Review Article

Ewe Ovarian Tissue Vitrification: A Model for the Study of Fertility Preservation in Women

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ABSTRACT

Emergency in vitro fertilization followed by embryo vitrification is one feasible fertility preservation option for cancer patients. However, its clinical application has several limitations. Hormonal stimulation delays the initiation of oncotherapy and it is contraindicated in hormone-sensitive cancers or for use in pre-pubertal females. Vitrification of ovarian cortical tissue prior to the start of cancer treatment could be utilized for autotransplantation or for in vitro maturation of follicles enclosed in ovarian tissue. Nevertheless, the main concern associated with autotransplantation is the risk of malignant cell re-introduction to the patient, which is non-existent with the use of follicular in vitro culture. Since obtaining ovarian tissues from women for research is challenging and experimental studies are difficult to complete due to ethical issues, exploring the alternative usage of animal models for fertility preservation may provide beneficial insight into the prospects of follicular culture as an alternative for fertility restoration following ovarian tissue vitrification. Similarities between ewe and human ovary structures, as well as in ovarian follicular development dynamics, make the ewe a possible animal model for the study of female fertility preservation. As vitrification of ovarian tissue has the potential to cryopreserve preantral ovarian follicles, the present review will describe the progress of ovarian tissue vitrification studies completed in ewes.

Keywords: Vitrification, ewes, preantral follicle, ovary, in vitro culture

INTRODUCTION

In general, the treatment indicated for women diagnosed with a malignant disease is often aggressive, and may include surgeries, such as oophorectomy. Additionally, other women undergo chemotherapy or radiotherapy; however, all unfortunately result in premature menopause and, consequently, infertility (Amorim et al., 2011a). Advances in assisted reproductive techniques (ART) have greatly increased the possibility of fertility preservation in women submitted to gonadotoxic treatments. The main alternatives routinely used for female fertility preservation are protection of the ovaries against radiation (oophoropexy), emergency in vitro fertilization (IVF) and oocyte vitrification. Although oophoropexy may provide some protection to germ cells, it may also considerably reduce the chances of successful future pregnancies (Wallace et al., 2005).

There are also limitations regarding the use of IVF in cancer patients, as the hormone stimulation protocols needed to obtain mature oocytes delay the beginning of cancer treatment. These hormonal treatments are also not recommended to young pre-pubertal patients or to adult women without a partner (Wallace et al., 2005). However, the damage caused to ovarian germ line cells by radio- and/or chemotherapy and the time needed for hormonal therapies would not be inconveniences with the removal and vitrification of ovarian tissue biopsies prior to therapy commencement.

In view of the low availability of ovarian tissue from women and of the ethical and legal aspects involved in these procedures, some animal models, such as sheep, for example, have been extensively utilized in cryopreservation studies (Gosden et al., 1994; Bordes et al., 2005), as well as in in vitro preantral follicular culture (Campbell et al., 2000).

Considering the importance of protecting female fertility, especially in women submitted to gonadotoxic treatments, and the prior usage of sheep as an animal model for humans, this review will: 1) establish a parallel between women and ewes regarding folliculogenesis, and 2) discuss the progress of ovarian tissue vitrification in both species.

Comparison of the main ovarian features between women and ewes

Women's ovaries are almond-shaped, measuring 3 cm in length, approximately 1.5-2 cm in width and are 1 cm thick. Similarly, in the ewe, the ovaries are also the approximate size of an almond (measuring approximately 1.7 cm in length, 1.2 cm in width and 1 cm thick) (Mohammadpour, 2007).

The formation and differentiation of female gametes, or oogenesis, commences in utero, with primordial germ cells (PGC) migrating from the vitelline sac to the primitive gonads (gonadal ridge). In women, PGC form in the vitelline sac sometime after the first month of pregnancy; in the ewe, the colonization of PGC occurs between days 18 and 28 of embryonic development (Lun et al., 1998). These cells undergo multiple mitotic divisions, populating the developing ovarian cortex. In women, these mitotic divisions occur until near the end of the fifth fetal month (Gartner & Hiatt, 2003); in ewes, the process ends at approximately 75 days of embryonic development (Smith et al., 1993). At that time, each ovary contains five to seven million oogonia. About one million oogonia are enveloped by follicular cells and survive until the time of birth. The remaining oogonia do not form ovarian follicles (Gartner & Hiatt, 2003) but instead, they undergo atresia.

The duration of ovarian folliculogenesis (period of

follicular growth from the primordial to the preovulatory stage) takes approximately 175 days in women (Gougeon, 1986; Wallace & Kelsey, 2010) and 170 days in ewes (Cahill & Mauleon, 1980; Bartlewski *et al*., 2011).

The primordial follicles, as well as the oocytes contained within, have similar diameters in both ewes and women, as depicted on Table 1. The oocyte nucleus is relatively large in both species and occupies a central position, clearly showing its nucleolus. In contrast, primary and secondary follicles and their corresponding oocytes are larger in sheep than in women (Table 1).

Granulosa cell numbers increase, as well as the size and protein content of the oocyte, and formation of the basal lamina and zona pellucida layers denote secondary follicle formation (van Den Hurk & Zhao, 2005). As shown on Table 1, ewe secondary follicles and their respective oocytes are larger than those of women. Secondary follicles are also observed in fetal ovaries, when the outer theca cells form from the interstitial stroma (van Den Hurk & Zhao, 2005). In contrast, the inner theca cells are defined after the follicles develop four or more layers of granulosa cells (Lucci *et al*., 2001).

With the continuous growth of secondary follicles and the organization of granulosa cells into various layers, a cavity filled with fluid is formed, denoted as the antrum. From this stage, follicles are termed tertiary, or antral, and their diameters increase considerably due to oocyte growth, the multiplication of granulosa cells and the increase in antral cavity fluid (Mcgee & Hsueh, 2000; Bartlewski *et al*., 2011).

With continuing growth and development, one or two antral follicles can potentially differentiate to become a Graafian, or preovulatory follicle. In response to the preovulatory gonadotropin surge occurring in each reproductive cycle, the Graafian follicle ovulates to release the mature oocyte ready to be potentially fertilized. However, throughout antral development, the majority of follicles undergo degeneration or atresia - the process responsible for the depletion of most of the ovarian follicles present in the ovary (Mcgee & Hsueh, 2000; Balasch *et al*., 2010; Bartlewski *et al*., 2011).

Sheep ovaries as a model for fertility preservation studies in women

Many variables can affect ovarian tissue viability during vitrification, such as the cryoprotectant type or concentration, cell exposure duration, tissue fragment size, cooling device, and speed of cooling. These variable effects must be exhaustively evaluated to maximize current procedures.

As previously mentioned, the detailed study of human female ovarian tissues is not feasible. Therefore, as a general rule, animal models should be biochemically, physiologically and anatomically comparable with human ovarian structures; for instance, ovary size, stroma layer thickness, ovulations per cycle, and estrous cycle (Vandeberg, 2004; Gerritse *et al*., 2008). Although there is no animal model that is a perfect human model, studies completed in bovines, pigs, equines, non-human primates, and ovines have provided tremendous amounts of valuable information that has been utilized in human medicine

With cow ovaries being easily accessible from abattoirs, bovine physiological functions are well documented. (Lavranos *et al*., 1994; Yang & Fortune, 2006; Irving-Rodgers *et al*., 2006). However, bovine ovaries are larger than the ovaries from women and the stromal tissue is much thicker (Gerritse *et al*., 2008).

Swine ovary size (approximately 7.3 cm3; Gerritse *et al*., 2008) is similar to that of women's ovaries (6.5 cm3; Munn *et al*., 1986) but the sow is a poly-ovulatory species (Soede *et al*., 2011). In fact, the sow often ovulates more than ten oocytes per estrus. In addition, the large lipid content within swine oocytes impairs proper vitrification. A detailed study conducted on both cows and sows demonstrated that the particularities in their ovarian structures make them not ideal species to be used as a model for women (Gandolfi *et al*., 2006).

The follicular fluid from human and mare dominant follicle contains greater estradiol and progesterone concentrations and lower androstenedione concentrations when compared to subordinate follicles. Similarities in follicular wave development in both species, as well as other features, have provided reasons for utilizing the mare as an additional model for studying ovarian follicular development in women (Schneyer *et al*., 2000; Donadeu & Ginther, 2002; Baerwald *et al*., 2012).

Non-human primates, like Rhesus macaques, have menstrual cycles that are analogous to women in duration and both steroid and protein hormone profiles. Anatomical similarities also exist when comparing oocytes between humans and non-human primates. Yet, the monkey mod-

Table 1. Folliculogenesis features in sheep and women			
	Sheep	**Women**	**References**
Primordial follicles	40.8 (28.1-60.5) μm	35.4 (29.2-41.6) μm	Lundy *et al*., 1999; Gougeon & Chainy, 1987
Oocytes from primordial follicles	34.6 (22.8-52.3) μm	32.1 (26.1-38.1) μm	Lundy *et al*., 1999; Gougeon & Chainy, 1987
Primary follicles	75.2 (49.7-118.2) μm	46.0 (39.8-52.2) μm	Lundy *et al*., 1999; Gougeon & Chainy, 1987
Oocytes from primary follicles	52.1 (31.0-80.0) μm	32.6 (27.7-37.5) μm	Lundy *et al*., 1999; Gougeon & Chainy, 1987
Secondary follicles	194.1 (164.2-256.3) μm	77.2 (66.3-88.1) μm	Lundy *et al*., 1999; Gougeon & Chainy, 1987
Oocytes from secondary follicles	72.9 (40.6-92.0) μm	47.8 (35.8-59.8) μm	Lundy *et al*., 1999; Gougeon & Chainy, 1987
Ovaries length, width and thickness	1.7 x 1.2 x 1 cm	3 x 1.5-2 x 1 cm	Mohammadpour, 2007; Gartner; Hiatt, 2003
Folliculogenesis duration	Approximately 170 days	More than 200 days	Cahill & Mauleon, 1980; Gougeon, 1986

el also has limits that must be considered. For example, a limited number of laboratories work with monkeys and there are ethical restrictions over the use of such highly sentient animals in biomedical research (Wolf *et al*., 2008).

However, ewe ovarian cortex has structure that is similar to ovaries in women (Munn *et al*., 1986, Gerritse *et al*., 2008). Sheep, like women, have an ovarian stroma rich in collagen, mainly located around the primordial follicles. These follicles are generally found in large clusters that represent the follicular reserve pool (Arav *et al*., 2005). It should be emphasized that the cryopreservation of either cortex pieces or the entire ewe ovary is being closely studied because of the extensive knowledge about their ovarian physiology. In addition, ewe ovarian endocrine function after auto-transplantation has been studied for more than 40 years (Goding *et al*., 1967). Because of the ovarian similarities between this species and women, the first publication of a human birth after cryopreserved ovarian tissue transplantation (Donnez *et al*., 2004) was based on a protocol developed and tested using sheep as a model (Gosden *et al*., 1994). However, it is still necessary to conduct further comparative studies of human and ewe ovaries as they are not identical and have subtle physiological differences.

Ovarian tissue cryopreservation

Hundreds of immature oocytes inside preantral follicles are located in the ovarian cortex and can be cryopreserved in situ, i.e., enclosed within the ovarian tissue. Immature oocytes within preantral follicles are normally more resistant to cryopreservation than mature oocytes. This is due to multiple factors such as the small size of the oocyte, low metabolic rate, cell cycle stage (quiescent or in prophase I), as well as fewer supporting cells, cortical granules, and the smaller quantity of intracytoplasmic lipids (Shaw *et al*., 2000; Kagawa *et al*., 2009).

The advantages of ovarian cortex cryopreservation, in addition to the large number of follicles, is that the material can be obtained regardless of the age or phase of the menstrual or estrous cycle. Moreover, this process involves fewer ethical and social questions than the cryopreservation of embryos, especially when applied to the human species (Shaw *et al*., 2000). This characteristic is extremely pertinent for human assisted reproduction, especially for women who must start cancer treatment immediately. Ovarian tissue cryopreservation, as mentioned early, is also an alternative for girls who have not yet reached puberty or women who do not have a partner for the donation of male gametes (Wallace *et al*., 2005). Preantral follicle oocytes are more resistant to cryopreservation in comparison to mature oocytes (Shaw *et al*., 2000; Kagawa *et al*., 2009). In fact, the transplantation of frozen-thawed ovarian tissue or the isolation of their enclosed preantral follicles for further in vitro maturation represent a promising alternative to female fertility restoration, specifically in women that require immediate therapy or to pre-pubertal girls (Woodruff *et al*., 2009).

The transplantation of frozen-thawed tissue has been successfully utilized, with reports of 30 human births (Donnez & Dolmans, 2013). The in vitro culture of ovarian follicles may be more viable since it eliminates the possibility of reintroduction of cancer cells back into the patient (Donnez *et al*., 2011). Although slow freezing has been successfully utilized for several years, the technique has some disadvantages compared to vitrification (Amorim *et al*., 2011a). The major disadvantage is ice formation during slow freezing, which results in cell structure damage. Since vitrification uses rapid cooling that results in solidification without crystallization, ice formation is prevented (Bagchi *et al*., 2008). Vitrification of ovarian tissue has been investigated in a variety of species by using several protocols,

as well as different tissue sizes (fragments, hemi-ovaries or even whole ovaries).

Tissue cryopreservation is substantially different from the cryopreservation of cells in suspension, such as isolated preantral follicles. Several cell types within a tissue contribute to its complex final physiological function and the survival of each cell type after cryopreservation is critical. Ovarian stromal tissue is very rich in regards to both cell number and variety. Accordingly, extra- and intracellular ice formation can easily damage these cells, hindering cellular communication needed for the resumption of appropriate tissue function after cryopreservation (Amorim *et al*., 2011a).

An alternative to reduce injuries caused by the ice formation would be the cryopreservation of preantral follicles isolated from ovarian tissue, since this system utilizes follicles that are removed from surrounding tissue (Amorim *et al*., 2011a). When the follicles are cryopreserved separately from ovarian tissue, they have the advantage of facing no risks of damage caused by ischemia and revascularization and greater facility of cryoprotectant perfusion due to the absence of tissue barriers. This technique also allows for the possibility of individual follicle monitoring during the procedures of in vitro culture (Amorim *et al*., 2011a). Vanacker *et al*. (2013) evaluated the survival and growth potential of human preantral follicles isolated before and after cryopreservation. Researchers reported that human preantral follicles can be successfully cryopreserved prior to or after isolation without impairing their ability to survive and grow in vitro. Since the preservation of preantral follicles enclosed in ovarian tissue via the vitrification method have resulted in positive outcomes, (Bordes *et al*., 2005; Lornage *et al*., 2006; Wang *et al*., 2011) we will describe here reports concerning ewe and human ovarian tissue cryopreserved by vitrification.

Advances in ovarian tissue vitrification in sheep

One of the first studies with sheep ovarian vitrified tissue utilized a vitrification solution originally tested in cow oocytes; a solution consisting of ethylene glycol (EG), fetal bovine serum (FBS), polyvinylpyrrolidone and trehalose. This solution enabled the vitrification of immature oocytes in ovarian tissue, as well as their retrieval and development during in vitro maturation up to the second meiotic division. In this study, the percentage of oocytes matured in vitro after vitrification was similar to non-cryopreserved oocytes (Al-aghbari & Menino, 2002).

Courbiere *et al*. (2005) demonstrated for the first time the possibility to maintain primordial follicle viability and morphology after sheep whole ovary vitrification, a fact that had been only demonstrated before in mice. In that study, two different vitrification solutions (VS) were used. One called VS1 and the other VS4, consisting of dimethylsulfoxide (DMSO), acetamide, polyethylene glycol, and propanodiol (PROH), or DMSO, formamide and PROH, respectively. VS1 was described by Rall & Fahy (1985) and utilized in murine embryos, while VS4 was first used for rabbit kidney vitrification (Kheirabadi & Fahy, 2000).

Ewe research on sheep progressed considerably with the publication of two successful reports, both after an orthotopic autotransplant of vitrified ovarian tissue in VS1, a solution previously described by Rall & Fahy in 1985 (Bordes *et al*., 2005; Lornage *et al*., 2006) (Table 2). The first of the two cited studies used six animals in which the cortical tissues were vitrified and later transplanted. Endocrine ovarian function resumption was detected in all animals four months after the transplant, with three of these ewes successfully producing offspring after natural mating (Bordes *et al*., 2005). In the second study, the orthotopic autotransplant of vitrified ovarian fragments resulted in three pregnancies with

Table 2. Advances in sheep ovarian tissue vitrification

Reference	Tissue	Vitrification device	Vitrification solution	Main outcomes
Al-aghbari; Menino, 2002.	Ovarian fragments (0.5cm x 0.5 cm)	Tissue dropped on the surface of a steel cube cooled by LN_2	35% EG, 5% polyvinylpyrrolidone, 0.4 mol/L trehalose and 20% FBS	Maintenance of oocyte recovery rate and percentage of oocytes developing to metaphase
Bordes et al., 2005.	Ovarian fragments (1 mm x 2 cm x 1 cm)	Cryogenic vials plunged directly into LN_2	2.62 mol/L DMSO, 2.60 mol/L acetamide, 1.31 mol/L 1.2 PROH, and 0.0075 mol/L PEG (gradual dehydration in: 12.5%, 25%, 50% and 100% of vitrification solution)	Pregnancies occurred after ovarian cortex autotransplantation, 4 lambs were born
Courbiere et al., 2005.	Whole ovaries with vascular pedicle	Samples plunged directly into LN_2	2.75 mol/L DMSO, 2.76 mol/L formamide, and 1.97 mol/L PROH (gradual dehydration in: 12.5%, 25%, 50% and 100% of vitrification solution)	Maintaining primordial follicle viability comparable to the fresh tissue
Courbiere et al., 2006.	Whole ovaries	Ethyl vinyl acetate cryobag plunged into LN_2	2.75 mol/L DMSO, 2.76 mol/L formamide, and 1.97 mol/L PROH (gradual dehydration in: 12.5%, 25%, 50% an 100% of vitrification solution)	Maintaining the aspect of the ovarian vein identical to the nonvitrified control
Lornage et al., 2006.	Ovarian fragments (1 mm thick and 1 cm² surface)	Cryotube plunged into LN_2	2.62 mol/L DMSO, 2.60 mol/L acetamide, 1,31 mol/L 1.2 PROH, and 0.0075 mol/L PEG	Pregnancies occurred after autotransplantation of vitrified warmed ovarian cortex and lambs were born
Baudot et al., 2007.	Whole ovary with blood vessels.	Ethyl vinyl acetate cryobag plunged into LN_2	2.7 5 mol/L DMSO, 2.76 mol/L formamide and 1.97 mol/L PROH (gradual dehydration in: 12.5%, 25%, 50% and 100% of vitrification solution)	Maintaining ovarian primordial follicle density and follicle membrane integrity similar to the fresh tissue
Courbiere et al., 2009.	Whole ovary with blood vessels	Ethyl vinyl acetate cryobag plunged into LN_2	2.75 mol/L DMSO, 2.76 mol/L formamide, and 1.97 mol/L PROH (gradual dehydration in: 12.5%, 25%, 50% and 100% of vitrification solution)	One sheep recovered ovarian endocrine function 6 months after transplantation
Fathi et al., 2011.	Ovarian fragments (1 x 2 x 2 mm³)	Cryopin (sample adhered in a needle) plunged into LN_2	60% HTCM, 15% EG, 15% DMSO, 0.25 mol/L SUC and 10% HSA	Maintaining intact antral follicles using 2-step dehydration protocol
Melo et al., 2011.	Ovarian fragments (1 mm³)	Solid-surface plunged into LN_2	40% EG, 0.5 mol/L SUC and 50 µg/mL AA	Maintaining follicular viability rates similar to the fresh tissue after 5 days of tissue culture
Lunardi et al., 2012.	Ovarian fragments (3 x 3 x 1: 9 mm³)	Macrotube plunged into LN_2	6 mol/L EG, 0.25 mol/L SUC and 10% FCS	Maintaining follicular viability similar to the cultured non-vitrified tissue after 2 days of tissue culture
Torre et al., 2012.	Whole ovaries	Ethyl vinyl acetate cryobags plunged into LN_2	2.75 mol/L DMSO, 2.76 mol/L formamide and 1.97 mol/L PROH (gradual dehydration in: 12.5%, 25%, 50% and 100% of vitrification solution)	Significant reduction in ovarian pedicle metabolism after vitrification

Note: LN_2 = Liquid nitrogen, EG = Ethylene glycol, FBS = Fetal bovine serum, DMSO = Dimethylsulfoxyde, PROH =: Propylene glycol, PEG = Polyethylene glycol, HTCM = HEPES tissue culture medium, SUC = Sucrose, HSA = Human serum albumin, AA = Ascorbic acid, FCS = Fetal calf serum.

the production of four lambs (Lornage *et al*., 2006).

Several other studies were conducted on the vitrification of sheep whole ovaries (Courbiere *et al*., 2006; 2009). Baudot *et al*. (2007) tested the VS4 solution previously described by Kheirabadi & Fahy (2000) for whole ovary vitrification and obtained reasonable rates of follicular viability (61.4 ± 2.2%) and normal morphology (48 ± 3.8%). Also in 2007, Courbiere *et al*. demonstrated that the reestablishment of hormone production was also maintained after the transplant of whole vitrified ovaries.

Fathi *et al*. (2011) compared vitrification techniques using two or four solutions with increasing EG and DMSO concentrations. Morphological evaluation was performed to quantify follicles in different developmental phases (primordial, primary, secondary, and antral) and differences in survival rates were observed among the different follicular classes. In general, vitrification performed with only two solutions yielded positive results with a lower incidence of cell death, as evaluated by the TUNEL technique. The vitrification technique used was called "cryopin" since ovarian fragments are picked up with an insulin needle, immersed in liquid nitrogen and then stored in cryotubes.

Although several studies have been conducted to develop a cryoprotectant, as well as a satisfactory vitrification technique (Fathi *et al*., 2011; Lunardi *et al*., 2012), until now, there has been no consensus regarding the best solution or even vitrification technique for sheep ovarian tissue. The few births reported in the literature thus far originated from ovarian tissue vitrified in the VS1 solution with direct immersion of ovarian tissue in liquid nitrogen, which was stored in cryotubes (Bordes *et al*., 2005; Lornage *et al*., 2006).

Advances in the in vitro culture of vitrified sheep ovarian tissue

Although the combination of vitrification and in vitro culture has resulted in the birth of mice (Hasegawa *et al*., 2006; Wang *et al*., 2011), there is no similar success documented in sheep.

The few studies conducted so far have demonstrated that the addition of antioxidant agents like ascorbic acid, considerably improves tissue viability after in vitro culture, both in the culture medium and in the vitrification solution. This may be due to their role in assisting collagen biosynthesis. It was also demonstrated that the vitrification solution containing EG associated with ascorbic acid promoted better results than the association with DMSO (Melo *et al*., 2011). The viability of ovarian vitrified tissue analyzed after 48 h of in vitro culture was similar to the fresh control with the use of a vitrification solution containing EG (6 M), fetal calf serum (FCS) (10%) and sucrose (0.25 M) (Lunardi *et al*., 2012).

Advances in ovarian tissue vitrification in women

Pioneering studies using vitrification methods were conducted on fetal ovaries obtained from elective abortions in China (Zhang *et al*., 1995) (Table 3). Despite relevant results obtained in this study, only years later other researchers performed vitrification in the women ovarian tissue.

After the vitrification of women's ovaries, ovarian follicles and the stromal cells were better morphologically preserved than in the slow-freezing group (Chang *et al*., 2011).

Rahimi *et al*. (2003) evaluated different vitrification protocols and reported that women's ovarian tissues that were cooled rapidly did not have statistically increased apoptosis compared with fresh controls. A later study demonstrated that both oocyte viability and granulosa cell proliferation were also maintained after vitrification (Kagawa *et al*., 2009). Although increased success has been ob-

tained using vitrification as a viable method for cryopreserving ovarian tissue, studies have shown that follicular apoptosis (Zhou *et al*., 2010) and disrupted morphology (Xiao *et al*., 2010, Amorim *et al*., 2011b) are still higher than those found in fresh tissue.

Ovary vitrification in women, combined with xenotransplantation is an ample field for research, but few studies have investigated xenografting after warmed, post-vitrified human ovarian tissue (Rahimi *et al*., 2004; Rahimi *et al*., 2009; Rahimi *et al*., 2010; Amorim *et al*., 2012). Rahimi *et al*. (2004) observed no increase in necrotic area proportion in the human vitrified-thawed ovarian tissue after 42 days of subcutaneous xenotransplantation in severe combined immunodeficiency (SCID) mice compared to fresh or slow human cryopreserved ovarian tissue. In contrast, the same team later reported that xenografts of vitrified-thawed human ovarian tissue in SCID mice after 30 days had a significantly greater amount of apoptotic cells, when compared to slow frozen (Rahimi *et al*., 2009).

Angiogenesis, the development of new blood vessels from preexisting ones, is delayed after tissue transplantation, triggering ischemia and hypoxia, which results in massive follicular loss until vascularization is reestablished. After human ovarian tissues were vitrified, thawed, and xenotransplanted for 30 days in SCID mice, the tissues were observed to have vascularization similar to tissues undergoing a similar process, but had been previously frozen (Rahimi *et al*., 2010). Amorim *et al*. (2012) observed a lower percentage of DNA damage in follicles within vitrified-thawed human ovarian tissue after 7 days of xenotransplantation in SCID mice. Since, in addition to these data, a single birth after ovarian vitrification was reported in women (Kawamura *et al*., 2013), further research should be directed to this area to find an ideal protocol to vitrify ovarian tissue from women.

Advances in the in vitro culture of vitrified women ovarian tissue

Since the beginning of the last decade, several studies have shown that the association of vitrification and in vitro culture can be successfully applied to human ovarian tissue, promoting stroma (Keros *et al*., 2009) and follicle morphology (Lee *et al*., 2000) preservation.

Follicle morphology has been evaluated after vitrified-thawed ovarian tissue in vitro culture for short or long periods (between one and 21 days) (Salehnia *et al*., 2012; Isachenko *et al*., 2003) and has been reported that it is possible to preserve morphology similar to that of fresh tissue (Salehnia *et al*., 2012; Isachenko *et al*., 2008; Lee *et al*., 2000; Isachenko *et al*., 2003) similar to slow freezing tissue (Huang *et al*., 2008), or even better than slow freezing tissue (Keros *et al*., 2009).

Li *et al*. (2007) found no significant differences in the proportion of morphologically normal primordial and primary follicles after in vitro culture (14 days). Ultra-strutural chromatin features of oocytes and follicular cells were normal after vitrified-thawed ovarian tissue and in vitro culture for one day, which was similar to the fresh tissue controls (Salehnia *et al*., 2012). These authors also demonstrated that the use of a vitrification solution based on the content of EG or a mixture of EG and DMSO did not affect primordial or primary follicle morphology after one day of tissue culture.

Follicle viability has been preserved so that it is similar to that of fresh tissue after vitrified-thawed ovarian tissue and in vitro culture for 14 days (Lee *et al*., 2000). Isachenko *et al*. (2006) showed that the in vitro culture of vitrified tissue in large volumes of culture medium (30 ml) under constant shaking guaranteed an increase in follicular viability. These results suggest that the combination of these procedures favors the maintenance of ovarian function.

Table 3. Advances in women ovarian tissue vitrification

Reference	Tissue	Vitrification device	Vitrification solution	Main outcomes
Zhang et al., 1995.	Fragments of fetal ovary (0.5 – 1 mm³)	Plastic cryo straws directly plunged into LN₂	4.2 mol/L DMSO, 0.35 mol/L SUC and 15 mg/ml BSA	Maintaining oocyte quality similar to the fresh tissue after 40 days of tissue culture
Isachenko et al., 2003.	Ovarian fragments (0.8 mm x 0.8 mm x 0.8 mm)	Straws or grids directly plunged into LN₂	40% EG, 0.35 mol/L SUC and 5% egg yolk extract	Maintaining the proportion of morphologically normal follicles similar to the fresh tissue
Rahimi et al., 2003.	Ovarian fragments (1 ± 0.5 mm³)	Straws, grids or metal filings directly plunged into LN2 or into nitrogen vapor	40% EG, 0.35 mol/L SUC and 10% egg yolk extract or 40% EG, 18% Ficoll and 0.35 mol/L SUC	Cooling using nitrogen vapor resulted in significantly elevated ROS levels and apoptosis after warming
Rahimi et al., 2004.	Ovarian fragments (0.5 x 1.0 x 4.0 mm)	Straws directly plunged into LN₂	25% Glycerol, 25% EG, 15% FCS and 1% Supercool X-100 (last solution from three steps)	No rise in the proportion of necrotic areas after 42 days of xenotransplantation compared to fresh or slow cryopreserved tissue
Gandolfi et al., 2006.	Ovarian fragments (1 mm³)	Straws directly plunged into LN₂	3.58 mol/L EG, 2.82 mol/L DMSO and 20% FCS	The addition of DMSO to the vitrification solution reduced primary follicles cryoinjuries
Isachenko et al., 2006.	Ovarian fragments (1 x 1 x 5 mm)	Cryovials directly plunged into LN₂	20% DMSO, 40% EG and 10% SSS (last solution from two steps)	Morphologically normal follicles were observed when vitrified ovarian tissue was cultured for 14 days in a large volume of culture medium in combination with stirring
Li et al., 2007.	Ovarian fragments (5 x 1 x 1 mm)	Minimum drop size directly plunged into LN₂	2 mol/L DMSO, 2 mol/L PROH, 0.2 mol/L SUC and 12% HSA	Maintaining estradiol and progesterone production during 14 days of tissue culture similar to the slow-frozen tissue
Huang et al., 2008.	Ovarian fragments (5 x 1 x 1 mm)	Solid-surface vitrification	20% DMSO, 20% EG, 25 mg/ml HSA (gradual dehydration in: 25%, 50%, 75% and 100% of vitrification solution)	Maintaining the percentage of intact primordial follicles and secretion of estradiol and progesterone within 10 days of tissue culture similar to the slow-frozen tissue
Isachenko et al., 2008.	Ovarian fragments (about 1 mm³)	Droplet directly plunged into LN₂	2.62 mol/L DMSO, 2.6 mol/L acetamide, 1.31 mol/L PROH and 0.0075 mol/L PEG (gradual dehydration in: 25%, 50%, 75% and 100% of vitrification solution)	Maintaining normally developed follicles similar to the fresh tissue after 12 days of tissue culture
Wang et al., 2008.	Ovarian fragments (~ 1–2.5 mm²)	Needle directly plunged into LN₂	15% EG, 15% DMSO and 0.5 mol/L SUC	Maintaining the ultrastructure of stromal cells better than the slow-freezing or the dropping vitrification group
Kagawa et al., 2009.	Ovarian fragments (1 x 10 x 10 mm)	Metal strip plunged into LN₂ (Cryotissue)	20% EG, 20% DMSO, 0.5 mol/l SUC	Maintenance of oocyte viability similar to the fresh tissue
Keros et al., 2009.	Ovarian fragments (1 x 1-2 x 5-8 mm)	Cryo straws (Hand-cut straw)	1.4 mol/L DMSO, 1.5 mol/L EG, 1.5 PROH, 10 mg/mL HSA, and 10% PVP (last solution from three steps)	Preservation of ovarian stroma morphology after 1 day of tissue culture better than slow freezing procedure

Rahimi *et al.*, 2010.	Ovarian fragments (~ 0.5 x 1 x 1 mm)	Droplet directly plunged into LN$_2$	2.62 mol/L DMSO, 2.6 mol/L acetamide, 1.31 mol/L PROH and 0.0075 mol/L PEG (gradual dehydration in: 25%, 50%, 75% and 100% of solution)	Maintaining the revascularization of ovarian tissue similar to the frozen tissue after 30 days of xenotransplantation
Xiao *et al.*, 2010.	Ovarian fragments (~ 2-3 mm²)	Needle directly plunged into LN$_2$	2.15 mol/L EG, 1.69 mol/L DMSO and 0.5 mol/L SUC (last solution from two steps)	The use of needle immersed vitrification method enabled the use of lower cryoprotectant concentration leading to improvements in tissue cryopreservation
Zhou *et al.*, 2010.	Ovarian fragments (1 x 1 x 1mm)	Direct cover vitrification (DCV) or conventional vitrification (CV)	15% EG and 15% DMSO or 20% EG and 20% DMSO	DCV showed a higher percentage of normal follicles and promoted less apoptotic cells compared with CV
Amorim *et al.*, 2011b.	Ovarian fragments (1 mm³)	Droplet directly plunged into LN$_2$	38% EG, 0.5 mol/L trehalose, 6% FBS in MEM-GlutaMAX at 10%	Maintaining a high percentage of normal follicles than solid-surface vitrification
Salehnia *et al.*, 2012.	Ovarian fragments (1–1.5 mm³)	Cryovials or cryovials pre-cooled to 0°C put in nitrogen vapour for 30 s and then immersed LN$_2$	40% EG, 30% Ficoll, 1 mol/l SUC and 1.2% BSA or increasing concentrations (2.5%, 5% and 10%) of DMSO, PROH and EG with 10% HSA	Maintenance of proportions of normal follicles, DNA fragmentation and ultrastructural characteristics similar to the fresh tissue after 1 day of ovarian cortex culture
Oktem *et al.*, 2011.	Ovarian fragments (0.25 cm)	Tissue loaded into vials and immersed in LN2	15% PROH , 15% EG, 0.2 mol/L SUC and 10% HSA	Maintaining estradiol production similar to the slow-frozen tissue after 3 days of tissue culture
Amorim *et al.*, 2012.	Ovarian fragments (1.0 x 1.0 x 1.0 mm)	Solid-surface vitrification or open cryo straws	20% DMSO, 20% EG and 25 mg/mL HSA or 10% DMSO, 26% EG, 2.5% PVP, 20 mg/mL HSA and 1 mol/L SUC	Percentage of follicles with DNA damage lower than in the slow-frozen tissue after 7 days of xenotransplantation
Kawamura *et al.*, 2013.				Pregnancy and birth

Note: LN$_2$ = liquid nitrogen, DMSO = Dimethylsulfoxide, SUC = Sucrose, BSA = Bovine serum albumin, EG = Ethylene glycol, ROS = Reactive oxygen species, FCS = Fetal calf serum, PVP = Polyvinylpyrrolidone, FBS = Fetal bovine serum, PROH = Propanediol, SSS = Serum substitute supplement, HSA = Human serum album

Estradiol and progesterone concentrations detected in the culture medium of vitrified-thawed ovarian tissue were similar to those found in slow-freeze preserved tissues after 10 (Huang *et al.*, 2008) and 14 (Li *et al.*, 2007) days of in vitro culture. Estradiol concentrations from slow-frozen and vitrified ovaries were similar (Oktem *et al.*, 2011) after three days of in vitro culture, and estradiol concentrations from vitrified ovaries were similar to those from fresh tissue after 21 days of in vitro culture (Isachenko *et al.*, 2003).

Other characteristics have also been evaluated in in vitro cultured ovarian tissues after vitrification such as apoptosis, DNA fragmentation (Salehnia *et al.*, 2012) and oocyte maturation (Zhang *et al.*, 1995). Apoptosis was similar when compared to slow freezing tissue after ten days of in vitro culture (Huang *et al.*, 2008). DNA fragmentation and oocyte maturation were similar to those reported from fresh tissue after one and 40 days of in vitro culture, respectively (Zhang *et al.*, 1995).

Perspectives of the in vitro culture of isolated early stage ovarian follicles to produce mature oocytes for embryo production

As discussed earlier, a marked follicular loss occurs naturally in vivo. Thus, oocyte availability is a limiting fac-

tor for this new reproductive technique development and expansion. The current methods for in vitro embryo production depends on a scarce supply of competent oocytes from large or preovulatory antral follicles, which are present in reduced numbers in the ovary (Telfer, 1998). Therefore, the use of preantral follicles, which represent 95% of the entire follicular reserve in the mammalian ovary, is an alternative to these limitations. In this regard, the development of an in vitro system that maximizes the oocyte potential of preantral follicles should be considered.

The isolation, cryopreservation and complete in vitro culture of preantral ovarian follicles may yield mature oocytes that can be utilized for further ART techniques, such as IVF and cloning, resulting in a greater number of in vitro produced embryos (Figueiredo *et al.*, 2008).

In veterinary medicine, the main objective of follicular culture is to increase the productivity of high genetic value animals and to enable the preservation of species threatened with extinction. In addition, the technique represents an excellent alternative for the encouragement and support of research related to the pharmaceutical industry by means of toxicological assays of the effect of substances on the reproductive function of females (Cortvrindt & Smitz, 2002).

In human medicine, follicular culture can be relevant

to clinical reproduction, since it enables the development of alternative strategies for the reestablishment of fertility in women at risk of premature ovarian failure, especially those submitted to cancer treatment. In this respect, cryopreservation associated with ovarian follicle culture in vitro can represent an excellent strategy for the reversal or reduction of the impact on follicular loss. A large stock of preantral ovarian follicles can be cryopreserved and maintained at a low temperature for long periods of time before they become atretic or degenerated. These follicles could be later thawed and cultured in vitro to obtain mature oocytes, thus guaranteeing reproductive function in women.

Until today, few studies using in vitro culture of isolated preantral follicles have been performed in human models (Xu *et al.*, 2009a; Vanacker *et al.*, 2013). The success of this technique has been acquired in the mouse model with the achievement of healthy offspring (Xu *et al.*, 2006). Promising results have also been shown in other animals such as sheep (Luz *et al.*, 2012; Luz *et al.*, 2013) and non-human primates (Zelinski *et al.*, 2008; Xu *et al.*, 2009b), but further studies are required in order to achieve increased mature oocytes for embryo production.

CONCLUSIONS AND PERSPECTIVES

Ovarian tissue vitrification has proved to be a valuable tool for female fertility preservation. The in vitro tissue culture method might be strategically used to restore reproductive capacity, especially in women submitted to cancer treatment. Although promising results regarding the combination of vitrification and in vitro culture of sheep or human ovarian follicles have been reported in the literature, further studies are needed to obtain viable embryos produced in vitro. Within this context, in view of the difficult execution of experiments with humans due to the limited material recovered from human reproduction clinics, there is a clear need to identify the most appropriate animal to be utilized as a model for human research.

Based on the reports described in the present review, sheep are believed to be a valid model for these studies proposal. It can be used as a model not only to improve the vitrification protocols, but also to achieve success in the production of embryos from immature oocytes grown in vitro.

CONFLICT OF INTERESTS
No conflict of interest have been declared.

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