0.5 mm^3	(1) 40% EG + 0.35 mol/l sucrose + 10% egg yolk extract (2) 40% EG + 18% Ficoll 70 + 0.35 mol/l sucrose	(1) Straws directly plunged into LN_2 (2) Inserted into metallic powder previously cooled in LN_2 (3) Inserted into LN_2 vapour prior to plunging into LN_2 (4) Ovarian tissue transferred onto copper grids prior to plunging into LN_2	ROS; apoptosis measurement	Ovarian tissue inserted into LN_2 vapour prior to plunging resulted in significantly higher ROS concentrations and apoptosis	Rahimi et al. (2003)

(*Table 2 continued on next page*)

Table 2 (continued)

Ovarian tissue	CPA	Vitrification method	After warming	Main results	Reference
0.5 × 1 × 4 mm	10% GLY + 20% EG + 15% FBS → 25% GLY + 25% EG + 1% Supercool X-1000 + 15% FBS	Straws directly plunged into LN_2	Xenotransplantation or IVC (6 weeks); necrosis measurement	No difference in necrosis after slow freezing and vitrification	Rahimi et al. (2004)
1 mm^3	(1) 0.64 mol/l EG + 20% FBS → 5.64 mol/l EG + 5% PVP + 0.4 mol/l trehalose + 20% FBS (2) 3.58 mol/l EG + 2.82 mol/l DMSO + 20% FBS	Straws directly plunged into LN_2	Histological analysis	Extensive follicular damage. Vitrification procedures were less efficient than slow freezing. The second vitrification procedure showed better results than the first	Gandolfi et al. (2006)
1 × 1 × 5 mm	50% EG + 10% SSS → 20% DMSO + 20% EG + 10% SSS	Cryovials directly plunged into LN_2	IVC (2 or 6 weeks with or without agitation); histological analysis	Best results after 2 weeks of IVC with agitation	Isachenko et al. (2006)
5 × 1 × 1 mm	2 mol/l DMSO + 0.1 mol/l sucrose + 12% HSA → 2 mol/l DMSO + 2 mol/l PROH + 0.2 mol/l sucrose + 12% HSA	Minimum drop size directly plunged into LN_2	IVC (14 days); histological analysis; hormone assay	Results were similar with both vitrification and slow freezing	Li et al. (2007)
5 × 1 × 1 mm	20% DMSO + 20% EG + 25 mg/ml HSA (gradual dehydration: 25 → 50 → 75 → 100%)	Solid-surface vitrification	IVC (10 days); histological analysis; TUNEL assay; hormone assay	Results were similar between vitrification and slow freezing	Huang et al. (2008)
∼1 mm^3	(1) 1.8 mol/l DMSO + 2.3 mol/l EG + 0.2 mol/l sucrose + 20% SSS (gradual dehydration: 12.5 → 25 → 50 → 100%) (2) 1 mol/l DMSO → 2 mol/l DMSO + 1 mol/l acetamide + 3 mol/l PROH	(1) 1 ml vitrification solution in the cryovials directly plunged into LN_2 (2) 100 μl vitrification solution in the cryovials directly plunged into LN_2	IVC (12 days); histological analysis; hormone assay	Best results were obtained with vitrification solutions (4), (5) and (6) and the 20 μl droplet directly plunged into LN_2	Isachenko et al. (2008)

(*Table 2 continued on next page*)

Table 2 (continued)

Ovarian tissue	CPA	Vitrification method	After warming	Main results	Reference
	(3) 1.36 mol/l GLY + 10% SSS → 1.36 mol/l GLY + 2.7 mol/l EG + 10% SSS → 3.4 mol/l GLY + 4.5 mol/l EG + 10% SSS (4) 2.75 mol/l DMSO + 2.76 mol/l formamide + 1.97 mol/l PROH + SSS (gradual dehydration: 12.5 → 25 → 50 → 100%) (5) 2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l PROH + 0.0075 mol/l PEG + SSS (gradual dehydration: 12.5 → 25 → 50 → 100%) (6) 2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l EG + 0.0075 mol/l PEG + SSS (gradual dehydration: 12.5 → 25 → 50 → 100%)	(3) ~20 μl droplet directly plunged into LN_2			
~1–2.5 mm^2	(1) 10% EG + 10% DMSO + 20% FBS → 20% EG + 20% DMSO (2) 7.5% EG + 7.5% DMSO + 20% FBS → 15% EG + 15% DMSO + 0.5 mol/l sucrose	(1) Minimum drop size directly plunged into LN_2 (2) Needle directly plunged into LN_2	Incubation (20 min); histological analysis; TEM; viability assay	After histology, results were similar with vitrification and slow freezing. However, follicular and stromal ultrastructures were better preserved with the needle method	Wang et al. (2008)
~1 mm^3	2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l EG + 0.0075 mol/l PEG + SSS (gradual dehydration: 12.5 → 25 → 50 → 100%)	~20 μl droplet directly plunged into LN_2	IVC (16 days); histological analysis; hormone assays; molecular evaluation	Results after conventional freezing were better than after vitrification	Isachenko et al. (2009b)
1 × 10 × 10 mm	7.5% EG + 7.5% DMSO + 20% SSS → 20% EG + 20% DMSO + 0.5 mol/l sucrose	Metal strip plunged into LN_2	Viability analysis; histological analysis	No difference in viability of oocytes from fresh and vitrified ovarian tissue	Kagawa et al. (2009)
1 × 1–2 × 5–8 mm	0.35 mol/l DMSO + 0.38 mol/l EG + 0.38 PROH + 10 mg/ml HSA → 0.7 mol/l DMSO + 0.75 mol/l EG + 0.75 PROH + 10 mg/ml HSA → 1.4 mol/l DMSO + 1.5 mol/l EG + 1.5 PROH + 10 mg/ml HSA + 10% PVP	Hand-cut straw	IVC (24 h); histological analysis; TEM	Results were similar with both vitrification and slow freezing for follicles. However, the stroma was better preserved after vitrification than slow freezing	Keros et al. (2009)

(Table 2 continued on next page)

Table 2 (continued)

Ovarian tissue	CPA	Vitrification method	After warming	Main results	Reference
0.5 × 1 × 1 mm	10% GLY + 20% EG → 25% GLY + 25% EG → 25% GLY + 25% EG + 1% Supercool X-1000	Straws directly plunged into LN_2	Xenotransplantation; apoptosis measurement; histological analysis	Vitrified ovarian tissue showed a significantly higher number of apoptotic cells compared with frozen tissue	Rahimi et al. (2009)
1 mm^3	30% EG + 0.5% trehalose + 6% FBS (gradual dehydration: 10 → 50 → 100%)	(1) Straws directly plunged into LN_2 (2) Minimum drop size directly plunged into LN_2 (3) Solid-surface vitrification	Histological analysis	The minimum drop size method resulted in a higher percentage of morphologically normal follicles	Amorim et al. (2011)
0.5 × 1 × 1 mm	2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l PROH + 0.0075 mol/l PEG (gradual dehydration: 12.5 → 25 → 50 → 100%)	~20 μl droplet directly plunged into LN_2	Xenotransplantation or IVC (3 days, 1, 2, 3 and 4 weeks); histological analysis; immunohistochemical assays;	Revascularization of ovarian tissue was similar between slow frozen and vitrified ovarian tissue. The percentage of growing follicles was higher in frozen tissue	Rahimi et al. (2010)
~2–3 mm^2	(1) 7.5% EG + 7.5% DMSO + 20% FBS → 15% EG + 15% DMSO + 0.5 mol/l sucrose (2) 7.5% EG + 7.5% DMSO + 20% FBS → 13.5% EG + 13.5% DMSO + 0.5 mol/l sucrose (3) 7.5% EG + 7.5% DMSO + 20% FBS → 12% EG + 12% DMSO + 0.5 mol/l sucrose (4) 7.5% EG + 7.5% DMSO + 20% FBS → 10.5% EG + 10.5% DMSO + 0.5 mol/l sucrose	Needle directly plunged into LN_2	Histological analysis; TEM; TUNEL; IVC (14 days); tissue damage assessment	No difference in morphology of primordial follicles from frozen and vitrified ovarian tissue, but inferior to fresh control. Vitrified tissue (solutions 2 and 3) yielded a better preservation of stroma cells, lower proportion of apoptotic primordial follicles	Xiao et al. (2010)
1 × 1 × 1 mm	(1) 10% DMSO + 10% EG → 20% DMSO + 20% EG% 0.5 mol/l sucrose (2) 5% DMSO + 5% EG → 10% DMSO + 10% EG (3) 7.5% DMSO + 7.5% EG → 15% DMSO + 15% EG (4) 10% DMSO + 10% EG → 20% DMSO + 20% EG	(1) Minimum drop size directly plunged into LN_2 (CV) or (2–4) Direct cover vitrification (DCV)	Histological analysis; TEM; TUNEL; xenotransplantation (24 days)	DCV showed a higher percentage of normal follicles and lower incidence of apoptotic cells compared with CV	Zhou et al. (2010)

BSA = bovine serum albumin; DMSO = dimethylsulphoxide; EG = ethylene glycol; FBS = fetal bovine serum; GLY = glycerol; HSA = human serum albumin; IVC = in-vitro culture; LN_2 = liquid nitrogen; PEG = polyethylene glycol; PROH = propylene glycol; PVP = polyvinylpyrrolidone; ROS = reactive oxygen species; SSS = synthetic serum substitute; TEM = transmission electron microscopy; TUNEL = terminal dUTP nick-end labelling.

Table 3 Ovarian tissue size, vitrification solution and procedure, evaluation after warming and main results of cryopreservation of ovarian tissue in mice.

Ovarian tissue	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Isolated preantral follicles	2 mol/l EG + 10% FCS → 6 mol/l EG + 0.3 mol/l raffinose + 10% FCS	Straw exposure to LN_2 vapour prior to plunging into LN_2	IVC (10 days); IVM; IVF; ET	Offspring	Dela Peña et al. (2002)
Whole ovary	30% Ficoll 70 + 0.5 mol/l sucrose + 10.7% acetamide + 40% EG + 4 mg/ml BSA	Cryovials directly plunged into LN_2	Unilateral autotransplantation	After 11 days of AT, the stroma contained normal primordial and primary follicles and was devoid of necrotic cells	Salehnia (2002)
Whole ovary	30% Ficoll 70 + 0.5 mol/l sucrose + 10.7% acetamide + 40% EG + 4 mg/ml BSA	Cryovials directly plunged into LN_2	Ultrastructural analysis	The ovaries showed no ultrastructural signs of damage or necrosis	Salehnia et al. (2002)
Whole ovary	1 mol/l DMSO → 2 mol/l DMSO + 1 mol/l acetamide + 3 mol/l PROH	Cryovials directly plunged into LN_2	Orthotopic transplantation; IVF; ET	Offspring, but the number was lower than with transplanted fresh ovaries	Migishima et al. (2003)
Half ovary	10% EG + 27% Ficoll 70 + 0.45 mol/l sucrose → 20% EG + 24% Ficoll 70 + 0.4 mol/l sucrose → 40% EG + 18% Ficoll 70 + 0.3 mol/l sucrose	Cryovial exposure to LN_2 vapour prior to plunging into LN_2	Follicle (100–130 μm) isolation; IVC; IVM; IVF	Fertilization rate in vitrified group was lower than in fresh group (53.5% versus 74.2%)	Segino et al. (2003)
Whole ovary	7.5% EG + 7.5% DMSO + 20% HSA → 15% EG + 15% DMSO + 0.5 mol/l sucrose	Vitrification kit soaked and capped in LN_2	Follicle isolation; IVC (10 days); IVM; IVF	After vitrification, oocytes retained the capacity for fertilization and development to preimplantation embryos	Hasegawa et al. (2004)
Whole ovary	(1) 7.5% EG + 7.5% DMSO + 20% FBS → 15% EG + 15% DMSO + 0.5 mol/l sucrose (2) 40% EG + 30% Ficoll 70 + 0.5 mol/l sucrose + 10.7% acetamide + 20% FBS	(1) Direct cover vitrification (DCV) (2) Straws directly plunged into LN_2	Histological analysis; follicular viability; ultrastructural evaluation; heterotopic transplantation	DCV showed a higher percentage of normal and viable follicles compared with straw vitrification and slow freezing. Similar results were obtained after grafting and pregnancy	Chen et al. (2006)
1 mm^3	1 mol/l DMSO → 2 mol/l DMSO + 1 mol/l acetamide + 3 mol/l PROH	Cryotubes directly plunged into LN_2	Orthotopic transplantation	Offspring	Hani et al. (2006)

(*Table 3 continued on next page*)

Table 3 (*continued*)

Ovarian tissue	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Whole ovary	40% EG + 30% Ficoll 70% + 1 mol/l sucrose	Straw exposition to LN_2 vapour prior to plunging into LN_2	Follicle isolation; IVC (12 days); IVM	Survival rate of fresh and vitrified follicles was similar	Haidari et al. (2006)
Whole ovary	7.5% EG + 7.5% DMSO + 20% SSS → 15% DMSO + 15% EG + 0.5 mol/l sucrose + 20% SSS	Polyester sheets directly plunged into LN_2	Histological analysis; follicle isolation; IVC (6 days); IVM; IVF; ET	Offspring	Hasegawa et al. (2006)
Half ovary	1 mol/l DMSO → 2 mol/l DMSO + 1 mol/l acetamide + 3 mol/l PROH	Cryotubes directly plunged into LN_2	Orthotopic transplantation; histological analysis	Offspring	Migishima et al. (2006)
Whole ovary	10% DMSO + 10% EG + 4 mg/ml HSA → 20% DMSO + 20% EG + 0.5 mol/l sucrose + 4 mg/ml HSA	Cryovials directly plunged into LN_2	Orthotopic transplantation	Offspring	Bagis et al. (2007)
Whole ovary	7.5% EG + 7.5% DMSO + 20% FBS → 15% DMSO + 15% EG + 0.5 mol/l sucrose	Electron microscopic grids directly plunged into LN_2	IVC (5 days); histological analysis; immunohistochemical assays; RT-PCR	Vitrification of ovarian tissue shown to inhibit development of primordial follicles	Choi et al. (2007a)
Isolated preantral follicles and cortex strips (2 × 4 mm)	20% EG + 10% FBS → 40% EG + 18% Ficoll 70 + 0.5 mol/l sucrose + 20% FBS	Electron microscopic grids directly plunged into LN_2	Follicle isolation (for the strips); IVC (10 days)	Follicles were able to develop up to the antral stage with both treatments. However, the survival rate was higher when isolated follicles were vitrified	Choi et al. (2007b)
Whole ovary	40% EG + 30% Ficoll 70 + 1 mol/l sucrose	Straws directly plunged into LN_2	Follicle isolation; IVC (4 days); TEM	Fresh and vitrified follicles had a similar ultrastructure	Haidari et al. (2007)
0.2–0.3 mm cubes	7.5% DMSO + 7.5% EG + 20% SSS → 15% DMSO + 15% EG + 20% SSS	Strips of Cryotop plunged into LN_2	Allotransplantation; IVM; IVF; ICSI; ET	Offspring	Kagawa et al. (2007)
Whole ovary	40% EG + 30% Ficoll 70 + 1 mol/l sucrose + BSA	Straws exposure to LN_2 vapour prior to plunging into LN_2	Follicular viability and morphology; TEM; ovarian apoptosis	Number of viable follicles after vitrification did not differ from fresh follicles. Follicle morphology and ultrastructure were not affected by vitrification	Mazoochi et al. (2008)

(*Table 3 continued on next page*)

Table 3 *(continued)*

Ovarian tissue	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Isolated follicles	(1) 2 mol/l EG $\rightarrow$ 6 mol/l EG + 0.3 mol/l raffinose + 10% FBS (2) 0.15 m raffinose $\rightarrow$ 0.3 m raffinose + 10% FBS $\rightarrow$ 6 mol/l EG + 0.3 mol/l raffinose + 10% FBS	Straws directly plunged into LN_2	IVC (10 days); IVM; histological analysis	Initial exposure to EG (2 mol/l) showed fewer morphological alterations compared with increased raffinose concentrations	Nagano et al. (2007)
$<0.5\ mm^3$	1.8 mol/l EG + 1.4 mol/l DMSO + 10% FBS $\rightarrow$ 3.6 mol/l EG + 2.8 mol/l DMSO + 1 mol/l sucrose + 10% FBS	Cryological solid surface vitrification	Viability analysis; heterotopic autotransplantation (7 days); histochemical analysis	Vitrification appeared to affect follicular growth after grafting compared with conventional freezing procedures	Aerts et al. (2008)
Whole ovary	40% EG + 30% Ficoll 70 + 1 mol/l sucrose	Straws directly plunged into LN_2	Follicle isolation; IVC (12 days); IVM; IVF; embryo IVC; hormone assay; histochemical analysis; TEM; dye exclusion assessment	Survival and development rates of fresh and vitrified follicles were similar	Haidari et al. (2008)
Isolated follicles, ovarian tissue slices and whole ovary	4% EG + 10% FBS $\rightarrow$ 6 mol/l EG + 0.4 mol/l trehalose + 10% FBS	Solid-surface method	Ovary transplantation; follicle isolation from ovarian tissue slices; IVC (12 days); IVM; IVF; ET	Best results were obtained with vitrification of isolated follicles and whole ovaries	Lin et al. (2008)
Whole ovary	(1) 10% EG + 10% DMSO + 20% FBS $\rightarrow$ 20% EG + 20% DMSO (2) 7.5% EG + 7.5% DMSO + 20% FBS $\rightarrow$ 15% EG + 15% DMSO + 0.5 mol/l sucrose	(1) Minimum drop size directly plunged into LN_2 (2) needle directly plunged into LN_2	Incubation (20 min); histological analysis; TEM; viability assay; heterotopic transplantation	After histology, the percentages of normal primary and secondary follicles were higher with the needle method compared with minimum drop size and slow freezing. The same result was observed after grafting	Wang et al. (2008)
Whole ovary	(1) 40% EG + 30% Ficoll 70 + 1 mol/l sucrose + 4 mg/ml BSA (2) 2 mol/l EG $\rightarrow$ 4 mol/l EG (3) 2 mol/l EG $\rightarrow$ 4 mol/l EG $\rightarrow$ 6 mol/l EG (4) 2 mol/l EG $\rightarrow$ 4 mol/l EG $\rightarrow$ 6 mol/l EG $\rightarrow$ 6 mol/l EG	(1) Cryotubes exposure to LN_2 vapour prior to plunging into LN_2 (for VS1) (2) DCV (for VS2–4)	Follicle isolation; viability assay; histological analysis; TEM	Survival rate and normality of primordial and preantral follicles was superior in the vitrification protocol 1 compared with DCV, as well as the follicular ultrastructure	Abedelahi et al. (2009)

(*Table 3 continued on next page*)

Table 3 *(continued)*

Ovarian tissue	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Whole ovary	40% EG + 30% Ficoll 70 (w/v) + 1 mol/l sucrose + FBS (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryovials plunged into LN_2	Allotransplantation (1 to 32 days); histological analysis; apoptosis assessment; viability analysis	Supplementation of melatonin increased follicular survival and development	Hemadi et al. (2009)
1/4 ovary	10% EG + 10% DMSO + 0.5% BSA → 17% EG + 17% DMSO + 0.5% BSA + 0.75 mol/l sucrose	Solid-surface vitrification	Heterotopic allotransplantation; IVM; IVF; ET	Recovery and survival rates of oocytes were similar between fresh, vitrified and frozen tissues, as well as blastocyst formation. Healthy pups were obtained in both vitrified and frozen treatments	Wang et al., 2009b
1 mm^3	(1) 10% EG + 10% DMSO + 15% FBS → 20% EG + 20% DMSO + 0.5% + 15% FBS (2) 10% EG + 10% PROH + 15% FBS → 20% EG + 20% PROH + 0.5% + 15% FBS (3) 10% PROH + 10% DMSO + 15% FBS → 20% PROH + 20% DMSO + 0.5% + 15% FBS	Straws directly plunged into LN_2	Allotransplantation (30 days); hormone assay; histological analysis; viability assay (proliferation assessment)	Follicular development, oestradiol concentrations and follicular density were higher with VS2	Zhang et al. (2009)
Isolated follicles	7.5% DMSO + 7.5% EG + 15% FBS → 15% DMSO + 15% EG + 0.5 mol/l sucrose + 15% FBS	Cryotop	SEM; assessment of DNA integrity; IVC; oocyte measurement; IVM; imprinting study	Cryotop method does not increase risks of abnormal imprinting	Trapphoff et al. (2010)
1 mm^3	(1) 10% EG + 10% PROH + 15% FBS → 20% EG + 20% PROH + 0.2% + 15% FBS (2) 10% EG + 10% PROH + 15% FBS → 20% EG + 20% PROH + 0.4% + 15% FBS (3) 10% EG + 10% PROH + 15% FBS → 20% EG + 20% PROH + 0.8% + 15% FBS (4) 10% EG + 10% PROH + 15% FBS → 20% EG + 20% PROH + 1.6% + 15% FBS	Straws directly plunged into LN_2	Allotransplantation (30 days); hormone assay; histological analysis	Resumption of the oestrous cycle varied according to versus Higher follicular density was observed with VS2 and VS3 were used	Zhang et al. (2010)

AT = autotransplantation; BSA = bovine serum albumin; DMSO = dimethylsulphoxide; EG = ethylene glycol; ET = embryo transfer; FBS = fetal bovine serum; FCS = fetal calf serum; HSA = human serum albumin; ICSI = intracytoplasmic sperm injection; IVC = in-vitro culture; IVM = in-vitro maturation; LN_2 = liquid nitrogen; PROH = propylene glycol; RT-PCR = real-time polymerase chain reaction; SEM = scanning electron microscopy; SSS = synthetic serum substitute; TEM = transmission electron microscopy; VS = vitrification solution.

et al., 2005), they set out to vitrify whole sheep ovaries as a model for humans (Baudot et al., 2007; Courbiere et al., 2006, 2009b) (**Table 4**). Although the results have been unsatisfactory so far, their work helps to elucidate many cryobiological aspects involved in the vitrification of an entire ovary and its further transplantation.

In general, vitrification of ovarian tissue from other animal species has produced promising results. After in-vitro culture, preantral follicles from vitrified monkey ovarian tissue developed similarly to follicles from frozen–thawed ovarian tissue (Yeoman et al., 2005). In dogs (Ishijima et al., 2006), rats (Kagabu and Umezu, 2000; Sugimoto et al., 2000), hamsters, monkeys and rabbits (Kagabu and Umezu, 2000), vitrified follicles were able to develop after xenografting. Autografting of bovine preantral follicles after vitrification of ovarian tissue also showed follicular development, indicating that such a procedure does not appear to affect follicles compared with fresh ovarian tissue (Kagawa et al., 2009). However, when Gandolfi et al. (2006) used a similar vitrification solution to preserve bovine follicles, they reported a high follicular injury rate. The difference in results could be due to the stepwise addition of CPA by Kagawa et al. (2009) or the carrier system used by Gandolfi et al. (2006). As previously mentioned, straws have thick walls and plastic is not a conductive material, which could decrease the cooling rate, negatively affecting follicular survival. **Table 4** summarizes studies published on vitrification of ovarian tissue from different animal species.

Final considerations

As previously stated, vitrification appears to offer a quick, easy and inexpensive means of cryopreserving ovarian tissue that does not require special equipment, and studies on human and different animal species have shown promising results. There is nevertheless room for improvement as most current vitrification protocols have been empirically created or adapted from protocols for embryos and oocytes, which are completely different structurally and functionally. Ovarian preservation currently involves removing a large fragment of the ovary, then cutting it into very small pieces to aid CPA equilibration and cryopreservation, whether by vitrification or slow freezing. This commonly yields more than 50 small cubes or slices of tissue. It may be easier with slow freezing than vitrification due to the precision required for the vitrification procedure (meticulous timing of exposure to vitrification solution and manual plunging into liquid nitrogen). A cryocycle tends to be performed with a few ovarian pieces at a time. Therefore, a large ovarian biopsy may require hours of manual work. In terms of cost, conventional freezing protocols usually employ expensive programmable freezers, but it is possible to carry out controlled cooling using Nalgene's Mr Frosty, a low-cost and simple cooling unit (Martinez-Madrid et al., 2004). However, not all vitrification procedures are low-cost as some commercially available carrier devices, such as the Cryotop, and cooling aids, like the VitMaster, are not exactly inexpensive.

In some respects, slow freezing is better understood than vitrification, having been more widely researched. Indeed, in the case of freezing, the permeation rate of the most common permeable CPA (DMSO, EG, PROH) is known at different temperatures. Many studies have assessed the impact of freezing on follicular development at the molecular, ultrastructural and morphological levels, and successful outcomes, such as fertility restoration and live births, have been reported in humans and animals (Andersen et al., 2008; Baird et al., 2004; Borges et al., 2009; Cecconi et al., 2004; Demeestere et al., 2006, 2007; Donnez et al., 2004, 2010; Eyden et al., 2004; Fabbri et al., 2003; Gook et al., 2003; Imhof et al., 2006; Meirow et al., 2005, 2007; Newton et al., 1998; Paynter et al., 1999; Sánchez-Serrano et al., 2010). All this information is not yet available with regard to vitrification. Many questions remain unanswered concerning, for example, the vitrifiability of most vitrification solutions, the permeation kinetics of different CPA 'cocktails' and whether a given equilibration period is long enough to allow penetration of the entire tissue, but not too long to cause damage due to CPA toxicity. To address these questions, different techniques, such as cryosubstitution (Dahl et al., 2006; Elder et al., 2005; Song et al., 2007), cryo-scanning electron microscopy (Gosden et al., 2010), proton nuclear magnetic resonance (Bateson et al., 1994; Newton et al., 1998; Taylor and Busza, 1992; Wusteman et al., 1995) and high-performance liquid chromatography (Carpenter and Dawson, 1991; Luz et al., 2009; Pinto et al., 2008), can be used. Salle's group have been engaged in important studies with differential scanning calorimetry, a thermoanalytical technique used to analyse the thermal properties of cryopreservation solutions (Courbiere et al., 2006; Lornage et al., 2006). Their unique studies have enabled in-depth analysis of the thermal properties of ovarian tissue impregnated with vitrification solution.

In addition, to progress in this field, it is necessary to apply different approaches and techniques to assess tissue functionality and follicle quality (Isachenko et al., 2009a, 2010). So far, the only reliable endpoints are pregnancy and birth, which may limit studies in humans. However, animals can be used as models for preliminary tests. For instance, cow and sheep ovaries are very similar to those of humans; they are almost the same size and show a similar texture and composition, as well as comparable follicle size and growth patterns (Newton et al., 1999; Oktay et al., 2000). Therefore, further experiments involving grafting of vitrified tissue might yield valuable results, allowing investigation of the impact of vitrification on follicular viability and developmental potential in humans.

The best approach to understand how vitrification can be used to cryopreserve ovarian tissue would probably be to focus on a single protocol, as suggested by Liebermann et al. (2002). After obtaining consistent results, the protocol could be improved step by step, changing one parameter at a time if necessary, adapting it according to tissue requirements and progressing toward a specific protocol for ovarian tissue. This is time-consuming work, but constitutes a realistic approach if vitrification is to replace conventional freezing in the future. Finally, it is also extremely important to investigate possible phenotypic and genotypic responses to high CPA concentrations.

Table 4 Ovarian tissue size, vitrification solution and procedure, evaluation after warming and main results of cryopreservation of ovarian tissue in different animal species.

Species	*Ovarian tissue*	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Rat	Whole ovary	20.5% DMSO + 15.5% acetamide + 10% PROH + 6% PEG 6000 (gradual dehydration: 12.5 → 25 → 50 → 100%)	Glass test tubes directly plunged into LN_2	IVC (4 days)	Number of viable follicles after vitrification and IVC was lower than fresh follicles in IVC	Sugimoto et al. (1996)
Mouse, hamster, rabbit, monkey and rat	Ovaries divided into 4 to 8 fragments	5.125% DMSO + 3.875% acetamide + 2.5% PROH + 1.5% PEG (gradual dehydration: 50 → 100%)	Cryovials directly plunged into LN_2	Transplantation to rats (uterine cavity)	After 7 days of transplantation, healthy follicles, including antral stages, were found in grafts	Kagabu and Umezu (2000)
Rat	Whole ovary	20.5% DMSO + 15.5% acetamide + 10% PROH + 6% PEG 6000 (gradual dehydration: 12.5 → 25 → 50 → 100%)	Glass test tubes directly plunged into LN_2	Autotransplantation	After 84 days of AT, small and antral follicles and CL were found in the grafts	Sugimoto et al. (2000)
Sheep	Half ovary	2.62 mol/l DMSO + 2.60 acetamide + 1.31 mol/l PROH + 0.0075 mol/l PEG (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryovials plunged into LN_2	Orthotopic transplantation; histological analysis; hormone assays	Offspring	Bordes et al. (2005)
Sheep	Whole ovary	2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l PROH + 0.0075 mol/l PEG or 2.75 mol/l DMSO + 2.76 mol/l formamide + 1.97 mol/l PROH	Cryobags plunged into LN_2	Follicle isolation; viability assessment; histological analysis	Number of primordial follicles was lower with the first VS	Courbiere et al. (2005)
Monkey	2 × 2 × 1 mm	10% GLY + 20% FBS → 10% GLY + 20% EG → 25% GLY + 25% EG	Minimum drop size directly plunged into LN_2	IVC (5 days); viability assay	Similar results between vitrification and slow freezing	Yeoman et al. (2005)
Sheep	Whole ovary	2.75 mol/l DMSO + 2.76 mol/l formamide + 1.97 mol/l PROH (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryobags plunged into LN_2	Histological analysis; viability assessment	No difference observed in the percentage of normal follicles between fresh and vitrified ovarian tissue. Ovarian vein after vitrification similar to fresh tissue	Courbiere et al. (2006)

(*Table 4 continued on next page*)

Table 4 (*continued*)

Species	*Ovarian tissue*	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Dog	1 mm^3; 3 mm^3; 5 mm^3	1 mol/l DMSO → 2 mol/l DMSO + 1 mol/l acetamide + 3 mol/l PROH	Cryotubes directly plunged into LN_2	Histological analysis; xenografting; proliferation assay	Ovarian tissue was morphologically normal after vitrification and follicular growth was observed after grafting	Ishijima et al. (2006)
Rabbit and pig	5 mm^3	7.5% EG + 7.5% DMSO + 20% SSS → 15% DMSO + 15% EG + 0.5 mol/l sucrose + 20% SSS	Polyester sheets directly plunged into LN_2	Histological analysis	Follicles showed similar morphology before and after vitrification	Hasegawa et al. (2006)
Pig and cow	1.0 mm^3	(1) 0.64 mol/l EG + 20% FBS → 5.64 mol/l EG + 5% PVP + 0.4 mol/l trehalose + 20% FBS (2) 3.58 mol/l EG + 2.82 mol/l DMSO + 20% FBS	Straws directly plunged into LN_2	Histological analysis	Extensive follicular damage. Vitrification procedures were less efficient than slow freezing. The first vitrification procedure showed better results than the second	Gandolfi et al. (2006)
Sheep	1 $cm^2 \times 1$ mm	2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l PROH + 0.0075 mol/l PEG (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryotubes plunged into LN_2	Autograft; hormone assays; histological analysis	Normal progesterone concentrations. Low density of primordial follicles. Offspring	Lornage et al. (2006)
Sheep	Whole ovary	(1) 2.62 mol/l DMSO + 2.6 mol/l acetamide + 1.31 mol/l PROH + 0.0075 mol/l PEG (gradual dehydration: 12.5 → 25 → 50 → 100%) (2) 2.75 mol/l DMSO + 2.76 mol/l formamide + 1.97 mol/l PROH (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryobags plunged into LN_2	Histological analysis; follicle isolation; viability assay	The percentage of viable follicles in VS1 and VS2 was lower than in fresh tissue. The percentage of normal primordial follicles was higher in VS2 than VS1	Lornage et al. (2006)
Sheep	Whole ovary	2.75 mol/l DMSO + 2.76 mol/l formamide + 1.97 mol/l PROH (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryobags plunged into LN_2	Dye exclusion assessment; histological analysis	61.4% small follicles were viable and 48% primordial follicles were normal after vitrification	Baudot et al. (2007)

(*Table 4 continued on next page*)

Table 4 *(continued)*

Species	*Ovarian tissue*	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Goat	1 mm^3	(1) 20% EG + 0.5 mol/l sucrose + 10% FBS → 40% EG + 0.5 mol/l sucrose + 10% FBS (2) 20% EG + 10% FBS → 40% EG + 10% FBS (3) 20% DMSO + 0.5 mol/l sucrose + 10% FBS → 40% DMSO + 0.5 mol/l sucrose + 10% FBS (4) 20% DMSO + 10% FBS → 40% DMSO + 10% FBS (5) 20% EG + 20% DMSO + 0.5 mol/l sucrose + 10% FBS → 40% EG + 40% DMSO + 0.5 mol/l sucrose + 10% FBS (6) 20% EG + 20% DMSO + 10% FBS → 40% EG + 40% DMSO + 10% FBS (7) 35% EG + 0.5 mol/l sucrose + 10% FBS (8) 35% EG + 10% FBS (9) 35% DMSO + 0.5 mol/l sucrose + 10% FBS (10) 35% DMSO + 10% FBS (11) 35% EG + 35% DMSO + 0.5 mol/l sucrose + 10% FBS (12) 35% EG + 35% DMSO + 10% FBS	(1) Straws directly plunged into LN_2 (for VS1–6) (2) Solid-surface vitrification (for VS7–12)	Histological analysis; viability assay	No difference in the percentage of normal follicles between fresh and vitrified ovarian tissue using VS7 and solid-surface vitrification	Santos et al. (2007)
Sheep	Whole ovary	2.75 mol/l DMSO + 2.76 mol/l formamide + 1.97 mol/l PROH (gradual dehydration: 12.5 → 25 → 50 → 100%)	Cryobags plunged into LN_2	Orthotopic transplantation; hormone assays; histological analysis	Total follicular loss after vitrification and grafting	Courbiere et al., (2009b)
Dasyurid marsupial	Isolated primary follicles	7.5% DMSO + 7.5% EG + 20% FCS → 18% DMSO + 18% EG + 1 mol/l sucrose	Stretched cryostraw exposure to LN_2 vapour prior plunging into LN_2	IVC (2 days); viability testing	Granulosa cells were more affected by vitrification than oocytes. However, they improve after IVC	Czarny et al. (2009)

(*Table 4 continued on next page*)

Table 4 *(continued)*

Species	*Ovarian tissue*	*CPA*	*Vitrification method*	*After warming*	*Main results*	*Reference*
Rat	1 × 2 × 2 mm	7.5% EG + 7.5% DMSO + 20% FBS → 15% DMSO + 15% EG + 0.5 mol/l sucrose + 20% FBS	Ovarian tissue plunged into LN_2	Heterotopic autotransplantation; hormone assay; histological analysis; vaginal cytology	Decrease in the follicular pool, preservation of ovarian and endocrine function after grafting	Deng et al. (2009)
Cow	1 × 10 × 10 mm	7.5% EG + 7.5% DMSO + 20% SSS → 20% EG + 20% DMSO + 0.5 mol/l sucrose	Metal strips plunged into LN_2	Autotransplantation; viability assay; histological analysis; hormone assay	No difference in the viability of oocytes from fresh and vitrified ovarian tissue. No loss of oocytes observed after AT of vitrified ovarian tissue and restoration of oestrous cycles	Kagawa et al. (2009)
Pig	2 × 1 × 0.5 mm	7.5% EG + 7.5% DMSO + 20% FBS → 15% EG + 15% DMSO + 0, 0.25 mol/l or 0.5 mol/l sucrose	Cryotops	Xenografting (60 days); histological analysis	Although follicles from vitrified ovaries can develop after xenografting, the developmental rate was lower than follicles from fresh ovarian tissue	Moniruzzaman et al. (2009)
Cow	Isolated secondary follicles and ovarian tissue	7.5% EG + 7.5% DMSO + 20% FBS → 15% EG + 15% DMSO + 0, 0.25 mol/l or 0.5 mol/l sucrose	Cryotops	Xenografting (4 weeks or 6 months); histological analysis	Isolated vitrified secondary follicles and primordial follicles from vitrified tissue can grow up to the antral stage after xenografting	Bao et al. (2010)
Monkey	10 × 10 × 1 mm	(1) 0.805 mol/l EG + 0.64 mol/l DMSO + 20% SSS → 1.61 mol/l EG + 1.28 DMSO + 20% SSS → 3.22 mol/l EG + 2.56 DMSO + 0.5 mol/l sucrose + 20% SSS (2) 1.61 mol/l EG + 20% SSS → 3.22 mol/l EG + 20% SSS → 5.64 mol/l EG + 5% PVP + 0.5 mol/l sucrose + 20% SSS	Cryosupport	Histological analysis; TEM	Morphology of preantral follicles can be preserved after vitrification using VS2	Hashimoto et al. (2010)

AT = autotransplantation; CL = corpora lutea; DMSO = dimethylsulphoxide; EG = ethylene glycol; FBS = fetal bovine serum; FCS = fetal calf serum; GLY = glycerol; IVC = in-vitro culture; LN_2 = liquid nitrogen; PEG = polyethylene glycol; PROH = propylene glycol; PVP = polyvinylpyrrolidone; SSS = synthetic serum substitute; TEM = transmission electron microscopy; VS = vitrification solution.

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