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.

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...
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. 2013).
The significant primordial follicle 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-Mullerian 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

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mechanism</th>
<th>Impact on PMF reserve</th>
<th>Graft species</th>
<th>Study</th>
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<tr>
<td>Increasing vascularization</td>
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<td>Stem cells</td>
<td>Angiogenesis</td>
<td>Non-statistical decrease in PMF after 30 days</td>
<td>Mouse</td>
<td>Damous et al. 2015</td>
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<td></td>
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<td>Increased PMFs (21 days)</td>
<td>Human</td>
<td>Xia et al. 2015</td>
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<td>Increased PMFs (7 days)</td>
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<td>Manabella et al. 2018</td>
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<td>Increased PMFs (10 days), no difference</td>
<td>Mouse</td>
<td>Shikanov et al. 2011</td>
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<td>Non-statistical increase in PMF (3 weeks)</td>
<td>Human</td>
<td>Wang et al. 2013</td>
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<tr>
<td>VEGF</td>
<td>Angiogenesis</td>
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<td></td>
<td></td>
<td>No assessment of PMF number</td>
<td>Human</td>
<td>Abir et al. 2011, Friedman et al. 2012</td>
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<td>Increased PMF (6 weeks)</td>
<td>Human</td>
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<td>Increased PMFs (7 days)</td>
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<td>Gao et al. 2013</td>
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<td>No difference</td>
<td>Human</td>
<td>Wang et al. 2013</td>
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<tr>
<td>FSH, Simvastatin, methylprednisolone</td>
<td>Angiogenesis, Anti-inflammatory</td>
<td>No assessment of PMF numbers</td>
<td>Mouse</td>
<td>Ma et al. 2017</td>
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<td>bFGF</td>
<td>Angiogenesis</td>
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<td>Increased PMF (21 days)</td>
<td>Mouse</td>
<td>Lee et al. 2015</td>
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<tr>
<td>Reducing ischemic injury</td>
<td>Ca channel blocker, Antioxidant</td>
<td>Increase PMFs (2 weeks)</td>
<td>Mouse</td>
<td>Saber et al. 2018</td>
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<td>No difference</td>
<td>Canine</td>
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<td>Erythropoietin</td>
<td>Antioxidant</td>
<td>Increased PMFs (28 days)</td>
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<td>Increased PMFs (28 days)</td>
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<td>N-acetylcysteine</td>
<td>Antioxidant</td>
<td>No significant difference (7 days)</td>
<td>Human</td>
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<td>No assessment of PMF number</td>
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<td>Vitamin E</td>
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<td>Decrease in PMFs (15 weeks)</td>
<td>Human</td>
<td>Maltaris et al. 2007</td>
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<td>S1p, GnRH agonist</td>
<td>Anti-apoptosis</td>
<td>No assessment of PMF number</td>
<td>Mouse</td>
<td>Soleimani et al. 2011</td>
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*Simvastatin and methylprednisolone increase vascularization and reduce ischemic injury.
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 heterotropic sites (Oktay et al. 2001, 2004, Kim et al. 2004, 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 heterotropic 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...
Follicle wastage in ovarian tissue grafts

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 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 3](https://rep.bioscientifica.com)
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 (bisperoxovanadium) 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 bisperoxovanadium 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 & Schoemakers 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
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, Meirow 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

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.
Author contribution statement

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

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Follicle wastage in ovarian tissue grafts

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