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Restoring fertility after ovarian tissue cryopreservation: a half century of research

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Summary

Tissue transplantation and *in vitro* ovarian follicle culture have been investigated as alternative techniques to restore fertility in young women who are facing fertility-threatening diseases or treatments following ovarian tissue cryopreservation. Although transplants of fresh or frozen ovarian tissue have successfully yielded healthy live births in different species including humans, the risks of reintroducing cancer cells back into the patient, post treatment, have limited its clinical purpose. The *in vitro* ovarian follicle culture minimizes these risks and provides a way to harvest more mature oocytes, however its clinical translation has yet to be determined. Not only is it possible for tissue cryopreservation to safeguard fertility in cancer patients, this technique also allows the maintenance of germplasm banks for animals of high commercial value or for those animals that are at risk of extinction. Given the importance of managing female genetic material, this paper reviews the progress of the methods used to preserve and restore female fertility in different species to demonstrate the results obtained in the past 50 years of research, the current achievements and the future directions on this field.

Keywords: Fertility preservation, *In vitro* follicle culture, Ovarian tissue transplant, Slow freezing, Vitrification

Introduction

Over the past decades remarkable advances in the techniques and protocols for cryopreservation of germinal tissues have contributed greatly to the establishment and maintenance of germplasm banks. The feasibility to preserve and restore fertility in species of high commercial value and those species at risk of extinction highlight the economical and ecological potential of cryotechnology (Demirci *et al*., 2003; Liu *et al*., 2008a). In addition, the association of ovarian tissue cryopreservation and assisted reproduction techniques have important clinical relevance as it permits the development of new strategies to restore fertility in women who are at risk of premature ovarian failure (Shea *et al*., 2008; West *et al*., 2009, Smitz *et al*., 2010).

The main alternatives for fertility preservation used in the clinical routine are limited to the protection of the ovaries against radiation (oophoropexy) or oocyte retrieval for *in vitro* fertilization (IVF) with subsequent cryopreservation of oocytes and embryos (Sonmezer & Oktay, 2004). Although oophoropexy may offer some protection to germ cells, this technique can reduce greatly the chances for successful future pregnancies (Wallace *et al*., 2005). There are also serious limitations in the use of IVF in cancer patients. This factor

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is due mainly to the hormonal stimulation protocol required to obtain mature eggs that may delay the beginning of cancer treatment and therefore is not recommended for prepubertal patients (Sonmezer & Oktay, 2004). Damage to the ovarian germline cells caused by radiotherapy and/or chemotherapy can be prevented by the removal and cryopreservation of ovarian biopsies before the initiation of therapies, by which immature tissue can be restored further for fertility therapy proposals (Shea *et al*., 2008).

Ovarian tissue transplantation and *in vitro* follicle culture are emerging as promising approaches to restore fertility, specifically in women who are undergoing cancer treatments and who may not have time or are not good candidates for the IVF option (Woodruff & Snyder, 2008). Although ovarian tissue transplantation has successfully yielded viable offspring in humans (Donnez *et al*., 2004; Meirow *et al*., 2005), the *in vitro* ovarian follicle culture approach has been considered more desirable because it eliminates the possibility of reintroduction of cancer cells into the patient (Shea *et al*., 2008). Even though the meiotic competence and developmental capacity of human oocytes grown from pre-antral stages *in vitro* have not yet been reported, animal studies indicate that *in vitro* follicle culture is a valid prospect for humans.

Given the importance of safeguarding female fertility, in the present work we review the progress of ovarian cryopreservation techniques followed by tissue transplantation or *in vitro* ovarian follicle culture experimentally applied in programmes of female fertility restoration. We also present an overview of the most relevant outcomes obtained to date, in different animal models as well as the future direction of this field.

Ovarian tissue cryopreservation

The first cell survival tests completed with freezing and thawing were carried out in the 1930s using sperm. Their success was achieved largely by prior cellular dehydration in hypertonic solution (Luyet & Hodapp, 1938). Years later, Polge *et al*. (1949) and Smith (1950) observed sperm and erythrocyte survival, respectively, using glycerol as a cryoprotectant (CPA). Lovelock (1953) found that blood cells were damaged after freezing without CPA, however remained preserved when frozen with glycerol. Based on this finding, the author hypothesized that this protection was related to the colligative properties of glycerol. The following year, this same author was responsible for discovering that the protective action of glycerol can be shared with a number of other neutral solutes of low molecular weight. Later, Lovelock verified that cells such as erythrocytes, particularly cattle erythrocytes, are not preserved when using just glycerol as a CPA. As glycerol has low penetration in this cell type it suggested that a smaller molecule, such as dimethyl sulphoxide (DMSO), would be an excellent CPA. This suggestion was confirmed by Lovelock (1954) in subsequent years.

The first studies on ovarian tissue cryopreservation were performed in rodents more than half a century ago (Parkes & Smith, 1953; Parkes, 1957). Since the emergence of this technique, encouraging results have been reported. These results include the maintenance of morphology (Parkes, 1955) and viability of the ovarian follicle structure (Parkes, 1956). Results have also shown the restoration of endocrine function (Parkes, 1955) after cryopreservation and transplantation of ovarian tissue. Several studies were conducted, years later, in the 1990s, with different cryoprotectants such as DMSO (Hovatta *et al*., 1996; Newton *et al*., 1996; Candy *et al*., 1997; Newton & Illingworth, 2001), ethylene glycol (Newton *et al*., 1996; Candy *et al*., 1997), propylene glycol (Hovatta *et al*., 1996; Newton *et al*., 1996; Candy *et al*. 1997; Newton & Illingworth, 2001) and even glycerol (Newton *et al*., 1996, Candy *et al*., 1997, Newton & Illingworth, 2001) in order to improve ovarian cryopreservation protocols. Several of these studies (Newton *et al*., 1996, Candy *et al*., 1997, Newton & Illingworth, 2001) showed that DMSO is one of the CPAs that stands out for its cryoprotective effect in female gonads.

The cryopreservation of ovarian tissue has also been investigated extensively in animal production. In goats, for example, the first studies carried out were based on the effect of different CPAs (DMSO or PROH, Rodrigues *et al*. (2004a); and EG or GLY, Rodrigues *et al*. (2004b)) on follicular morphology. The effect of these CPAs was investigated to ascertain the viability of ovarian follicles in the early stages of folliculogenesis (primordial follicles) as described by Amorim *et al*. (2004). Recent studies in cattle reported the follicle survival in different stages of development (primordial, primary and secondary) after ultralow temperatures (Celestino *et al*., 2008). Currently, other studies have been conducted in order to define the best protocol for cryopreservation of ovarian tissue collected from goats (Luz *et al*., 2009; Carvalho *et al*., 2011; Castro *et al*., 2011), sheep (Faustino *et al*., 2010) and sow (Borges *et al*., 2009) using the methods of slow freezing or vitrification.

Slow freezing is considered to be the classic method of cryopreservation. After a period of exposure to low concentrations of CPA, which can vary from 5 to 60 min, the biological material is kept in a programmable freezer stable between 0°C (Donnez *et al*., 2004) and 20°C (Rodrigues *et al*., 2004a,b). The temperature is then gradually (about 2°C/min) reduced to –5 to –9°C (Rodrigues *et al*., 2004a,b; Andersen *et al*.,

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2008). At this temperature, the seeding procedure is performed, causing the induction of extracellular formation of ice crystals. This procedure aims to guide the beginning of the freezing solution to keep the biological sample from disordered freezing and super cooling (Zhang *et al*., 2011). This practice is designed to dehydrate the biological material before cryopreservation, thus reducing the chances of ice forming inside the cell (Zhang *et al*., 2011). The vitrification method is characterized by an ultrafast cooling rate, which uses high concentrations of cryoprotectants in order to increase the viscosity of the extender solution. This method also promotes the transition from liquid to the amorphous glassy state, thus avoiding the formation intracellular ice crystals (Vajta *et al*., 1998).

Ovarian tissue cryopreservation and fertility restoration

The cryopreservation of a biological material is intended to keep it viable for a long time under low temperature. This material can later be used with minimal damage to its function. The ovarian tissue, for example, after cryopreservation can be used with great potential for assisted reproduction, whether in procedures involving transplantation or *in vitro* culture.

Transplantation of ovarian tissue

After an indeterminate period of cryopreservation, ovarian tissue can be removed from liquid nitrogen and transplanted for the purpose of recovering its activity. According to the graft recipient or transplanted tissue, the transplant can be classified as xenotransplantation (when performed between different species), allotransplantation (when performed within the same species) or autotransplantation (if performed in the same animal). The transplant may also be identified according to the site of implantation. Orthotropic implantation is defined as tissue transplanted to its place of origin. The other possibility is heterotopic implantation, in which tissue is transplanted to a different region (Sonmezer & Oktay, 2010).

The transplantation of cryopreserved ovarian tissue is already used in human clinical assisted reproduction (Oktay *et al*., 2011). Several studies have shown that women (even before reproductive age) who are affected by cancer, before undergoing chemotherapy treatments and/or radiotherapy, could have the ovarian cortex removed and preserved. This material can be cryopreserved indefinitely and, after medical therapy, can be used as grafts in transplantation techniques for the re-establishment of the endocrine and reproductive functions (Andersen *et al*., 2008; Oktay *et al*., 2011). The association between ovarian cryopreservation and ovarian transplantation has been performed successfully in some species. Several studies have shown that ovarian function can be restored, including follicular growth and development (Demeestere *et al*., 2006) and steroidal hormone production (Andersen *et al*., 2008), as well as term pregnancies (Salle *et al*., 2003) and the live birth of both animals (Gosden *et al*., 1994) and humans (Donnez *et al*., 2004).

However, the clinical applicability to human transplantation is not always a viable option. For patients in whom cancer has metastasized, there is a risk of reintroducing cancer cells by transplanting previously harvested tissue, even after the cancer is cured (Aubard *et al*., 2001). As an alternative to transplantation, the ovarian tissue can be grown *in vitro* in order to obtain mature oocytes suitable for fertilization and *in vitro* production of embryos. This principle has already been demonstrated using fresh follicles in mice (Wang *et al*., 2011) and goats (Magalhães *et al*., 2011), respectively.

Live births from transplanted frozen ovarian tissue

The ovarian tissue can be transplanted by either heterotopic or orthotropic procedures after freezing/thawing. After the first type the patient can have a spontaneous pregnancy but heterotopic transplantation requires the use of supplementary techniques. Techniques such as *in vitro* culture of ovarian follicles followed by *in vitro* maturation and fertilization of oocytes in order to produce and transfer embryos and obtain offspring may be necessary. The use of freezing/thawing of ovarian tissue has resulted in births in mice, hares, sheep and humans (Table 1). The first births from frozen/thawed ovarian tissue were possible after orthotopic transplantation. This site provides optimal conditions for the deployment of folliculogenesis, as well as adequate blood supply and temperature for normal development and functioning of the organ (Nisolle *et al*., 2000; Oktay *et al*., 2004).

After whole ovary orthotopic transplantation previously frozen and thawed, Parrott (1960) described the first birth, a single offspring in mice. Using similar protocols in the same species, Cox *et al*. (1996) and Gunasena *et al*. (1997) obtained a pregnancy rate of 33% and 73%, respectively, and about three and four births per litter. Other researchers have also obtained promising results (23 births) after orthotopic transplantation of frozen/thawed half ovaries (Sztein *et al*., 1998). Takahashi *et al*. (2001) proposed that ovarian tissue after the death of an animal could be perfectly viable after a period of storage at low

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Table 1 Live births from transplanted frozen ovarian tissue

Species	Tissue	Device/tool	Assisted reproduction technique	Results	Cryoprotectant solution	References
Mice	Whole ovary	Glass ampoules	Orthotopic allotransplantation	1 live birth	12% Glycerol and horse serum	Parrott, 1960
	Whole fetal ovary	Plastic straws	Orthotopic allotransplantation	8 live births	1.5 M DMSO and 10% fetal calf serum	Cox *et al.*, 1996
	Whole ovary	Cryotube	Orthotopic autotransplantation	Live births	1.4 M DMSO	Gunasena *et al.*, 1997
	$^1/_2$ Ovary	Cryotube	Orthotopic allotransplantation	23 live births	1.5 M DMSO and 10% fetal bovine serum	Sztein *et al.*,1998
	Whole ovary	Cryotube	Orthotopic allotransplantation	Live births	1.5 DMSO	Candy *et al.*, 2000
	$^1/_2$ And $^1/_4$ ovary	Cryotube	Orthotopic allotransplantation	65 live births	1.5 M DMSO and 10% fetal bovine serum	Takahashi *et al.*, 2001
	Whole ovary	Plastic straws	Orthotopic allotransplantation	Live births	1.5 M DMSO or EG.	Snow *et al.*, 2003
Ewes	Whole ovary	Cryotube	Orthotopic allotransplantation	30 live births	1.5 M PROH, 0.1 M SUC and 20% fetal bovine serum.	Chen *et al.*, 2006
	Whole ovary and $^1/_2$ ovary	Cryotube	Orthotopic allotransplantation	435 live births	1.5 M DMSO and 10% fetal bovine serum	Liu *et al.*, 2008b
	Ovarian cortex fragment (1–1.5 mm)	Plastic straws	Heterotopic allotransplantation (renal capsule); IVM, IVF, IVPE and ET	4 live births	10% DMSO and 0.1 M SUC	Wang *et al.*, 2009
	Ovarian cortex fragment (≈ 1 mm)	Cryotube	Orthotopic autotransplantation	1 live births	1.5 M DMSO and 10% of donor calf serum	Gosden *et al.*, 1994
	$^1/_2$ Ovary	Cryotube	Orthotopic autotransplantation	6 live births	2 M DMSO	Salle *et al.*, 2002
Rabbits	$^1/_2$ Ovary	Cryotube	Orthotopic autotransplantation	6 live births	2 M DMSO	Salle *et al.*, 2003
Humans	Ovarian cortex fragment (1.5 mm)	Cryotube	Orthotopic autotransplantation	4 live births	1.5 M DMSO and 10% fetal calf serum	Almodin *et al.*, 2004
	Whole ovary	Cryotube	Orthotopic autotransplantation	1 live births	1.5 M DMSO and 10% autologous sheep blood serum	Imhof *et al.*, 2006
	Ovarian cortex fragment (∼1 mm^2)	Cryotube	Orthotopic autotransplantation	7 live births	1.5 M DMSO and 10% fetal calf serum	Almodin *et al.*, 2004
	Ovarian cortex fragment (2 × 2 mm)	Cryotube	Orthotopic autotransplantation	1 live births (girl)	1.5 M DMSO and human serum albumin	Donnez *et al.*, 2004
	Ovarian cortex fragment (0.5 × 1.5 × 0.1–0.2 cm)	Cryotube	Orthotopic autotransplantation; FP, IVF/ICSI and ET	1 live births (girl)	1.5 M DMSO and human serum albumin	Meirow *et al.*, 2005
	Ovarian cortex fragment	Cryotube	Orthotopic autotransplantation	1 live births (girl)	1.5 M DMSO, 0.1 M SUC and 10% patient serum	Demeestere *et al.*, 2007
	Ovarian cortex fragment (5 × 5 × 1–2 mm)	Cryotube	Orthotopic autotransplantation; FP, IVF and ET	2 live births (boy/girl)	1.5 M EG and 1.0 M SUC	Andersen *et al.*, 2008
	Ovarian cortex fragment (≈ 1 mm)	Cryotube	Orthotopic autotransplantation	1 live births (boy)	1.5 M PROH and 0.2 M de SUC	Silber *et al.*, 2008
	Ovarian cortex fragment (∼3 × 2 mm)	Cryotube	Orthotopic autotransplantation	7 live births	1.5 M DMSO and 10% fetal calf serum	Almodin *et al.*, 2004

DMSO: dimethylsulphoxide; EG: ethylene glycol; ET: embryo transfer; FP: follicular puncture; ICSI, intracytoplasmic sperm injection; IVF: *in vitro* fertilization; IVM: *in vitro* maturation; IVPE: *in vitro* production of embryos; PROH: propylene glycol; SUC: sucrose.

temperatures. These researchers aimed to preserve animals of high genetic value, such as founders to a transgene of interest, in case of death before the establishment of a germplasm bank. For this process, the researchers froze the ovaries from transgenic mice (lineage α_{1A}6.3-lacZ-15 that carried a 6.3 kb fragment in the 5′ upstream gene *cacna1a*) 2 h after the death of animals. Subsequently, the ovaries were allografted to non-transgenic ovariectomized recipients. These animals were also mated with non-transgenic males, resulting in 10 offspring, all carriers of the gene *cacna1a*.

Encouraging results were obtained in farm animals by Gosden *et al*. (1994). These authors reported the resumption of cyclic activity and pregnancy, achieving a birth after orthotopic autotransplantation of contralateral fragments of a frozen/thawed ovarian cortex in sheep. Eight years after these first births, Salle *et al*. (2002) also documented the birth of healthy offspring. In this study, ovaries from six animals were frozen/thawed and after autologous transplantation, resulting in four pregnancies and the birth of six lambs. The sheep that received the ovarian graft after delivery continued to be monitored for 2 years after transplantation, in which time other lambs were conceived (Salle *et al*., 2003). Almodin *et al*. (2004a) using 18G hypodermic needles filled with fragments of ovarian tissue – frozen–thawed ovary and contralateral irradiated ovary – were grafted into two ewes. Six months after grafting the two sheep were pregnant. The lambs were conceived and were healthy, showing that the females' fertility even after ovarian failure caused by radiation could be restored. Similar results were also reported in rabbits (Almodin *et al*., 2004b). Demonstrating the feasibility of contralateral whole freezing/thawing ovary autotransplantation, with microvascular anastomoses of the ovarian pedicle in nine sheep, Imhof *et al*. (2006) reported the birth of a healthy lamb. In this study it was shown that microvascular anastomosis of whole ovaries and orthotopic transplantation after cryopreservation is technically feasible.

The above studies revealed that cases of young women who needed immediate chemotherapy and who wished to preserve fertility, could rely only on ovarian cryopreservation. Once treatment is completed and the patient is free of disease it is possible to transplant ovarian tissue in hopes of pregnancy and birth. To date, 15 births have occurred after orthotropic transplantation of frozen ovarian tissue (Table 1). In 2004, Donnez and colleagues published a landmark study in human reproductive medicine, which brought hope to thousands of women who have suffered from and overcome cancer (Donnez *et al*., 2004). In this study, one patient had their endocrine and gametogenic function restored and reported a pregnancy after 11 months post orthotopic autotransplantation of ovarian fragments. This procedure showed that after treatment and cure of cancer other women could become pregnant and bear healthy children without the use of assisted reproduction techniques such as IVF (Silber *et al*., 2008, 2010; Demeestere *et al*., 2007). A recent study by Ernst *et al*. (2010) also showed that it is possible to keep implantation endocrine and gametogenic activities for an extended period. One patient in this study underwent both cancer treatment and ovarian tissue transplantation, similar to cases described previously. However, this individual was the first to give birth to two children at different times (the first birth in 2008 and second in 2010) from two pregnancies and only one graft. Similarly, orthotopic autotransplantation of ovarian cortex fragments cannot only be applied to reverse infertility in patients after cancer treatment, but also in women with health problems such as sickle cell anemia. This disease is sometimes treated with curative allogeneic bone marrow, which is generally preceded by chemotherapy and the use of the drug cyclophosphamide, which can damage oocytes and granulosa cells (Meirow *et al*., 1999). Donnez & Dolmans (2010) and Roux *et al*. (2010) reported cases in which patients were treated for sickle cell anemia, thereby posing premature loss of ovarian function. These women received orthotopic autologous transplantations of ovarian tissue and after a few months showed follicular growth and ovulation, which later allowed term pregnancies and births.

Although the orthotopic implantation spot provides optimal folliculogenesis conditions, possibly due to good conditions such as temperature, adequate blood supply and increased angiogenic capacity (Nisolle *et al*., 2000; Oktay *et al*., 2004), heterotopic autotransplantation allows the retrieval of oocytes and IVF to improve the chance of pregnancy. Assisted reproductive techniques can be applied subsequently to orthotopic autologous transplantation. In this type of procedure, the graft can be stimulated with hormones and can accelerate follicular growth, reducing the time between transplantation and the first subsequent pregnancy (Meirow *et al*., 2005; Andersen *et al*., 2008). Meirow *et al*. (2005) described, in woman, treatment and cure from lymphoma and the restoration of fertility after receiving an autograft. The tissue, 9 months after implantation, showed spontaneous growth of the ovarian follicle, by 15 mm. From this observation they performed ovarian stimulation with the gonadotropins (GnRH antagonist) and human chorionic gonadotrophin (hCG). After 34 to 36 h of hormonal stimulation, a single oocyte was recovered, fertilized by intracytoplasmic sperm injection (ICSI), and developed into a 4-cell embryo. This embryo was

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transferred and resulted in both a term pregnancy and birth.

Live births from transplanted vitrified ovarian tissue

While slow freezing has been used successfully for several years, this technique has some disadvantages in relation to vitrification. Among the disadvantages, the key lies in the fact that the slow freezing process can cause intracellular ice formation (IIF), which then causes irreversible damage to cellular structures. In this context, vitrification is advantageous, as it uses fast cooling, which results in solidification without crystallization, thus avoiding damage resulting from the IIF (Bagchi *et al.*, 2008). To date, there have been investigations in vitrification of ovarian tissue in a variety of species with different protocols and different sizes of tissue, such as fragments (Bordes *et al.*, 2005; Lornage *et al.*, 2006; Kagawa *et al.*, 2007; Wang *et al.* 2009), halves (Takahashi *et al.*, 2001) or even whole ovaries (Migishima *et al.*, 2003; Chen *et al.*, 2006; Hasegawa *et al.*, 2006; Bagis *et al.*, 2008; Liu *et al.*, 2008a). Some of these studies reported births, which are summarized in Table 2, and protocols are described below.

Migishima *et al.* (2003) vitrified whole ovaries of female mice and found that the ovary is capable of restoring their physiological condition and maintaining a pregnancy. In this study, ovarian fragments of transgenic female mice for green fluorescent protein (GFP) were transplanted into ovariectomized non-transgenic mice. After this procedure 33 pups were born, of which 48.5% were able to express the gene for GFP and thus the authenticity of the progeny. Years later, Chen *et al.* (2006) compared conventional vitrification, in a straw, with direct coverage vitrification (DCV) in a cryotube, getting 10 and 64 pups, respectively. Liu *et al.* (2008a) found that the recipients of vitrified whole ovaries have their reproductive activity regained after transplantation. In addition, pups had their origin proven by the polymerase chain reaction (PCR) technique. That same year, and in order to improve mice ovarian tissue cryotolerance, Bagis *et al.* (2008) investigated the effect of antifreeze protein (AFP) type III during vitrification of whole ovaries of female mice. Antifreeze protein was found originally in marine organisms that inhabit regions overwhelmed by conditions of extreme cold, and is characterized by giving natural freeze tolerance. After vitrification, the ovaries were transplanted into non-transgenic recipients who give birth to live pups.

In sheep there are two successful reports, both after orthotopic autotransplantation of ovarian tissue (Bordes *et al.*, 2005; Lornage *et al.*, 2006). The first study utilized six animals whose hemi-ovaries had the medulla removed and cortical fragments were vitrified and transplanted. Four months after transplantation, resumption of endocrine function in all animals was detected. Also three of these sheep gave birth to four lambs after natural mating (Bordes *et al.*, 2005). The following year, Lornage *et al.* (2006) studied whole and fragment ovary vitrification, as well as the physical properties involved in the formation of ice crystals, and found that vitrification of whole ovaries using solution with DMSO, propanediol and formamide, is important for follicular morphology preservation. Paradoxically, this team has obtained three pregnancies from which four lambs were born after transplantation of ovarian cortex fragments.

Kagawa *et al.* (2007) vitrified ovarian fragments by the cryotop technique and completed allogeneic transplantation to mice kidney capsules. The recipients were stimulated, after 9 days of transplantation, with 7.5 IU of equine chorionic gonadotropin (eCG). After 48 h, the tissue was removed and antral follicles were grown *in vivo* and punctured. The recovered oocytes were matured and underwent ICSI. In total, 57 embryos were transferred to six recipients who gave birth to 10 pups. Similarly, Wang *et al.* (2009) vitrified ovaries of adult mice using the solid surface technique. Subsequently, the ovaries were transplanted to the kidney capsules of recipient mice. After 10 days of transplantation cumulus–oocyte complexes (COCs) were recovered, underwent IVF and were cultured to the blastocyst stage. Eight pups were born after the transfer of 32 blastocysts.

In situations in which a transplant is not feasible for the restoration of fertility, the alternatives of *in vitro* culture of ovarian tissue or artificial ovary can be considered. Over the past year progress in this area has been seen and it is now possible to grow pre-antral follicles in several species (mice: O'Brien *et al.*, 2003; humans: Telfer *et al.*, 2008; sheep: Arunakumari *et al.*, 2010). This practice allows the recovery of oocytes from these follicles, which can also be matured and fertilized *in vitro* to generate embryos (Magalhães *et al.*, 2011).

The association between vitrification and *in vitro* culture resulted in the birth of mice (Hasegawa *et al.*, 2006, Wang *et al.*, 2011). In the study by Hasegawa *et al.* (2006), 36 embryos were produced *in vitro* and transferred to two recipients, resulting in the pregnancy and births of two offspring in one of the recipients. Wang *et al.* (2011) obtained 16 blastocysts, which were transferred and resulted in pregnancy and two births.

***In vitro* culture of ovarian pre-antral follicles**

The association between ovarian tissue cryopreservation and ovarian pre-antral follicle culture might offer a value alternative to preserve and restore fertility by reducing the chances of reintroduction of

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Table 2 Live births from transplanted or *in vitro* cultured vitrified ovarian tissue

Species	Tissue	Device/tool	Assisted reproduction technique	Results	Cryoprotectant solution	References
Mice	½ and ¼ Ovary	Cryotube	Orthotopic allotransplantation	89 live births	20.5% DMSO, 10% PROH, 6% PEG and 15.5% acetamide	Takahashi *et al.*, 2001
	Whole ovary	Cryotube	Orthotopic allotransplantation	33 live births	2 M DMSO. 1 M acetamide and 1 M PROH	Migishima *et al.*, 2003
	Whole ovary	Polyester sheets	OGCs; IVG; IVM; IVF; IVPE and ET	2 live births	7.5% EG, 7.5% DMSO and 10% SSS / 15% EG, 15% DMSO, 0.5 M SUC and 20% SSS	Hasegawa *et al.*, 2006
	Whole ovary	Cryotube	Orthotopic allotransplantation	64 live births	7.5% EG, 7.5% DMSO and 20% and fetal bovine serum	Chen *et al.*, 2006
	Whole ovary	Plastic straws	Orthotopic allotransplantation	10 live births	40% EG, 10.7% acetamide, 30% Ficoll, 0.5 M SUC and 20% and fetal bovine serum	Chen *et al.*, 2006
	¼ Ovary	Cryotube	Orthotopic allotransplantation	418 live births	2 M DMSO, 3 M PROH and 1 M acetamide	Hani *et al.*, 2006
	Ovarian cortex fragment (0.2–0.3mm)	Cryotop	Heterotopic allotransplantation (Renal capsule); IVM; IVF/ICSI; IVPE and ET	10 live births	7.5% EG, 7.5% DMSO and 20% SSS / 15% EG and 15% DMSO	Kagawa *et al.*, 2007
	Whole ovary	Cryotube	Orthotopic allotransplantation	117 live births	20% DMSO, 20% EG and 0.5 M SUC	Bagis *et al.*, 2008
	Whole ovary	Cryotube	Orthotopic allotransplantation	Live births	1 M DMSO / 4 M DMSO, 2 M acetamide, 6 M PROH	Liu *et al.*, 2008a
	Ovarian cortex fragment (1–1.5mm)	Fiberplug	Heterotopic allotransplantation (Renal capsule); IVM; IVF; IVPE and ET	8 live births	10% EG, 10% MSO / 17% EG, 17% DMSO and 0.75 M SUC	Wang *et al.*, 2009
	¼ Ovary and Whole ovary	Fiberplug	Follicle isolation, follicle IVG; IVF; IVM; IVPE and ET	2 live births	1.8 EG, 1.4 M DMSO / 3 M EG, 2.3 M DMSO and 0.75 M SUC	Wang *et al.*, 2011
Ewes	Ovarian cortex fragment (1mm × 2cm × 1cm)	Cryotube	Orthotopic autotransplantation	4 live births	2.62 M DMSO, 1.31 M PROH, 0.0075 M PEG, 2.60 M acetamide and 10 g/mg human albumin	Bordes *et al.*, 2005
	Ovarian cortex fragment (1mm × 1cm^2)	Cryotube	Orthotopic autotransplantation	4 live births	2.75 M DMSO, 1.97 M PROH, 2.76 M formamide	Lornage *et al.*, 2006

DMSO: dimethylsulphoxide; EG: ethylene glycol; ET: embryo transfer; IVF: *in vitro* fertilization; IVG: *in vitro* growth; IVM: *in vitro* maturation; IVPE: *in vitro* production of embryos; OGCs: Oocyte–granulosa cell complexes; PEG: polyethylene glycol; PROH: propylene glycol; SSS: serum substitute supplement; SUC: sucrose.

cancer cells derived from the transplanted tissue to the patient (Shea *et al.*, 2008; Woodruff & Snyder, 2008). Furthermore, this method may also ensure a greater number of mature oocytes for subsequent fertilization (West *et al*., 2009). So far, this technology is available for human follicles; however, *in vitro* maturation of oocytes derived from cultured pre-antral follicles does not show significant results. Although the use of a traditional *in vitro* (bidimensional) systems has supported the full growth and differentiation of immature mice follicles, as well as meiotic competent oocytes (Eppig & O'Brien, 1996), similar results have not yet been reported for other species of mammals, including bovine (Gutierrez *et al*., 2000), sheep (Tambe & Nandedkar, 1993) and humans (Abir *et al*., 1997). This fact is due mainly to the difficulty in maintainance of the architectural structure of the follicle in large mammals, a problem similar to that found *in vivo* (Picton *et al*., 2002). *In vivo* and *in vitro* studies have shown the importance of maintainance of follicular architecture to the process of growth and differentiation of the oocytes (Matzuk *et al*., 2002), which reaffirms the structural dependency component to the success of follicular cultivation.

Unlike the *in vitro* bidimensional culture system, in which somatic cells disconnected from the oocyte and spread through the area of culture, the architecture of the follicle is kept intact when performed on a three-dimensional *in vitro* culture system (Smitz *et al*., 2010). In this system, individual immature follicles are encapsulated within an extracellular matrix of hydrogels of sodium alginate (West *et al*., 2007). Divalent cations, such as calcium, bind cooperatively between the blocks of adjacent alginate chains, creating ionic inter-chain bridges. This action causes gelling of aqueous alginate solutions while maintaining cellular viability without causing adsorption of non-specific proteins and cellular adhesion (Rowley *et al*., 1999). In addition to mechanical array support, the alginate is highly porous, thereby facilitating diffusion of soluble hormones and factors between the culture medium and the follicle, a process necessary for the survival and growth of follicles (West *et al*., 2007). In fact, this system was able to guarantee the growth and complete differentiation of immature mouse follicles, furthermore allowing the oocyte maturation and development of healthy progeny (Xu *et al*., 2006). The use of this technique for non-human primate follicular culture has generated promising results. The alginate hydrogel matrix was also able to maintain the three-dimensional structure of secondary follicles in monkeys, from fresh and frozen ovarian tissue, and allowed follicular growth and steroidogenesis by more than 30 days *in vitro* (Xu *et al*., 2009a; Ting *et al*., 2011).

Other studies from this same research group have examined the role of endocrine and paracrine factors on *in vitro* follicular growth, as well as the influence of age on the viability of follicular tissue (Xu *et al*., 2010; Xu *et al*., 2011). So far, only two studies have tested the feasibility of the alginate matrix in the cultivation of human immature follicles. The results of these two studies are still inconclusive and insufficient for the standardization of the *in vitro* culture of follicles to produce eggs that are able to be fertilized (Amorim *et al*., 2009; Xu *et al*., 2009b). However, *in vitro* cultivation can be considered as a possibility for use in cryopreserved pre-antral follicles, especially when the transplantation of ovarian tissue is not the most appropriate alternative.

Conclusions

The reviewed data presented here reinforce the feasibility of ovarian tissue cryopreservation to safeguard fertility, specifically for cancer patients who want to protect their germ line cells from the destructive effects of chemotherapy and/or radiotherapy. The combination of cryopreservation, tissue transplantation, and *in vitro* ovarian follicle culture emerged as an alternative option to restore female fertility. Although ovarian tissue transplants have successfully yielded viable offspring in different species, the data obtained so far using the *in vitro* ovarian follicle culture approach are still preliminary and more efforts are needed to generate an adequate milieu for complete *in vitro* development of the immature ovarian follicles in large species, especially human.

Disclosure statement

The authors have nothing to disclose.

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