FERTILITY PRESERVATION

Follicle reserve loss in ovarian tissue transplantation

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Abstract

Ovarian tissue cryopreservation and transplantation (OTCP-TP) has progressed over the past decade from a revolutionary experimental procedure to a well-accepted treatment in many centers for young patients with a high risk of ovarian failure after cancer treatment. The procedure is remarkably successful, with studies reporting return of ovarian function in up to 95% of graft recipients and pregnancy rates of between 30 and 50%. The most significant limitation of OTCP-TP is the massive loss of follicles that occurs following transplantation, which is primarily attributed to ischemic damage and follicle activation. We review the current approaches to reducing follicle loss and maximizing graft lifespan via pharmacological agents which reduce ischemic damage and follicle activation. We further discuss the value and disadvantage of inducing follicle activation in the graft as a means of generating mature follicles in the immediate short term.

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Introduction

Since the first successful live births post transplantation of cryopreserved stored ovarian tissue (Donnez *et al.* 2004, Meirow *et al.* 2005), ovarian tissue cryopreservation and transplantation (OTCP-TP) has progressed from a revolutionary experimental procedure to a well-accepted treatment in many centers for young patients with a high risk of ovarian failure after cancer treatment. Hundreds of graft transplantations have been performed to date, with a pregnancy rate of between 30 and 50% and over 130 live births (Gellert *et al.* 2018). In some countries OTCP has become standard care covered by national health authorities (Meirow *et al.* 2016). There has been no reported increase in rate of birth defects or other abnormalities in children born from transplanted ovarian tissue (Rodriguez-Wallberg *et al.* 2016). Ovarian tissue cryopreservation is most commonly performed for medically indicated fertility preservation in young cancer patients facing potentially ovotoxic treatments. Although return of ovarian function occurs in 95% of ovarian tissue transplantations, the duration of graft survival is variable; in some cases, a single graft has maintained function for years, producing as many as four consecutive pregnancies, while other grafts cease functioning within a few months (Andersen *et al.* 2012, Donnez & Dolmans 2015, Meirow *et al.* 2016). The total number of follicles present in any particular graft is

influenced by patient age, natural variability, exposure to chemotherapy treatments prior to tissue harvesting as well as graft dimensions. However, while these factors define the initial starting reserve in the graft, many studies have shown that the vast majority (approximately 80%) of these initial follicles are lost through the process of OTCP-TP (Liu *et al.* 2002, Dolmans *et al.* 2007, Hancke *et al.* 2009, Dath *et al.* 2010, Gavish *et al.* 2018), such that what ultimately defines the lifespan of the graft is the final number of primordial follicles in the graft after transplantation that survive the procedure. This dramatic loss of follicle reserve is considered a significant limitation of OTCP-TP (Gosden *et al.* 1994, Nugent *et al.* 1998, Dolmans *et al.* 2007); therefore, analysis of the causes of this loss is necessary for the development of new techniques to maximize graft lifespan and optimize OTCP-TP (Kawamura *et al.* 2015, Silber 2016).

Follicle loss in ovarian transplantation

OTCP-TP involves a multitude of steps that each contribute to a different extent to the overall decline in follicle numbers (Fig. 1). Briefly, a section of the ovary, or the entire ovary, is removed from the patient, following which the medulla is removed, and the cortex is mechanically thinned so that almost all the larger growing follicles are removed and the remaining tissue

Figure 1 Loss of ovarian reserve with each stage of ovarian tissue cryopreservation and transplantation. Different degrees of follicle loss occur at each stage of the cryopreservation and transplantation procedure and are influenced by specific factors. At each stage of the cryopreservation and transplantation procedure, various factors affect the degree of follicle loss that occurs. However, it is estimated that the vast majority of the 80% of primordial follicles lost during the procedure are lost in the final post grafting stage.

comprises cortical stroma containing mostly primordial as well as some primary and secondary follicles (Donnez & Dolmans 2010, Lee *et al.* 2016). The tissue is then equilibrated with cryoprotectants prior to being frozen and stored in liquid nitrogen (almost all OTCP conducted to date has used the slow-freezing protocol, and a comparison of the advantages or disadvantages of slow freezing vs vitrification is beyond the scope of this review). At the time of transplantation the tissue is thawed, washed from the cryoprotectants media and placed into the non-functioning ovary or intraperitoneal pocket, the method used by the first studies which achieved live births using OTCP-TP (Donnez *et al.* 2004, Meirow *et al.* 2005).

In order to evaluate graft follicle loss or survival, it is important to differentiate between two different populations of follicles; the dormant primordial follicles that comprise the reserve and the fertility potential of the graft, and the larger, developing follicles. The largest growing follicles, the antral follicles, are lost by mechanical injury during tissue preparation, and smaller growing follicles are lost in the process of cryopreservation since they are highly vulnerable to freezing and thawing (Candy *et al.* 1997, Baird *et al.* 1999, Aubard 2003, Liu *et al.* 2008). This may explain why the return of endocrine function and the production of mature follicles occurs on average 4–5 months after transplantation (Donnez *et al.* 2013, Silber 2016), a time period that aligns with the 4–6 months that it is believed to take for PMFs to develop to that stage in the human ovary (Gougeon 1986), although this estimate is not absolute. The population of dormant follicles is less susceptible to cryopreservation injury than the developing follicles (Newton *et al.* 1996, Gosden *et al.* 2002, Camboni *et al.* 2008), and studies comparing transplanted fresh and frozen-thawed tissue show similar rates of follicle survival (Amorim *et al.* 2011) and functioning (Silber 2015), which strengthens the inference that cryopreservation does not contribute greatly to the

loss of primordial follicles in tissue grafts. These data are based on small sample sizes, and additional study is needed to clarify this point since it is otherwise possible that the steps of tissue preparation and cryopreservation might induce damage that subsequently predisposes the follicles to die after transplantation, in which case changes in these earlier stages could have a significant impact on follicle survival and graft lifespan.

Studies assessing follicle loss in ovarian tissue grafts indicate that while each step of the OTCP-TP procedure contributes to the loss of primordial follicle reserve, the most significant loss occurs in the post-transplantation period (Fig. 1) (Gosden *et al.* 1994, Nugent *et al.* 1998, Aubard *et al.* 1999, Baird *et al.* 1999, Dolmans *et al.* 2007, von Schonfeldt *et al.* 2012, Gavish *et al.* 2018). It is estimated that at least 75% of the follicles which are lost during the process of OTCP-TP are lost during transplantation as the graft re-vascularizes and regains homeostasis in a new environment. For this reason, optimization efforts on the post-transplantation stages are likely to have the greatest impact on the final follicle reserve.

Causes of transplantation-induced primordial follicle loss

Two mechanisms have been proposed to contribute to the massive follicular loss that occurs post transplantation: ischemia and activation.

Initial removal of the ovary from its blood and oxygen supply begins a period of ischemia that is temporarily paused during cryopreservation, only to continue upon thawing and transplantation (McCord 1985, Van Eyck *et al.* 2010, Donnez *et al.* 2013). Follicle survival depends on rescue of the tissue from the initial period of ischemia and hypoxia with neovascularization. The beginnings of neovascularization have been observed 3 days following transplantation of human ovarian tissue onto a chick chorioallantoic membrane (Martinez-Madrid

et al. 2009), and it is estimated that the transplanted graft exists in a hypoxic environment with nutrients and oxygen supplied via diffusion from surrounding tissue for a period of approximately 3–5 days, (Van Eyck *et al.* 2009). It can take up to 10 days before revascularization is able to provide full oxygenation and nutrients to the graft (Aubard *et al.* 1999, Israely *et al.* 2004, Cacciottola *et al.* 2018). Angiogenesis from both the host and the graft itself have been shown to contribute to neovascularization of the graft (Van Eyck *et al.* 2010).

During this period of angiogenesis the grafted tissue is subjected to ischemic reperfusion injury, resulting in the production of inflammatory mediators, free radicals and reactive oxygen species (Demeestere *et al.* 2009, Commin *et al.* 2012). Ischemic reperfusion injury has been shown to cause follicle loss primarily via effects on the granulosa cells of growing follicles, while primordial follicles are thought to be less vulnerable to ischemic injury since as dormant cells they have a low metabolic rate (Kim *et al.* 2004b, Harris *et al.* 2009, Bols *et al.* 2010) and are accustomed to a relatively hypoxic environment in the poorly vascularized outer ovarian cortex (Gosden & Byatt-Smith 1986). Primordial follicles are not entirely resistant to the effects of ischemia; however, as some methods aimed at reducing the ischemic period, there have been improvements in primordial follicle survival in transplanted grafts (Table 1).

The significant primordial follicles loss that occurs shortly after transplantation has been shown to be simultaneously accompanied by a significant increase in early growing follicles populations and increased proliferation of granulosa cells in transitional and early growing follicles (Oktay *et al.* 2000b, Dolmans *et al.* 2007, Dath *et al.* 2010, Amorim *et al.* 2011, Ayuandari *et al.* 2016, Gavish *et al.* 2018) (Fig. 2). Although only proportional increases in growing follicle populations were shown in some of these studies, which could be explained by the loss of PMFs rather than any actual increase in growing follicle numbers, this is unlikely. The process of transplantation causes death of growing follicles, so that if even a similar number of growing follicles are seen in the transplanted tissue this would indicate that some process of activation had occurred. Moreover, most of these studies present evidence of significant proliferation in transitional and early growing follicles (80–95% of these follicles were shown to be actively proliferating in the transplanted graft, compared to only 18–40% in untransplanted tissue (Dolmans *et al.* 2007, Dath *et al.* 2010)). A significant rise in anti-Müllerian hormone (AMH) was also observed in patients who underwent transplantation of ovarian tissue in the immediate post-transplantation period before subsequently returning to low, sometimes undetectable levels (Silber 2016). These data suggest a phenomenon of mass activation of the PMF pool that occurs immediately

Table 1 Agents tested for use as pharmacological fertility preservation agents in animal studies.

Substance	Mechanism	Impact on PMF reserve	Graft species	Study
Increasing vascularization				
Stem cells	Angiogenesis	Non-statistical decrease in PMF after 30 days	Mouse	Damous <i>et al.</i> 2015
VEGF	Angiogenesis	Increased PMFs (21 days)	Human	Xia <i>et al.</i> 2015
		Increased PMFs (7 days)	Human	Manavella <i>et al.</i> 2018
		Increased PMFs (10 days), no difference	Mouse	Shikanov <i>et al.</i> 2011
		Non-statistical increase in PMF (3 weeks)	Human	Wang <i>et al.</i> 2013
bFGF	Angiogenesis	No assessment of PMF number	Sheep	Labied <i>et al.</i> 2013
		Increased PMF (6 weeks)	Human	Abir <i>et al.</i> 2011, Friedman <i>et al.</i> 2012
		Increased PMFs (7 days)	Human	Tanaka <i>et al.</i> 2018
FSH	Angiogenesis	No difference	Mouse	Gao <i>et al.</i> 2013
Simvastatin, methylprednisolone*	Angiogenesis, anti-inflammatory	No assessment of PMF numbers	Human	Wang <i>et al.</i> 2013
		No increase in PMFs (21 days)	Mouse	Ma <i>et al.</i> 2017
			Mouse	Lee <i>et al.</i> 2015
Reducing ischemic injury				
Verapamil	Ca channel blocker	Increase PMFs (2 weeks)	Mouse	Saber <i>et al.</i> 2018
Erythropoietin	Antioxidant	No difference	Canine	Commin <i>et al.</i> 2012
N-acetylcysteine	Antioxidant	Increased PMFs (28 days)	Mouse	Mahmoodi <i>et al.</i> 2014
		Increased PMFs (28 days)	Mouse	Mahmoodi <i>et al.</i> 2015
Vitamin E	Antioxidant	No significant difference (7 days)	Human	Nugent <i>et al.</i> 1998
S1p	Anti-apoptosis	No assessment of PMF number	Human	Abir <i>et al.</i> 2011, Friedman <i>et al.</i> 2012
		No assessment of PMF number	Human	Soleimani <i>et al.</i> 2011
GnRH agonist		Decrease in PMFs (15 weeks)	Human	Maltaris <i>et al.</i> 2007

*Simvastatin and methylprednisolone increase vascularization and reduce ischemic injury.

Figure 2 Increased proliferation seen in early growing follicles in transplanted tissue grafts. (A) Total (including all follicular stages) follicular proliferation rate measured by evaluating the percentage of Ki-67-positive follicles in ungrafted tissue and grafted fragments. (B) Follicular proliferation rate for each follicular stage in the three groups (c – significantly different from d, $P < 0.05$. e significantly different from g, $P < 0.001$). Adapted with permission from Dolmans *et al.* (2007).

post transplantation and could be a significant contributing cause of the loss of reserve in ovarian tissue grafts (Roness *et al.* 2013, Meirow *et al.* 2015). This over-recruitment of PMFs in the graft may be triggered by the extensive loss of the growing follicles that results from the mechanical preparation of ovarian tissue strips, the freezing-thawing procedure as well as post-transplantation ischemia. Growing follicles play a vital role in maintaining PMF suppression via the excretion of suppression factors, primarily AMH (Durlinger *et al.* 2002), and therefore, the loss of growing follicles and the resulting absence of AMH suppression could be responsible for the mass

PMF activation and follicle growth that occurs post transplantation (Roness *et al.* 2013).

Mechanical changes to the extracellular environment have also been shown to be involved in regulation of follicle activation. Mechanical fragmentation of ovarian tissue grafts was shown to cause extracellular actin polymerization, inducing disruption of Hippo signaling in the granulosa cells, increasing nuclear localization and decreasing phosphorylation of YAP (Kawamura *et al.* 2013). The decrease in pYAP-induced proliferation and growth of granulosa cells and follicle activation in fragmented grafts. The importance of the physical environment provided by the extracellular matrix is also highlighted by another recent study which demonstrated that mechanical stress plays a key role in maintaining follicle dormancy and that reduction of this stress triggers follicle activation and growth (Nagamatsu *et al.* 2019).

Prevention of PMF loss in transplanted grafts

Different approaches have been used in attempts to reduce follicle loss and optimize OTCP transplantation outcomes addressing different stages of the procedure.

Transplantation site

While almost all OTCP is performed at orthotopic sites; in the atrophic cortex of the existing ovaries or in peritoneal pockets created in the pelvis, the broad ligament or ovarian fossa, there have been studies which investigated the use of heterotopic sites (Oktay *et al.* 2001, 2004, Kim *et al.* 2004a, Demeestere *et al.* 2007). Heterotopically transplanted grafts have been reported to produce a high number of empty follicles, arrested follicle development (<15 mm), poor oocyte recovery and low fertilization rates (Dolmans *et al.* 2009, Greve *et al.* 2012), possibly due to factors such as environmental temperature, local pressure, paracrine factors and blood supply (Donnez & Dolmans 2010). Very few live births have been achieved from grafts implanted at heterotopic sites; in one case, transplantation to the abdominal wall actually invaded the abdominal cavity (Stern *et al.* 2013) and in the second case the graft was placed in the pelvis (Kristensen *et al.* 2017). Animal studies comparing xenotransplantation into different sites including muscle, fat pad, kidney capsule and subcutaneous (Dath *et al.* 2010, Youm *et al.* 2015) uniformly show that orthotopic sites are most favorable and retain the highest percentage of PMFs, and this is supported by clinical results from the centers around the world which demonstrate high success rates performing transplantation to orthotopic sites (Donnez & Dolmans 2015, Jensen *et al.* 2015, Meirow *et al.* 2016).

Graft dimensions

Graft dimensions have been shown to play a significant role in reducing follicle loss. Thinning the cortical strip

prior to freezing is critical in order to enable good penetration and evacuation of cryoprotective agents, reduce the likelihood of ice crystal formation and injury and to provide a shorter distance for new blood vessels to traverse to reduce the ischemic period (Gavish *et al.* 2008). Commonly, (Oktay *et al.* 2000a) the ovarian cortex is prepared in strips of 1–2 mm thickness; however, in terms of diffusion of oxygen distances greater than 0.2 mm are generally poorly tolerated (Loffredo & Lee 2008), and for larger molecules like growth factors and nutrients, diffusion distances are even shorter. It was therefore assumed that reducing graft dimensions and thickness would reduce ischemia by expediting graft vascularization, (Kagawa *et al.* 2009, Ferreira *et al.* 2010). Attempts have been made to create thinner grafts – ‘micro-organs’ – to reduce this distance even further (Revel 2011); however, a xenograft study which directly compared the impact of graft dimensions (Gavish *et al.* 2014) found that reducing graft thickness does not increase revascularization of the graft. Reducing the thickness of the graft had the additional adverse effect of increasing follicle activation, resulting in increased loss of dormant follicles (Fig. 3). Massive activation related to very small tissue dimensions was highlighted by studies which used fragmentation of graft tissue to increase follicle activation and demonstrated that cutting of the tissue disrupts the Hippo pathway, triggering follicle growth (Kawamura *et al.* 2013).

Pharmacological methods of reducing transplantation-induced follicle loss

Reducing ischemic injury

To reduce ischemic damage during the post-transplantation period, studies have investigated the use of pharmacological treatments which aim either

Figure 3 Total follicle (black) and primordial follicle (gray) in thin vs conventional grafts. Comparison of follicle counts in fresh or frozen-thawed thin or conventionally prepared bovine ovarian tissues recovered 7 days post transplantation. Values represent the mean per graft $\pm$ SE. $P < 0.05$: a = compared with control, b = compared with fresh conventional, c = compared with frozen conventional. (Reprinted with permission from Gavish *et al.* 2014.)

to reduce the time period of ischemia by accelerating graft re-vascularization (with angiogenesis agents) or by reducing the harmful effects of ischemia (with antioxidants, anti-inflammatory and anti-apoptotic agents) (see summary, Table 1). However, only a few of these have been shown to increase PMF survival. Mahmoodi *et al.* tested two antioxidants; erythropoietin (Mahmoodi *et al.* 2014) and N-acetylcysteine (Mahmoodi *et al.* 2015) administered just before and during the first week following transplantation of autografts in mice. They demonstrated almost identical results with each of the treatments, including reduced apoptosis and increased survival of PMFs in grafts which received antioxidant treatment. Both mesenchymal (Xia *et al.* 2015) and adipose tissue-derived (Manavella *et al.* 2018) stem cells were shown to increase vascularization and increase PMF survival in xenotransplantation models using human ovarian tissue grafts. Antigenic growth factor basic fibroblast growth factor (bFGF) has been shown to increase angiogenesis and follicle survival in mouse (Gao *et al.* 2013) and human ovarian tissue grafts (Tanaka *et al.* 2018). A number of studies looked at another angiogenic growth factor, vascular endothelial growth factor (VEGF), with mixed results; only one study reported an increase in PMF population in VEGF-treated grafts (Shikanov *et al.* 2011). Culture of ovarian tissue with FSH prior to transplantation was shown to increase VEGF and bFGF expression and vascular density; however, no assessment of follicle numbers was made (Ma *et al.* 2017).

Other agents which aimed to reduce ischemic injury, such as Vitamin E, S1p, simvastatin and methylprednisolone, were reported in some cases to shorten the ischemic period, reduce apoptosis and improve certain outcomes, but in many cases PMF numbers were not assessed and in others the treatment did not increase follicle survival. The only agent which did result in increased survival of PMFs was verapamil, a calcium channel blocker (Saber *et al.* 2018); however, the mechanism of action is unclear. The least efficacious were GnRH agonists, which were hypothesized to prevent PMF loss via downregulation of LH/FSH secretion or via angiogenesis (upregulation of VEGF) and in fact increased PMF loss in the grafts (Maltaris *et al.* 2007).

Reducing follicle activation and loss

Since the discovery that a contributing factor in follicle loss after transplantation is dysregulated follicle activation (Dath *et al.* 2010, Amorim *et al.* 2011), some groups have investigated the possibility of protecting the graft ovarian reserve by preventing this activation (Fig. 4). AMH is a highly specific suppressor of follicle activation and two recent studies have used recombinant human AMH in human and mouse models of transplantation with varying degrees of success. One study pretreated the tissue prior to transplantation or injected AMH

Figure 4 Potential molecular mechanisms and targeted manipulation of follicle activation in ovarian tissue grafts after transplantation. Studies have investigated the role of transplantation-induced activation and loss of dormant follicles in ovarian tissue grafts via three molecular pathways; activation of the PI3K/PTEN and the Hippo pathway and via reduction in AMH. Targeted manipulation of each of these pathways at the time of transplantation has been shown to impact on the extent of follicle activation in the graft, and thereby on the final dormant follicle reserve remaining in the tissue after transplantation. Fragmentation of the tissue activates the Hippo pathway, phosphorylating YAP which then localizes to the nucleus, increasing follicle activation. Culture of the tissue in the presence of PTEN inhibitors or PI3K pathway activators induces upregulation of the PI3K pathway also resulting in increased follicle activation. Conversely, administration of AMH during transplantation reduces dormant follicle activation, resulting in increased follicle reserve in the graft following transplantation.

into the recipient mouse but found no increase in the primordial follicle reserve as a result of either treatment up to 28 days after transplantation, possibly due to the fact that they injected a relatively low dose of AMH only every 48 h (Kong *et al.* 2016). A second study used continuous treatment of recipient mice with AMH in intra-abdominal pumps, but also saw no change in graft reserve compared to untreated mice (Detti *et al.* 2018). Both studies observed reduced apoptosis in AMH-treated grafts, but the data are not strong and additional study is needed since there is no previous indication that AMH plays any direct role in apoptosis signaling. AMH did, however, prevent transplantation-induced follicle loss in a study which transplanted human ovarian tissue together with exogenous endothelial cells (ExECs) engineered to express AMH (Man *et al.* 2017). ExECs alone promoted formation of blood vessels at the interface of graft and host tissue, improving vascularization and reducing fibrosis, but tissue grafts transplanted with AMH-ExECs retained the highest numbers of primordial follicles with fewer growing follicles, indicating that AMH was suppressing follicular activation. This combined

approach which both increases vascularization and reduces follicle activation shows particular potential for reducing follicle loss after transplantation.

Programmed acceleration of follicle activation in grafts

Rather than reduce follicle loss, an alternative manipulation of OTCP-TP has focused on increasing the activation of dormant follicles within the graft cortical tissue in the immediate short term prior to transplantation to enable collection of mature oocytes for *in vitro* fertilization. Termed '*in vitro* activated' (IVA) OTCP (Kawamura *et al.* 2013, Suzuki *et al.* 2015), this method involves fragmentation of the tissue grafts into small cubes of 1–2 mm², and *in vitro* culture of the graft fragments with PTEN inhibitor (bisphosphonate) and PI3K activator (740YP). Fragmentation promotes actin polymerization within the graft, disrupting Hippo signaling, which promotes follicle growth (Kawamura *et al.* 2013). Incubation of the tissue with a PTEN inhibitor and a PI3K activator, aims to induce upregulation of the PTEN-PI3K signaling pathway, which has been shown to stimulate activation and growth of dormant primordial follicles both in animal models (Adhikari & Liu 2009) and in humans (Li *et al.* 2010) (Fig. 4). While two live births have been achieved using this method (Kawamura *et al.* 2013, Suzuki *et al.* 2015), there are safety concerns regarding chemical exposure of the tissue and a recent study reported that ovarian graft exposure to bisphosphonate increased DNA damage in oocytes of growing and PMFs and decreased expression of DNA repair proteins (Maidarti *et al.* 2019). More recent use of IVA has removed the step of chemical activation and focused on tissue fragmentation only, which was found to be sufficient to induce activation (Fabregues *et al.* 2018). IVA has been conducted on 51 patients, with a total of 5 (10%) resulting pregnancies and 3 (6%) live births. Given that in general, POI patients have unpredictable ovarian function, and reports indicate a 5–10% rate of spontaneous pregnancy (van Kasteren & Schoemaker 1999), the success of IVA may not exceed that of no intervention at all.

The promotion of dormant follicle activation, while producing a short-term burst of mature follicles, will also by necessity reduce the PMF population and therefore the lifespan of the graft (Fig. 5). OTCP is currently indicated for use primarily in cancer patients who will autotransplant the tissue once they are in remission. The aim of graft transplantation in such patients is to restore ovarian function for as long as possible, providing the greatest benefit in terms of endocrine function and fertility from each piece of cortex. For this purpose, the loss of large numbers of primordial follicles via activation is counterproductive (Meirow *et al.* 2015). In contrast, IVA OTCP is currently aimed at treating women with extremely low ovarian reserve and who are therefore

Figure 5 The magnitude of follicle activation directly impacts PMF reserve in ovarian grafts. (A) Inducing activation in ovarian follicle grafts increases the number of growing follicles in the immediate short term at the expense of the PMF population, resulting in decreased graft lifespan. This may be suitable for immediate IVF retrieval but comes at the expense of the overall fertility potential and long-term functioning of the graft. (B) Limiting activation at the time of transplantation reduces PMF loss thereby extending graft lifespan. It is preferable to reduce the activation that occurs with transplantation in order to maximize the fertility outcomes of the procedure. (Reprinted with permission from Gavish *et al.* 2018.)

poor responders to controlled ovarian stimulation (Kawamura *et al.* 2015). In these women, the acceleration of activation aims to stimulate the few remaining follicles to grow during the immediate time post transplantation.

Intra- and extra-ovarian regulation of follicle recruitment in ovarian grafts

Given the low numbers of follicles that survive OTCP and transplantation, it is surprising that ovarian grafts can and do last for periods of years, generating repeated pregnancies and live births (Jensen *et al.* 2015, Meiorow *et al.* 2016). The rate of follicle activation and loss over the course of the human reproductive span is non-linear and regulated by both intra- and extra-ovarian signaling, controlling a complex interplay between rates of recruitment, growth, selection and atresia (Gougeon 1996, Hansen *et al.* 2008, Wallace & Kelsey 2010, Wilkosz *et al.* 2014). Intra-ovarian regulation of follicle recruitment derives from numerous signals, including the PI3K-PTEN pathway, kit ligand, AMH, nerve growth factor (NGF) and members of the BMP family of proteins. In addition, several studies have suggested that the architecture of the ovary and the density of the population of primordial follicles plays a self-regulating role, such that a reduction in the pool of dormant follicles reduces the rate of recruitment (Krarup *et al.* 1969, Hirshfield 1994, Gaytan *et al.* 2015). The influence of extra-ovarian factors is evidenced in studies which look at the age of menopause in patients who underwent unilateral oophorectomy, effectively halving their ovarian reserve. The age at menopause in these patients is surprisingly unaffected by this radical reduction in reserve, with onset only 1–2 years before the normal age at menopause (Bjelland *et al.* 2014, Rosendahl *et al.* 2017), rather than the expected 10-year reduction in fertility that would be predicted if there were no adjustment in follicle recruitment (Wilkosz *et al.* 2014). This ability of the body to ‘adjust’ the rate of follicle recruitment to conserve follicles in the case of sudden loss of a large proportion of the ovarian reserve

may also play a role in OTCP. It is possible that following the initial acute phase of transplantation, both intra-ovarian and extra-ovarian regulation factors identify and respond to the limited reserve in the transplanted graft by adjusting the rate of recruitment in order to reduce superfluous follicle activation and loss and maximize the lifespan of that reserve.

Conclusion

OTCP-TP is increasingly offered as a fertility preservation option to young women undergoing ovotoxic treatments, resulting in a growing number of pregnancies and live births around the world. The objective of OTCP is to restore ovarian function for as long as possible, both in order to enable multiple ovulations and pregnancies and for restoration of endocrine function. Optimization of the procedure therefore focuses on reducing follicle loss in the graft as the basis for extending graft lifespan and productivity. As the vast majority of dormant follicles in the graft are lost following transplantation, developing methods that will reduce follicle loss at this stage is key. Techniques such as reducing graft dimensions or heterotopic grafting have been shown to negatively impact follicle survival and graft functionality. Pharmacological methods that decrease ischemic damage and/or reduce follicle activation can potentially reduce follicle loss after transplantation. Techniques which act to increase follicle activation have been used in specific cases, such as recent onset premature ovarian insufficiency, but with little success. In cases of fertility preservation, IVA will reduce graft lifespan and is therefore not recommended for the majority of women undergoing OTCP. Reducing follicle wastage and thereby increasing the productivity of the procedure will provide greater long-term benefit to these patients.

Declaration of interest

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H R and D M both conducted the review and wrote the paper.

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