

Expectations and limitations of ovarian tissue transplantation

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Summary

Constant progress in the diagnosis and treatment of cancer disease has increased the number and prognosis of cancer survivors. However, the toxic effects of chemotherapy and radiotherapy on ovarian function have resulted in premature ovarian failure. Patients are, therefore, still expecting methods to be developed to preserve their fertility successfully. Several potential options are available to preserve fertility in patients who face premature ovarian failure, including immature or mature oocyte and embryo cryopreservation. However, for children or prepubertal women needing immediate chemotherapy, cryopreservation of ovarian tissue is the only alternative. The ultimate aim of this strategy is to implant ovarian tissue into the pelvic cavity (orthotopic site) or in a heterotopic site once oncological treatment is completed and the patient is disease free. Transplantation of ovarian tissue with sufficiently large numbers of follicles could potentially restore endocrine function and allow multiple cycles for conception. However, the success of ovarian tissue transplantation still has multiple challenges, such as the low number of follicles in the graft that may affect their longevity as well as the survival of the tissue during *ex vivo* processing and subsequent transplantation. Therefore, this review aims to summarize the achievements of ovary grafting and the potential techniques that have been developed to improve ovarian graft survival.

Keywords: Cryopreservation, Fertility preservation, Neovascularization, Oncofertility, Transplantation

Introduction

Advances in oncological diagnosis and treatments have provided a considerable increase in survival of cancer patients (Jemal *et al.*, 2012), however, chemo-

therapy and/or radiotherapy treatments, including alkylating agents may compromise their future fertility (Anderson & Wallace, 2013). Furthermore, as the number of young cancer survivors augments, the demand for fertility preservation before cancer therapy increases progressively (Donnez *et al.*, 2006b). Cryopreservation and transplantation are, therefore, the main and most viable options to preserve and thus regenerate the fertility of women who will undergo cancer treatment (Donnez *et al.*, 2013).

Especially for children or young patients, the available option to preserve fertility is the cryopreservation of ovarian tissue (slices/fragments) that allows the storage of a large number of primordial and primary follicles (Meirow *et al.*, 2007) and can ensure the restoration of the ovarian endocrine function (Donnez *et al.*, 2013). The cryopreserved ovarian tissue needs, therefore, to be grafted to the patient after a period of storage at low temperatures.

More than a half century ago, a study conducted in mice described the first birth after a whole ovary orthotopic transplantation with previous cryopreservation

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(Parrot, 1960). In humans, the first birth obtained using ovarian tissue cryopreservation and grafting was reported by Donnez *et al.* (2004) and was a landmark in human reproductive medicine. Since then, the birth of 70 healthy babies (Silber, 2016) has been reported after transplantation of cryopreserved ovarian tissue. Despite these encouraging results, the techniques of cryopreservation and ovarian transplantation to restore reproductive function in women are still considered experimental. New studies are therefore being performed around the world to investigate the best way to restore fertility, either in humans (Burmeister *et al.*, 2013), non-human primates (Amorim *et al.*, 2013), in domestic animals (Fassbender *et al.*, 2007) or in laboratory animals (César *et al.*, 2015).

The natural plasticity of the ovary facilitates grafting to different sites in which they can be revascularized and rapidly restore its normal physiology. Furthermore, ovarian tissue can be transplanted orthotopically to the pelvis (Demeestere *et al.*, 2010) or heterotopically in subcutaneous areas, kidney capsule or fat pad (Youm *et al.*, 2015) as well as in other sites (the rectus muscle: Kim *et al.*, 2009; subperitoneal tissue: Stern *et al.*, 2011). Regardless of the site, the ovarian graft can undergo ischemia and potentially follicular atresia that represent a challenge to the success of this technique. It is necessary, therefore, to make sure that there is good cell communication between graft and host tissue when the ovary is grafted.

The practice of cryostorage followed by transplantation has been restoring fertility and hormone production (in humans: Silber, 2016; and others species: Amorim *et al.*, 2013; Campbell *et al.*, 2014). This option is elective for patients who have high risk of premature ovarian failure (POF) in which oncologists and physicians work together to achieve the patient's global well-being (Revelli *et al.*, 2013).

Thus, this review will provide an insight into the different factors that affect ovarian functionality after transplantation and some relevant advance to date.

Ovarian tissue transplantation

The main goal of transplantation of ovarian tissue is the restoration of ovarian endocrine function and fertility especially in young and adult women undergoing cancer treatment (Donnez *et al.*, 2013). The ovary is abundant in primordial follicles in the quiescence stage or at rest that constitute the ovarian reserve. Only a few of these will be activated and develop to an advanced follicular stage (Kim, 2012). Primordial follicles can be cryopreserved and stored at any stage of the female reproductive life, without the need for hormonal treatment.

The ovary is a well suited place for transplantation due to its naturally abundant angiogenic factors that favor the neovascularization process. According to the site of transplantation the procedure is classified as: *orthotopic* or *heterotopic implantation*. Orthotopic implantation is defined as tissue transplanted to its place of origin or into the pelvic cavity. In the case of heterotopic implantation, it is defined as tissue transplanted in a different site or in an extra-ovarian region (Sonmezer & Oktay, 2010), such as the abdominal wall (Rodriguez-Wallberg & Oktay, 2012), forearm (Oktay *et al.*, 2001), kidney capsule (Youm *et al.*, 2015) or breast (Kim *et al.*, 2004) following the cryopreservation process.

According to the graft recipient, transplantation can be classified as *xenotransplantation* (performed in different species), *allograft transplantation* (performed in the same species) or *autotransplantation* (performed in the same individual).

Site of ovarian tissue implantation

Orthotopic site

In this type of transplantation, the tissue is reimplanted in its original physiological surroundings and the development of transplanted tissue is very effective. The main advantage of orthotopic transplantation of ovarian tissue is that natural conception could occur without the intervention of assisted reproductive techniques. According to Donnez & Dolmans (2015), the pelvic cavity (orthotopic site) would provide the optimal environment for follicular development compared with heterotopic sites, as temperature, pressure, paracrine factors and blood supply are similar to those observed in a physiological situation. Conversely, the disadvantage would be the limited number of fragments able to be transplanted due to ovary size. In addition, orthotopic transplantation is an invasive procedure that may cause severe pelvic adhesions (Demeestere *et al.*, 2009).

In animal production, the first encouraging results were obtained by Gosden *et al.* (1994) in sheep. These authors reported the resumption of cyclic activity, pregnancy, and producing a live birth after orthotopic autotransplantation of samples of ovarian tissue cryopreserved by slow freezing. Subsequently, Salle *et al.* (2002, 2003) and Bordes *et al.* (2005) also reported live birth using orthotopic transplantation of a frozen-thawed and vitrified ovary in ewes. Imhof *et al.* (2006) also reported a live birth after orthotopic transplantation of frozen-thawed ovarian tissue in ovine. Moreover, Santos *et al.* (2009) demonstrated complete follicular development and the recovery of endocrine function after cryopreservation and orthotopic autotransplantation of small ovarian

fragments in bilaterally ovariectomized goats without administration of hormones.

It should be emphasized that the first live birth in humans obtained using the combination of ovarian tissue cryopreservation and orthotopic transplantation was reported by Donnez *et al.* (2004), and was a landmark in human reproductive medicine. Since then, various authors have reported live birth after ovary transplantation (Andersen *et al.*, 2008; Silber *et al.*, 2008; Donnez *et al.*, 2011, Silber, 2016).

Two techniques were successfully used to reimplant fresh or frozen–thawed ovarian tissue in an orthotopic site, within a specially created window on the peritoneum (Donnez *et al.*, 2004) or on the remaining ovary (Donnez *et al.*, 2006a). The large tissue strips (8–10 × 5 mm) of ovarian fragments could be sutured into the remaining ovary after the removal of the native cortex. However, the transplantation of small pieces (2 × 2 mm), in the medulla space was difficult because it could not be sutured (Donnez *et al.*, 2008). A normal reproductive lifespan was demonstrated after orthotopic grafting of vitrified ovary in the mouse (Liu *et al.*, 2008). Furthermore, Silber *et al.* (2008) reported reinitiation of ovulatory menstrual cycles and normal serum FSH levels after 77–142 days after transplantation of fresh and cryopreserved ovarian tissue between a series of monozygotic (MZ) twin pairs. Moreover, two live births were reported following fresh ovary transplantation between two identical twin sisters and another one live birth after autotransplantation of cryopreserved ovarian tissue (Silber *et al.*, 2008). Burmeister *et al.* (2013) also reported human pregnancy after ovarian tissue cryopreservation and subsequent orthotopic autotransplantation.

Heterotopic site

The potential advantages of heterotopic implantation include: avoidance of invasive procedures; easy accessibility of the graft; increased capacity for cortical slices; and feasibility for grafting even if severe pelvic adhesions preclude orthotopic transplantation (Kim, 2012). In addition, the use of general anaesthesia is not required and the removal of the transplanted fragments is not difficult (Filatov *et al.*, 2016). However, unlike orthotopic transplantation, natural conception cannot be expected after transplantation of ovarian tissue to heterotopic sites and, therefore, *in vitro* fertilization (IVF) is required for conception.

As mentioned above, ovarian grafts can be transplanted in several different sites, including the bursa cavity, the kidney capsule, and subcutaneous sites. Transplantation in the kidney capsule has been often used due its excellent blood supply that would enhance graft survival (Youm *et al.*, 2015). Graft recovery and oocyte yield are significantly higher from the bursal cavity and kidney capsule compared with

subcutaneous site in murine species (Youm *et al.*, 2015). Regardless of the site of transplantation, the ovarian graft will undergo ischemia and potential follicular atresia during the period after transplantation before tissue revascularization; this process remains a challenge to the success of this technique (Wang *et al.*, 2012). However, by virtue of their low metabolic rate, primordial follicles are relatively resistant to the effects of oxygen deprivation (Schmidt *et al.*, 2003).

Heterotopic ovarian transplantation experience in humans and non-human primates has been very limited (Amorim *et al.*, 2013). Several heterotopic sites have been tested in humans, including the uterus broad ligament (Gosden *et al.*, 2010), the rectus muscle (Kim *et al.*, 2009), the forearm (Oktay *et al.*, 2001), breast tissue (Kim *et al.*, 2004), the subcutaneous tissue of the abdomen (Youm *et al.*, 2015) and subperitoneal tissue (Stern *et al.*, 2011). In studies with primates, other heterotopic sites (omentum, retroperitoneal iliac fossa, uterine serosa, mesosalpinx, and the pelvic wall) were also used (Suzuki *et al.*, 2012). In addition to intraperitoneal (omentum) or subperitoneal sites, the subcutaneous tissue of the abdomen could also provide an adequate environment for follicle growth in both humans and primates (Kim, 2014). However, heterotopic sites may not provide an optimal environment for follicular development due to differences in temperature, paracrine factors and blood supply compared with the intraperitoneal environment (Donnez *et al.*, 2010a). Primate models have been used to find suitable locations to overcome some of these challenges (Igarashi *et al.*, 2010). High rates of follicular survival after heterotopic xenotransplantation of human cryopreserved prepubertal ovarian tissue in mice have been recently demonstrated with the retention of a large pool of dormant primordial follicles in the graft (Luyckx *et al.*, 2013). Furthermore, Stern *et al.* (2013) reported the first clinical pregnancy following heterotopic grafting of cryopreserved ovarian tissue in a woman after a bilateral oophorectomy.

Therefore, the choice of the transplantation sites constitutes an essential factor involved in future graft viability and in the subsequent oocyte competence (Demeestere *et al.*, 2009).

Graft recipient of ovarian tissue implantation

Xenotransplantation

Complex biological processes often require *in vivo* analysis, and several important research studies have been made using mice as a model for the study of various biological systems. Humanized mice, or mouse–human chimeras were developed to overcome these constraints and are now an important research tool for *in vivo* study of human cells and tissues

(Shultz *et al.*, 2007). Immunodeficient mice have been used to evaluate follicle development and survival after cryopreservation of ovarian tissue. Some studies (Table 1) have shown that xenografting of human ovarian tissue into mice has been an effective model to study ovarian function and follicle development *in vivo* (Van Eyck *et al.*, 2009). Since 1960, athymic mice (nude) have been a standard for establishing *in vivo* models of human malignancies. Due to the lack of a thymus, nude mice cannot generate mature T lymphocytes and therefore are unable to mount most types of immune responses (Fransolet *et al.*, 2015). This absence of functioning T cells prevents nude mice from rejecting not only allografts, but even xenografts (Fransolet *et al.*, 2015). Another attractive model is the use of severe combined immunodeficient (SCID) mice (Fransolet *et al.*, 2015). SCID mice were the first and are the most commonly used model for ovarian xenografts (Aubard, 2003) and were characterized by their *scid* mutation, which leads to a defect in the recombination of antigen receptor genes, impairing their capacity to generate functional B and T lymphocytes (Custer *et al.*, 1985). Thus, SCID mice can maintain tissues from foreign species without demonstrating a graft-versus-host response (Bosma *et al.*, 1983) and, consequently, they can serve as a good model for transplantation studies. A few years after SCID mouse generation, the *scid* mutation was transferred onto a non-obese diabetic (NOD) background. This transfer leads to NOD-SCID mice, which have reduced natural killer cell (NK cell) activity in addition to the deficiency in functional B and T cells (Prochazka *et al.*, 1992). Moreover, their ability to activate some components of the complement system is impaired, and these mice are markedly deficient in macrophages (Shultz *et al.*, 2007).

In order to develop a novel protocol for the establishment of human ovarian stroma within a mouse model subcutaneously, normal human ovarian tissues were subcutaneously implanted into SCID mice and then the implants were identified by immunohistochemistry. The results demonstrated that human ovarian tissue successfully survives in a SCID mouse host and retains the properties of the original normal ovarian tissues (Fu *et al.*, 2014). Previously, Luyckx *et al.* (2013) reported that frozen-thawed preantral follicles from prepubertal patients can successfully survive and develop after long-term ovary xenografting. The study conducted by Henry *et al.* (2015) after xenotransplantation of sheep ovary to SCID and NOD-SCID showed that they were both suitable for studying graft recovery, however, based on histologic analysis, the overall tissue morphology was better preserved in SCID mice. A significant alteration of the gene responsible for ovarian metabolism and function was reported after xenotransplantation of rat ovary into the kidney capsule of immune-deficient

mice (Agca *et al.*, 2009). Ishijima *et al.* (2009) also showed high follicular loss after xenotransplantation of canine cryopreserved ovary in immune-deficient mice. Moreover, it was demonstrated that encapsulation of ovarian tissue with VEGF165 in a collagen matrix during xenografting in SCID mouse produces a more rapid onset of functional vessel formation and earlier revascularization of the transplant (Henry *et al.*, 2015). Lotz *et al.* (2014) reported antral follicle formation after 122 days post xenotransplantation in a single human ovarian fragment of 6 years old without exogenous hormone stimulation. Moreover, it was shown that isolated human follicles were able to survive after encapsulation in fibrin clots and short-term xenotransplantation (Paulini *et al.*, 2016).

Autotransplantation/allotransplantation

As mentioned before, the main goal of ovarian tissue transplantation is the restoration of fertility and endocrine function of women who undergo cancer treatments (chemo-/radiotherapy). In this context, the efforts made so far by research teams from at least 10 countries have proved that transplantation of cryopreserved ovarian tissue is a promising option to preserve fertility in female patients with cancer. These procedures allow immediate initiation of cancer treatment, as it does not require prior ovarian stimulation nor sperm donation compared with other technologies such as embryo or oocyte cryopreservation.

It is very difficult to know the exact number of attempts made to transplant ovarian tissue (fresh and cryopreserved), especially when it is not directly involved with the clinic or even when the main focus of research is the biotechnology of animal production. In fact, according to Donnez & Dolmans (2015), the number of reimplantations performed worldwide is not known. However, excellent results have been seen in the USA, Brussels, Paris, Spain, Denmark, and Israel. In addition, successes are reported by research teams in Japan, Italy, Germany, and Australia (Stoop *et al.*, 2014). In addition to the excellent restoration rate of ovarian function (95%) and a reasonable gestation rate (23%) obtained when this strategy is applied, it has been observed that the duration of ovarian function has been maintained for a period from 4 to 5 years (Donnez & Dolmans, 2013, 2014).

Autotransplantation of frozen and thawed ovarian tissue is only possible if absence of cancer cells in the graft is confirmed and there is a legitimate concern for the reseeding of malignant cells when carrying out ovarian transplantation (Rodriguez-Wallberg & Oktay, 2012). Disadvantages of ovarian tissue cryopreservation and autotransplantation include the limited life span of the ovarian grafts due to the potential post-transplantation window of ischemia responsible

Table 1 Summary of some relevant results obtained after xenotransplantation of ovarian tissue in different species

Species involved	Graft number and size	Graft site	Ovary preparation	Reproductive stage of ovary's donor	Exogenous substance	Graft recovery date	Outcome	References
Rat to Mice	1 mm ³	Kidney capsule	Fresh and frozen/thawed	Adult (3 weeks)	No	10–12 weeks	Alteration of genes responsible for ovarian metabolism and function, upregulated of genes responsible for graft rejection	Agca <i>et al.</i> , 2009
Sheep to Mice	1 pc (2.5×2.5×1 mm)	Ovary	Frozen/thawed	Adult	VEGF 165	3 days or 3 weeks	functional vessel formation and earlier revascularization	Henry <i>et al.</i> , 2015
	1 pc (2.5×2.5×1 mm)	Ovary	Frozen/thawed	Adult	VEGF 111	3 days or 3 weeks	SCID mice is best murine model for studying ovarian tissue xenografts	Fransolet <i>et al.</i> , 2015
Bovine to Mice	1 pc (1–2 or 0.5–0.9 mm)	Subcutaneous	Frozen/thawed	Adult	No	7 days	Follicle activation and 'burn out' induce follicles loss after transplantation	Gavish <i>et al.</i> , 2014
Human to Mice	1 pc (1×2×1mm)	Intramuscular	Frozen/thawed	Children (6 year)	No	122 days	Antral follicle formation	Lotz <i>et al.</i> , 2014
	2 pc (5×1.5×1 mm)	Intraperitoneal	Fresh and frozen/thawed	Adult (20–30 years)	FSH and hCG	28 weeks	Antral follicles obtained after grafting of frozen–thawed human ovarian tissue showed a thinner theca cell layer compared with those from fresh grafts	Amorim <i>et al.</i> , 2011
	1–2 pc (1×3 or 8–10 mm)	Intraperitoneal	Frozen/thawed	Children (7.2–12.2 years)	FSH	21 weeks	Antral follicles formation	Luyckx <i>et al.</i> , 2013
	2 pc (2×4mm)	Intraperitoneal	Frozen/thawed	22–38 years	No	2 and 7 days	Alteration of gene expression after human ovarian tissue xenografting	Van Langendonck <i>et al.</i> , 2014
	6 pc (1×1×1 mm)	Intramuscular	Fresh and vitrified	Adult (21–35 years)	No	30 days	Evidence on the significant effect of vitrification on follicular apoptosis of grafted human ovarian tissue at mRNA level	Jafarabadi <i>et al.</i> , 2015
Human to Rabbit	2 pc	Back muscle	Fresh	Adult (30–35 years)	VEGF, bEGF and hMG	6 weeks	VEGF and bFGF, especially the combination of them, triggered angiogenesis, reduced apoptosis and fibrosis, increased survival in transplanted human ovarian tissue	Wang <i>et al.</i> , 2013

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for follicular atresia. In addition, for patients with autoimmune or genetic disorders, gonadal autotransplantation is ineffective in preserving fertility. In such cases, allotransplantation might be the solution (Yi-Hsin *et al.*, 2011). Silber and colleagues reported a series of monozygotic twins (Table 2) who underwent ovarian isograft transplantation to rescue the sterile sister (Silber *et al.*, 2005, 2008). Furthermore, orthotopic ovarian allotransplantation has been performed in patients diagnosed with Turner's syndrome. This technique was able to restore regular menstruation and ovulation, it raised hormonal concentrations and led to the development of secondary sexual characters (Mhatre & Mhatre, 2006). However, Scott *et al.* (1987) have shown that immunosuppressive therapy, such as cyclosporine A plus steroid, is needed for ovarian allografts to survive.

Factors affecting graft function

Follicle loss is one of the major limitations after ovary transplantation. This phenomenon occurs in several steps of the procedure as cited below.

Ovarian tissue size

The thickness of ovarian cortical strips prior to freezing is critical to enable the perfusion of cryoprotectants and the ensuring of the graft survival. The surface-to-volume ratio of the graft has to be high in order to ensure good penetration and removal of cryoprotectants agents. It reduces the probability of damage caused by ice crystals during freezing and thawing, and by the ischemia and hypoxia that occurs during the initial steps of graft acceptance (Gavish *et al.*, 2008). Currently, ovarian cortical tissue is cryopreserved in strips of 1–2 mm thickness either by slow freezing or vitrification protocols (Kagawa *et al.*, 2009). It has been suggested (Gavish *et al.*, 2008; Revel *et al.*, 2011) that reducing graft dimensions might enhance diffusion of oxygen, growth factors and nutrients into the ischemic graft and improve follicle's survival. However, a study conducted by Gavish *et al.* (2014) showed an extensive neovascularization 1-week post xenotransplantation of bovine ovarian tissue in mice. No beneficial effect was found when reducing the graft thickness beyond 1 mm, including extensive primordial follicle loss and increased follicle 'burn out' without improving neovascularization. Moreover, the transplanted fragments of ovarian cortex contain only a fraction of an individual's ovarian reserve and as such can only provide the recipient with a relatively brief fertile window before the supply of oocytes contained within their graft is depleted (Andersen *et al.*, 2008; Ernst *et al.*, 2010; Silber, 2012; Donnez *et al.*,

2013). This limitation means that cryopreservation and autografting of pieces of cortex appear to be less effective as a means to restore the fertility of older patients in whom follicle density is already low at the time of tissue preservation and in which it is associated with endocrine disturbance (Campbell *et al.*, 2004). In this context, cryopreservation of the whole ovary (complete with vascular pedicle) for later autotransplantation provides an attractive alternative strategy for fertility preservation as it involves restoration of all of the primordial follicles within the ovary. Furthermore, as transplantation requires vascular anastomosis rather than cortical revascularization, this intervention should result in no marked reduction of ovarian reserve due to ischemia, preventing endocrine imbalance and reducing the age constraint which limit the efficacy of this fertility preservation technology (Campbell *et al.*, 2014). However, autotransplantation of frozen-thawed whole ovary has also some disadvantages including:

- (1) It has a higher risk of cryoinjury during freezing due to inadequate diffusion of cryoprotectants throughout the entire ovary and non-homogenous cooling rate between the core and the periphery of the ovary as well.
- (2) Cryoinjury of ovarian vasculature
- (3) Surgical difficulty of vascular anastomosis due to the small size of ovarian artery (~0.5 mm in diameter), short ovarian vascular pedicle (~5 cm in length), discrepancy between the diameters of graft and recipient vessels, and possible failure of microvascular anastomosis.
- (4) Higher risk of post-operative vascular complications including anastomotic bleeding, pseudoaneurysm, stenosis, or microvascular thrombosis.
- (5) Its vascular complications can severely affect the survival of the entire ovary leaving no other attempt for transplantation (Bedaiwy & Falcone 2010; Kim, 2010; Zhang *et al.*, 2011).

Ischemia

Ischemia is defined as the insufficient perfusion and reduction of the arterial or venous blood flow, depletion of cellular energy storages, and the accumulation of toxic metabolites which can lead to cell death (Ingec *et al.*, 2011). Ischemic times that extend for several days most likely induce profound and irreversible ischemia-reperfusion injuries. They are responsible for follicle loss, as the graft needs 4–5 days to be reoxygenated (Van Eyck *et al.*, 2009). A study with sheep ovarian tissue reported that 65% of the follicles are lost after grafting of fresh tissue while subjecting the tissue to cryopreservation and thawing

Table 2 Summary of some relevant results obtained after auto/allotransplantation of ovarian tissue in different species

Species	Transplantation type	Graft number and size	Graft site	Reproductive stage of ovary's donor	Ovary preparation	Exogenous substance injected	Outcome	References
Mice	Allotransplanted	Whole ovary (2 mm ³)	Ovarian bursa	Immature (10 days)	Fresh and frozen/thawed	No	15 Live birth	Candy <i>et al.</i> , 2000
	Allotransplanted	1, 2 or 4 pc Hemi-ovary	Kidney capsule, subcutaneous, bursa	Adult (3 weeks)	Fresh	PMSG	19 live birth	Yang <i>et al.</i> , 2006
Goat	Autotransplanted	15 slides (1 mm ³)	Uterus	Adult	Frozen-thawed	No	Restoration of ovarian function	Santos <i>et al.</i> , 2009
Sheep	Autotransplanted	Hemi-ovary	Hilus	Immature (6 months to 1 year)	Frozen/thawed	No	6 live birth	Salle <i>et al.</i> , 2003
	Autotransplanted	Whole ovary	Uterus	Immature (6 months)	Frozen/thawed	No	1 live birth	Imhof <i>et al.</i> , 2006
	Autotransplanted	Whole ovary	Rectus muscle	Adult (2–3 years)	Frozen/thawed	Chronogest sponge	Restoration of ovarian function	Grazul-Bilska <i>et al.</i> , 2008
	Autotransplanted	Whole ovary	Uterine horn	Adult (3–6 years)	Frozen/thawed	Aspirin, S1P, Vit E	7 live birth	Campbell <i>et al.</i> , 2014
Non-human Primates	Autotransplanted	1 mm	Subcutaneous	Adult (4–6 years)	Fresh and frozen/thawed	VEGF	Restoration of ovarian function	Schnorr <i>et al.</i> , 2002
	Autotransplanted	8×3×1 mm	Decorticate ovary	Adult	Vitrified/warmed	No	successful ovulation	Amorim <i>et al.</i> , 2013
	Allotransplanted	1–2 mm	Ovary medulla	Adult (24 years)	Fresh	No	1 live birth	Silber <i>et al.</i> , 2005
	Autotransplanted	5×5×(1–2 mm)	Subperitoneal wall	Adult (26–35 years)	Frozen/thawed	No	2 live birth	Andersen <i>et al.</i> , 2008
	Allotransplanted	1 mm, whole ovary	Ovary medulla, uterine horn	Adult (24–40 years)	Fresh and frozen/thawed	No	3 live birth, 3 pregnancy	Silber <i>et al.</i> , 2008
	Allotransplanted	10×6 mm and 2×1 cm	Ovary medulla	Adult (32 an 35 years)	Fresh	No	Restoration of ovarian function	Donnez <i>et al.</i> , 2010b
	Allotransplanted	2×1 cm	Ovary medulla	Adult (36 years)	Fresh	rec-hFSH, GnRH	1 live birth	Donnez <i>et al.</i> , 2011
	Autotransplanted	10 pc (5×5mm ²)	Subcapsular	Adult (37 years)	Frozen/thawed	Choriogonadotr-opin	1 live birth	Burmeister <i>et al.</i> , 2013
	Autotransplanted	30 pc (1×2×4 mm)	Intraperitoneal	Adult (21 years)	Frozen/thawed	FSH, LH, GnRH	pregnancy	Stern <i>et al.</i> , 2013
Autotransplanted	10–12pc 1×3×5 mm	Peritoneal and subperitoneal pockect, abdominal wall	Adult (23 years)	Frozen/thawed	Gonadotropin	1 live birth	Rodriguez-Wallberg <i>et al.</i> , 2015	

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would add another 7% of follicle loss (Baird *et al.*, 1999). Moreover, fresh or frozen human ovarian grafts transplanted into nude mice showed similar reduction in follicular density, demonstrating that ischemia is the main factor behind follicular depletion (Demeestere *et al.*, 2009). Thus, duration of warm ischemia before neovascularization is crucial for follicular survival and may be responsible for the major loss of follicles after grafting rather, than after freezing.

The mechanisms behind ischemic injury involves energy depletion and reperfusion oxidative stress (Demeestere *et al.*, 2009), which can cause damage to lipids, DNA, enzymes and structural proteins, therefore leading to cell death (Kim *et al.*, 2003). Gene expression of several inflammatory factors is initiated by hypoxia-sensitive response elements and nuclear factor-kappa beta (NF- κ B), resulting in transmigration of neutrophils and macrophages into the tissue (Vollmar *et al.*, 1995) that causes tissue destruction and fibrosis. However, primordial follicles still survive for relatively long periods of ischemia after avascular transplantation due to their very low metabolic demands. Moreover, they are distributed just under the ovarian surface and they may be the first to benefit from the ingrowth of new vessels. In a study with cryopreserved bovine ovarian cortex it was demonstrated that stromal cells are more vulnerable to ischemia compared with primordial follicles (Kim *et al.*, 2004). Due to the negative effects of ischemic time in the follicular pool, angiogenesis is the key mechanism for follicle survival.

Addition of exogenous substances to improve angiogenesis and ovarian graft

Many animal experiments have focused on improving ovarian tissue survival. Transplantation of frozen-thawed ovarian tissue in animal models led to antral follicle development and live birth (Schubert *et al.*, 2008). However, despite the successful results obtained with cryopreservation and autotransplantation of ovarian tissue, it is important to highlight the large follicle loss resulting from the latter procedure (Gavish *et al.*, 2014). In this context, many studies have been conducted with different substances such as antioxidants agents or angiogenic factors. Local antioxidant injection of vitamin E before grafting could improve follicular survival rate (Martinez-Madrid *et al.*, 2009). Ischemia and oxidative stress could be reduced by using drugs to stimulate revascularization of the graft, such as vascular endothelial growth factor or sphingosine-1-phosphate (S1P), as described by Donnez & Dolmans, (2013). A study carried out by Zelinski *et al.* (2011) showed that S1P and its analogues

protected the primate ovary against radiation-induced damage. In addition, Scalercio *et al.* (2015) showed that trolox, an analogue of vitamin E improved follicular quality and avoided apoptosis in stromal cells when ovarian tissue is pre-treated with this substance. Moreover, a study showed that autografted mice ovary followed by daily administration of L-carnitine until day 7 inhibited follicle apoptosis, relieved oxidative damage, and increased follicular survival and function in ovarian graft (Zhang *et al.*, 2015).

To reduce the hypoxic period after tissue transplantation and improve follicular preservation, angiogenesis can be stimulated by vascular endothelial growth factor (VEGF), which is the main signaling protein that regulates new vessel formation from pre-existing vessels (Henry *et al.*, 2015).

Ovarian cryopreservation and transplantation procedures have so far been almost exclusively limited to avascular cortical fragments. The small cortical pieces grafted without vascular anastomosis are completely dependent on the establishment of neovascularization after grafting. Consequently, the cells in the graft undergo significant ischemic and reperfusion damage, which can induce a high rate of follicular loss (Zhang *et al.*, 2016). Therefore, these cells remain quiescent until there is an angiogenic stimulus such as hypoxia or wounding, which then up-regulates proangiogenic factors, such as VEGF (Gerhardt & Betsholtz, 2003). Angiogenesis is regulated in the reproductive tract and elsewhere by at least 20 angiogenic growth factors and inhibitors identified to date. A key player is VEGF also known as vascular permeability factor (Ferrara and Davis-Smyth, 1997). It has been shown that VEGF acts via two tyrosine kinase family receptors, namely flt-1 (VEGF receptor; VEGFR-1) and flk-1/KDR (Ferrara and Davis-Smyth, 1997), which are expressed in granulosa cells (Greenaway *et al.*, 2004). The effects of two VEGF-A isoforms, VEGF111 and VEGF165, have been tested in a xenograft model using SCID mice (Commin *et al.*, 2012). VEGF111 is soluble and resistant to proteolysis, whereas VEGF165 is additionally anchored to the extracellular matrix. Both VEGF isoforms increase blood vessel recruitment and functional angiogenesis. The use of this isoform in a collagen matrix, which encapsulates the ovarian cortex at the time of transplantation was found to improve angiogenesis and decrease hypoxia, thereby enhancing the preservation of primary follicles (Labied *et al.*, 2013). Moreover, it was demonstrated (Henry *et al.*, 2015) that encapsulation of ovarian tissue with VEGF165 in a collagen matrix during xenografting in mice produces a more rapid onset of functional vessel formation and earlier revascularization of the transplant. In addition, Campbell *et al.* (2014) used a post-operative regime of anti-thrombotic (aspirin) agents to reduce post-transplant ischemia.

This substance prevents clot formation in the ovarian vasculature induced by cryo- and cyto-toxic damage to the arterial endothelial cells.

Final considerations

This review reinforces the fact that cryopreservation of ovarian tissue for further transplantation is helpful for prepubertal and women of reproductive age or patients for whom cancer treatment cannot be delayed to perform another assisted reproductive technique such as oocyte or embryo cryopreservation. Because preparation and stimulation for oocyte retrieval usually requires 2 to 3 weeks or longer, it is generally not feasible to freeze embryos from an adult female cancer patient for potential future use. Additionally, not all patients have partners with whom they can produce embryos to cryopreserve. Moreover, in contrast with oocytes and embryos, cryopreservation of ovarian tissue does not depend on the age and phase of the patient's estrous cycle, it also involves fewer ethical and social issues especially when this process is performed in humans.

Most of the experimental studies conducted with the transplant of cryopreserved ovarian tissue have the aim to re-establish fertility. However, in special situations, when it is necessary to remove the normal ovaries due to cancer or infectious processes of the pelvic organs, the absence of ovarian hormones may lead to several endocrine and functional disorders such as osteoporosis, reduction of libido, sexual dysfunction, and enhancement of lipoprotein levels. In such conditions, transplantation of cryopreserved ovarian tissue is the main solution to overcome this problem. However, despite the good results obtained by ovary cryopreservation following transplantation, it appears that the main obstacle to successfully restore fertility from frozen-thawed ovarian cortex are adhesions and the massive ischemic damage to follicles until neovascularization develops. Most follicles surviving cryopreservation will undergo ischemic loss during the time required for neovascularization.

Therefore, the optimization of ovary transplantation may require preparing the right thickness of tissue and finding the best site of transplantation. This could avoid ischemia and promote rapid revascularization. Angiogenesis is a key factor for graft survival because right after transplantation the lack of connective tissue between the graft and surrounded tissue is leading to hypoxia resulting in cell death. In this context reducing the ischemia period could enhance the success of transplantation. In this case, further animal model studies are required to develop optimized cryopreservation/transplant protocols for women.

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