Testicular transplantation for fertility preservation: clinical potential and current challenges

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Abstract

Transplantation of testicular tissues and cells has been proposed as a future clinical option for patients who have had testicular tissue cryopreserved prior to receiving gonadotoxic therapies. Whilst this approach remains experimental, success using animal models and successful transplantation of ovarian tissue resulting in live births in female patients provides optimism for the development of clinical applications involving transplantation of testicular tissue in males. Careful consideration must be given to patient groups that may benefit from this approach in the future. Current research is focused on optimising patient selection, methods for tissue cryopreservation and development of transplantation techniques that might restore sperm production or future fertility in males. Crucially, attention must be focused on ensuring safety of transplantation, including eliminating the potential for infection or re-introducing malignancy. Furthermore the genetic/epigenetic integrity of any gametes generated must be ensured to allow generation of normal offspring. This review will provide an overview of the current status of transplantation of testicular tissue and cells for fertility preservation in males.

Introduction

Cryopreservation of testicular tissue and/or cells prior to gonadotoxic treatment is increasingly used for fertility preservation in prepubertal boys (Picton et al. 2015). The ultimate goal is to use stored tissue to restore fertility or generate mature gametes for use in assisted reproduction technologies (ARTs). Transplantation of tissue or cells into the patient after treatment represents an important potential approach for fertility restoration. Prepubertal testicular cryopreservation for future transplantation has been advocated for a number of patient groups; however, there remain significant challenges before this becomes a reality for fertility restoration in clinical practice. Importantly, there are currently no studies reporting development of sperm following transplantation of prepubertal human testis tissue or spermatogonial stem cells (SCCs). Therefore, this approach remains experimental and careful consideration is required regarding patient selection, optimisation of surgical and cryopreservation procedures and development of effective techniques to generate sperm from transplanted tissue or cells. Whilst development of gametes is the primary objective, attention to patient safety should remain of paramount importance. This review will outline the need for fertility preservation in young males and the options available for specific groups of patients. We will focus primarily on studies involving immature human testis tissues and testicular transplantation in selected patient groups. We will describe the current status for each of the key stages from patient selection, through biopsy, to transplantation. Importantly, we will highlight the challenges in translating the process of testicular tissue cryopreservation and transplantation into viable future clinical applications.

Fertility preservation in males: establishing the need and potential options

A number of groups of young males are at risk of infertility in adulthood. These can be broadly classified into (1) those with abnormal testicular development as a result of an underlying clinical condition (e.g. Klinefelter, cryptorchidism) or (2) those in whom testicular development and function is considered normal but are due to receive gonadotoxic treatments. The latter group includes cancer patients and those requiring haematopoietic stem cell transplantation (HSCT) for a variety
of malignant and non-malignant conditions. Whilst reduced intensity HSCT regimens have been introduced in childhood cancer, there is evidence to suggest that the deleterious effect on germ cells, as indicated by a rise in FSH, is similar to that of traditional regimens (Panasiuk et al. 2015). Importantly, the number of patients who may benefit from cryopreservation of testicular tissue and cells has increased significantly in recent decades with childhood cancer survival rates now in excess of 80% (Ward et al. 2014).

For adults at risk of infertility, semen cryopreservation is an effective and established option to retain the potential to father biological children (van Casteren et al. 2008). This should be routinely offered, along with appropriate counselling and suitable facilities, to individuals facing gonadotoxic treatments (Anderson et al. 2015). However, fertility preservation is dependent on the clinician recognising the need for fertility preservation and providing appropriate counselling. This can often be a limiting factor for permitting patients to access fertility preservation services (Anazodo et al. 2019). However, this option is dependent on prior establishment of spermatogenesis, the ability to obtain a semen sample and acceptability of this approach to the patient (Anderson et al. 2015). For patients in whom gametes are present, testis tissue may be removed and stored for future use of sperm in ART. For prepubertal boys at risk of infertility in whom spermatogonia are the only germ cell type present, an alternative approach is required (Fig. 1). Modification of treatment regimen offers the possibility of using less gonadotoxic agents (Viviani et al. 1985), whilst shielding the gonad may be used to protect the gonad from radiotherapy damage (Wallace & Thomson 2003). Protecting the in situ testis from chemotherapy-induced damage using hormone suppression or cytoprotective agents would also be an attractive option. However, whilst such approaches have been shown to work in animal models, no such therapies have been shown to work in humans (Mitchell et al. 2009, Allen et al. 2018). Therefore, removal of testicular tissue for future generation of sperm using in vitro maturation or transplantation should be considered in boys due to receive gonadotoxic therapies (Medrano et al. 2018). For the remainder of this review we will focus specifically on obtaining testicular tissue, for storage as tissue or cells and subsequent use in transplantation (Fig. 2).

Patient selection for testicular cryopreservation

For individuals facing future infertility, counselling regarding the fertility risk should be provided. Options for fertility preservation should be discussed and offered where appropriate. Testicular tissue cryopreservation requires invasive surgery and therefore consideration to patient selection is required to ensure that this experimental approach is offered to those considered most likely to benefit from future clinical applications. Whilst criteria for patient selection have been published (Anderson et al. 2015), there remains variation between centres and there are no definitive clinical recommendations for patient selection. Furthermore, for the majority of centres where testicular tissue cryopreservation is being performed, it is conducted as part of an ethically approved clinical research study (Picton et al. 2015). Currently, the majority of patients for whom testicular cryopreservation is offered are those due to receive gonadotoxic treatments for a variety of malignant and non-malignant conditions (Picton et al. 2015, Ho et al. 2017), with selection criteria often excluding patients with known underlying testicular pathology (Anderson et al. 2015, Uijldert et al. 2017).

Pre-existing testicular pathology

Pre-existing testicular pathology may have an impact on the potential success of subsequent transplantation. Cryptorchidism is a common condition resulting in impaired testicular growth, reduction in spermatogonial numbers and impaired germ cell differentiation (Kollin et al. 2012), resulting in an increased risk of infertility (Cortes et al. 1996). Whilst testicular biopsy for
cryopreservation at the time of orchidopexy has been advocated in patients with cryptorchidism (Thorup et al. 2018), pre-existing impairment of germ cell development may affect the potential for generating sperm following transplantation. Furthermore, pre-existing impairment to the testicular somatic environment of the recipient testis may prevent further germ cell development. Similarly, for patients with Klinefelter syndrome, apparent loss of germ cells has been reported prior to puberty (Van Saen et al. 2018). An overall reduction in spermatogonial numbers have also been reported prior to puberty, with the appearance of fibrosis in peri-puberty (Van Saen et al. 2018). An overall reduction in spermatogonial numbers have also been reported prior to puberty. However, the timing of the loss of spermatogonia cannot be accurately determined from these studies. In addition, whether the process of biopsy, cryopreservation and transplantation in prepubertal patients with cryptorchidism or Klinefelter will further reduce spermatogonial number compared with leaving the tissue in situ remains to be determined.

Whilst the vast majority of pre- and peripubertal patients undergoing testicular biopsy for fertility preservation will have spermatogonia present in the testis (Wyns et al. 2011), evidence for reduced number of spermatogonia has been reported in biopsies obtained from prepubertal boys with cancer who have received no prior treatment (Pietzak et al. 2015). Whilst total germ cell numbers were either normal or increased in 27 patients with various malignant and non-malignant conditions, in 5 (19%) of the patients, no Type A (dark) spermatogonia (putative SSCs) were identified. In those over the age of 6 years, primary spermatocytes were identified in only 9/16 (56%) patients, whereas these cells can be identified in a small proportion of seminiferous tubules in the majority of normal testes at this age (Nistal & Paniagua 1984).

Pre-existing testicular pathology may also be present in patients who have already received treatments previously considered to be of no (or unknown) risk of gonadotoxicity. Testicular cryopreservation may be offered to patients with sickle cell disease (SCD) due to undergo HSCT; however, recent evidence demonstrates that patients with SCD have a significantly lower number of spermatogonia in their testes compared with patients undergoing testicular biopsy for a variety of other indications who have not received prior treatments (Stukenborg et al. 2018). Whether the reduction in spermatogonia is due to the hydroxyurea treatment they receive or the disease itself is currently unknown.

**Planned treatments and regimen**

For patients due to receive chemotherapy or radiotherapy, criteria for testicular tissue cryopreservation have been established based primarily on a high risk (>80%) of infertility after the proposed treatment (Brougham et al. 2003). However, it must be recognised that the predicted risk of subsequent infertility in childhood cancer survivors is based on limited data and can only be considered an estimation of the actual risk. The high threshold for patient selection is due to the fact that this remains experimental (Anderson et al. 2015). Whilst the majority of young patients with cancer do not receive such treatments, for certain diagnoses, highly gonadotoxic regimens are used. In addition, whilst initial treatment may involve agents which are considered low risk for gonadotoxicity, subsequent treatment for relapse may require treatment that re-classifies the patient into the ‘high-risk’ category (Jahnukainen et al. 2015), making it challenging to limit patient selection to those who have received no prior chemotherapy. Selection criteria in some centres do therefore permit inclusion of patients who have already received treatments that are considered low risk of gonadotoxicity (Anderson et al. 2015).

Validation of selection criteria, to ensure that testicular transplantation is offered to those who are likely to become infertile and not to those in whom future fertility is retained, is an important aspect of ongoing research.
This can be achieved by prospective follow-up of patients and comparison of the future fertility of those offered tissue cryopreservation compared to those who are not. This approach has been utilised to validate selection criteria for ovarian tissue cryopreservation in females (Wallace et al. 2014). Similar follow-up studies of males would include monitoring of prepubertal patients through puberty and biochemical assessment of gonadal function (e.g. LH/FSH, inhibin B) and semen analysis in adulthood. Whilst current selection criteria involve patients at high risk of gonadotoxicity, it is likely that this threshold will change as experimental approaches for fertility preservation are developed into proven clinical therapies; therefore, frequent re-assessment of the selection criteria should be employed to take account of new therapeutic options.

### Intra-operative and early post-operative complications of testicular biopsy

Testicular biopsy is considered minimally invasive. However, for patients requiring gonadotoxic therapy, there may be additional risks of bleeding and infection as a result of their underlying condition, particularly in those with haematological disorders such as leukaemia or aplastic anaemia. Due to the experimental nature of this form of fertility preservation, it is important to ensure that the risk of complications is low. To minimise the anaesthetic risk, the biopsy should ideally be performed alongside another planned procedure requiring a general anaesthetic, for example, bone marrow aspirate or central line insertion (Picton et al. 2015). Intra-operative and early post-operative complications of testicular biopsy specifically for fertility preservation in prepubertal boys have been reported in a small number of studies (Table 1). Overall, the reported rate of complications is ~2% (6/284 procedures) and primarily consists of infection in the biopsied testis or wound (Ginsberg et al. 2014, Uijldert et al. 2017, Ming et al. 2018), with a single patient reported to have had a wound dehiscence (Ho et al. 2017). However, this may underestimate the potential for specific complications such as post-operative testicular pain, which is only reported in three of the studies (Ginsberg et al. 2010, 2014, Ming et al. 2018). The use of retrospective data in some studies (Ming et al. 2018) may also lead to underestimation of the complication rates. Whilst the evidence to date is reassuring with respect to the safety of testicular biopsy, larger studies using standardised systems for prospective recording of intra- and post-operative complications should be considered for this patient group.

### Long-term post-operative complications of testicular biopsy

The medium- to long-term effects of biopsy on testicular development and function have been assessed in a cohort of 64 boys with cancer (Uijldert et al. 2017). Ultrasound scans showed the volume of both testes decreased in the month after the biopsy, likely due to chemotherapy (Uijldert et al. 2017). However, overall, there were no significant differences in testicular volume in biopsied testes compared to the contralateral (unbiopsied) testes at any time point following biopsy, suggesting that there are no additional effects of biopsy over the short to medium term (Uijldert et al. 2017). Small fibrotic lesions were identified in the testis of 4/55 (6.3%) boys at 12 months, the significance of which is unclear and requires further follow-up of these patients (Uijldert et al. 2017). Long-term studies are required to assess the effect of biopsy on testicular growth through puberty and the impact on testis volume in adulthood in this patient population.

### Transport and storage of immature testicular biopsy material

Ensuring that the tissue remains viable during transport is critical. Tissue viability may be influenced by media, temperature, transport duration and tissue size. In a study using human adult tissues stored at 4°C or room temperature or 37°C, temperature did not affect the overall morphology, viability or spermatogonial number, whilst apoptosis was only increased after storage at 37°C, suggesting that there are no additional effects of biopsy over the short to medium term (Uijldert et al. 2017). Small fibrotic lesions were identified in the testis of 4/55 (6.3%) boys at 12 months, the significance of which is unclear and requires further follow-up of these patients (Uijldert et al. 2017). Long-term studies are required to assess the effect of biopsy on testicular growth through puberty and the impact on testis volume in adulthood in this patient population.

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**Table 1** Studies reporting intra- and early post-operative complication rate from testicular biopsy for fertility preservation in prepubertal boys at risk of gonadotoxicity.

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Age at harvest</th>
<th>Cancer diagnosis</th>
<th>Biopsy details</th>
<th>Complications</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>7.0 ± 1.5</td>
<td>4/9 (45%)</td>
<td>≤15%</td>
<td>0/9 (0%)</td>
<td>No complications</td>
<td>Babayev et al. (2013)</td>
</tr>
<tr>
<td>14</td>
<td>5.8 (0.3–11)</td>
<td>14/14 (100%)</td>
<td>~80 mm³</td>
<td>0/14 (0%)</td>
<td>No complications</td>
<td>Ginsberg et al. (2010)</td>
</tr>
<tr>
<td>34</td>
<td>6.9 ± 4.4</td>
<td>27/34 (79%)</td>
<td>~80 mm³</td>
<td>2/34 (6%)</td>
<td>1 epididimo-orchitis, 1 torted appendix testis</td>
<td>Ming et al. (2018)</td>
</tr>
<tr>
<td>78</td>
<td>8.3 (0.5–15.5)</td>
<td>61/64 (92%)</td>
<td>≤1 mL</td>
<td>3/78 (3.8%)</td>
<td>2 wound infection</td>
<td>Uijldert et al. (2017)</td>
</tr>
<tr>
<td>48</td>
<td>7.5 (0.9–16)</td>
<td>48/48 (100%)</td>
<td>~80 mm³</td>
<td>1/48 (2.1%)</td>
<td>1 scrotal cellulitis</td>
<td>Ginsberg et al. (2014)</td>
</tr>
<tr>
<td>62</td>
<td>7.9 (0.3–16.8)</td>
<td>27/44 (61%)</td>
<td>&lt;5%</td>
<td>0/52 (0%)</td>
<td>No complications</td>
<td>Wyns et al. (2011)</td>
</tr>
<tr>
<td>44</td>
<td>7.9 (0.3–16.8)</td>
<td>5/78 (6%)</td>
<td>5–10 mm</td>
<td>1/44 (2.3%)</td>
<td>1 scrotal wound dehiscence</td>
<td>Ho et al. (2017)</td>
</tr>
</tbody>
</table>

larger tissue fragments (50–80 mm³) (Faes & Goossens 2017). Media composition is also important with reduced tissue quality described after the addition of human serum albumin (HSA) (Faes & Goossens 2016). Storage for >5 days resulted in significant tissue deterioration (Faes & Goossens 2016). Validation of these results using human prepubertal testis tissues is required and should include histology, germ cell quantification and functional assessment of the somatic cell populations, including protein expression or hormone production following in vitro or xenotransplantation. Ideally, assessment of the tissue would include testing potential for germ cell development; however, this is challenging due to limited tissue availability for research.

A summary of the progress, gaps in knowledge and future work for patient selection and procedure for testicular biopsy is provided in Table 2.

### Cryopreservation of prepubertal human testicular tissue and cells

Biopsy material may be stored as tissue fragments or may be dissociated to isolate specific cell types for cryostorage as a cell suspension. The aim of cryopreservation for prepubertal patients is to maintain the long-term viability and function of the SSCs through freezing, storage and thawing processes.

### Cryoprotectants and methods for cryopreservation of prepubertal testicular tissue

Several studies have investigated methods for cryopreservation of testicular tissue from children and adults with various diagnoses (Onofre et al. 2016, Zarandi et al. 2018). Studies using animal tissues and limited studies using human tissues generally advocate a DMSO-based cryomedium for cryopreservation of immature tissues and a glycerol-based medium for mature testicular tissues (Picton et al. 2015, Onofre et al. 2016, Zarandi et al. 2018). Cryopreservation may involve one of the three freezing methods. Slow rate freezing is most commonly used either as a controlled rate programme or using an uncontrolled approach, whilst vitrification involves ultra-rapid cooling aimed at preventing ice crystal formation which may cause membrane damage (Onofre et al. 2016). For testis material cryopreserved as intact tissue, non-uniform transfer of temperature and variable penetration of the cryoprotectant may be important. In this regard, the size of tissue pieces may be important. Tissue pieces cryopreserved from prepubertal boys typically range from 1 to 9 mm³ (Keros et al. 2007, Wyns et al. 2007); however, a study directly measuring the impact of tissue size on viability of tissue post-cryopreservation has not been performed. Controlled slow-freezing protocols are most commonly used for immature human tissue and are associated with spermatogonial survival, whilst uncontrolled slow rate freezing has also resulted in spermatogonial survival in immature human testis tissues (Onofre et al. 2016, Zarandi et al. 2018). For vitrification, two studies have shown spermatogonial survival using immature human testis (Curaba et al. 2011, Poels et al. 2013), with proliferation of spermatogonia in vitrified testis tissues after xenografting (Poels et al. 2013). Further studies are required to determine optimal methods for cryopreservation of prepubertal testicular tissue and methods for thawing following cryopreservation in order to ensure viability of spermatogonia for future clinical use.

### Cryopreservation of immature testis: tissue or cell suspension?

For future tissue transplantation, material must be cryopreserved as tissue fragments, whilst for SSC transplantation, tissue may be stored either as whole tissue or as a SSC suspension. Limited data exist on cryopreservation of testicular cell suspensions using tissue from immature animals or humans (Onofre et al. 2016, Zarandi et al. 2018) and given that tissue could be dissociated and SSCs isolated after cryopreservation, current evidence suggests that tissue should be cryopreserved as fragments to maximise the chances of successful preservation of SSCs and retain all potential long-term impact of biopsy on testicular function and fertility post-biopsy.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Progress</th>
<th>Current gaps in knowledge and future work</th>
</tr>
</thead>
</table>
| Patient selection for testis cryopreservation | (1) Several patient groups at risk of infertility identified | • Absolute risks of infertility for patient groups  
• Availability of testicular cryopreservation programme  
• Impact of pre-existing pathology  
| | | • Experimental studies to determine the gonadotoxic effects in human tissues  
• Increased access to clinical research programmes for patients at risk of infertility  
• Long-term follow-up of patients including fertility outcomes  
| Procedure for testicular biopsy | (1) Performed in many centres*  
(2) Post-biopsy complications reported as rare | • Optimal biopsy procedure  
• Long-term impact of biopsy on testicular function and fertility unknown  
| | | • Develop standardised approach to biopsy procedure  
• Long-term studies of testicular function and fertility post-biopsy |

*Clinical research programmes.

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clinical options for generating gametes (Zarandi et al. 2018). This recommendation may change as techniques for cryopreservation and options for clinical use of the tissue evolve.

**Functional assessment of cryopreserved testicular tissue and cells**

Loss of a proportion of spermatogonia is inevitable as a result of the freeze-thaw process (Onofre et al. 2016); however, minimising cell loss and retaining function of the remaining SSCs should be the goal for optimisation of clinical cryopreservation protocols. Survival of spermatogonia has been described in cryopreserved prepubertal human testis in several studies (reviewed in Onofre et al. 2016, Zarandi et al. 2018). The importance of the efficiency of spermatogonial stem cell preservation in cryopreserved testicular tissue may depend on which method is subsequently used to generate gametes from the tissue. From a practical clinical perspective, for SSC transplant, maximising the number of viable SSCs in the tissue may be important for SSC transplantation in which the primary goal is to repopulate the testis and restore natural fertility. However, for testis tissue transplantation, removal of transplanted tissue would be required and therefore small numbers of sperm would be sufficient for use in ART.

Table 3 provides a summary of the progress, gaps in knowledge and future work for tissue transport, storage and cryopreservation for fertility preservation in prepubertal boys.

**Generation of gametes from transplanted testicular tissue or cells**

**Transplantation of gonadal tissues**

Transplantation of cryopreserved testicular tissue back to the patient following the completion of gonadotoxic therapy has been proposed for restoring fertility in prepubertal boys (Anderson et al. 2015, Medrano et al. 2018). This concept is supported by numerous reports of livebirths following cryopreservation and re-transplantation of human ovarian tissue in females who had tissue stored prior to gonadotoxic therapy (Donnez & Dolmans 2017), including the first livebirth following re-transplantation of ovarian tissue harvested during prepuberty (Matthews et al. 2018). Whilst there are clearly differences between ovarian and testicular development and function, this provides proof of principle that the general approach to removal, storage and re-transplantation of gonadal tissue is feasible.

**Generation of functional sperm in animal models of testicular transplantation**

For males, animal studies have provided proof of principle for successful generation of functional gametes in re-transplanted testis tissue. This has been shown using testis tissue from several species transplanted subcutaneously into castrate-recipient mice (Hutka et al. 2017). This includes sperm generated in immature non-human primate xenografts (Honaramooz et al. 2004, Liu et al. 2016). In juvenile rhesus monkeys, xenografted tissues demonstrated accelerated initiation of spermatogenesis (5- and 7 months after xenografting), compared to ungrafted controls (Honaramooz et al. 2004, Ehmcke et al. 2011), which may be important considering that cryopreserved tissues may be re-transplanted in adulthood, without the long prepubertal period. Hormonal stimulation is also likely to play a key role in the sperm development within transplanted tissues. Endogenous gonadotrophins in pubertal recipients may be sufficient to drive spermatogenesis in transplanted tissues, whilst exogenous administration of gonadotrophins can accelerate and sustain spermatogenesis in rhesus monkey testicular xenografts (Rathi et al. 2008). The endocrine status of the host

**Table 3** Summary of the progress, gaps in knowledge and future work for tissue transport, storage and cryopreservation.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Progress</th>
<th>Current gaps in knowledge and limitations</th>
<th>Future work</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport and storage of biopsy material</td>
<td>(1) Studies comparing protocols are published for human adult tissues</td>
<td><em>Lack of studies on cellular function</em>&lt;br&gt;<em>Lack of studies using prepubertal tissues</em>&lt;br&gt;<em>Limited human tissue availability</em></td>
<td><em>Functional studies involving cell development, measurement of hormone production</em>&lt;br&gt;<em>Determine optimal transport and storage conditions for prepubertal tissues</em>&lt;br&gt;<em>Increased access to clinical research programmes for patients at risk of infertility</em>&lt;br&gt;<em>Conduct controlled studies comparing cryopreservation protocols in human prepubertal testis tissues</em>&lt;br&gt;<em>Determine functional capacity of germ and somatic cell populations after cryopreservation</em></td>
</tr>
<tr>
<td>Cryopreservation protocol</td>
<td>(1) Studies comparing cryopreservation of animal and human adult testis published&lt;br&gt;(2) Limited studies using prepubertal tissues</td>
<td><em>Lack of studies comparing protocols in prepubertal testicular tissues</em>&lt;br&gt;<em>Current studies limited to germ cell histology and survival</em></td>
<td></td>
</tr>
</tbody>
</table>
animal may also be important as indicated by a recent study using marmoset prepubertal testis xenografts. When comparing intact males with castrate males or intact females as host mice, the most favourable results for germ cell development were achieved in the intact males (Sharma et al. 2018).

Ultimately, the true test of functionality of sperm generated from transplanted tissue is generation of normal offspring. Birth of live offspring following intracytoplasmic sperm injection (ICSI) of sperm generated from immature mouse, rabbit and porcine testicular transplants has been demonstrated (Shinohara et al. 2002, Schlatt et al. 2003, Nakai et al. 2010, Kaneko et al. 2013). Importantly, blastocyst formation has been shown using sperm generated from immature rhesus Macaque xenografts (Honaramooz et al. 2004); and more recently, offspring have also been reported using sperm generated using fresh and cryopreserved testicular xenografts from immature cynomolgus monkey (Liu et al. 2016).

Transplantation of human testicular tissue

Research into the development and function of human testis xenografts has increased over recent decades, including the use of fetal, prepubertal and adult tissues (Hutka et al. 2017). For prepubertal xenografts, in which spermatogenesis are the only germ cell type identified in pre-biopsy tissue, the most advanced germ cell type identified were spermatocytes (Wyns et al. 2008, Sato et al. 2010, Poels et al. 2013, Van Saen et al. 2013), whilst for some studies no differentiation of spermatogonia was identified (Wyns et al. 2007, Goossens et al. 2008, Van Saen et al. 2011). Differences in patient age, cryopreservation status of the tissue, graft size and grafting duration may account for the variation in germ cell differentiation between these studies (Hutka et al. 2017); however, it should be noted that the maximum duration of grafting was 39 weeks, which may be insufficient for further progression to post-meiotic stages. Several of the studies involving xenografting of prepubertal human testis tissue also xenografted tissues from (peri)pubertal patients, in which spermatocytes were already present in the pre-graft material. Spermatocytes remained the most advanced germ cell type in the xenografts (Van Saen et al. 2011, 2013). Attempts to stimulate further maturation of germ cells in pre- and peripubertal human testis xenografts using exogenous FSH did not result in additional benefit in terms of germ cell survival or progress through meiosis (Van Saen et al. 2013). Xenografts of testicular tissue containing post-meiotic cells consistently show regression of spermatogenesis and frequent degeneration of the tissue (Geens et al. 2006, Schlatt et al. 2006, Wyns et al. 2008), which may be important when considering the potential future application of transplantation of testicular tissue from pubertal patients.

Autoolgous testicular tissue transplantation in primates

Whilst xenografting of testicular tissue offers an experimental approach to develop strategies for fertility preservation in males, development of this approach for clinical use carries risks including transmission of zoonosis (Mitchell et al. 2009). Therefore, strategies for generating functional sperm in autologous transplants would be preferable. This has been demonstrated in non-human primate models including rhesus monkey (Jahnukainen et al. 2012) and marmoset (Luetjens et al. 2008, Ntemou et al. 2019), which also highlight the importance of the grafting site. For marmoset transplants, spermatogenesis occurred in orthotopic (intratesticular) tissues, but did not occur in tissues grafted ectopically (subcutaneous). This may be related to differences in temperature regulation at the two sites, an important factor for testicular function (Luetjens et al. 2008). Alternatively, the presence of local testicular factors that may drive maturation of germ or somatic cells may be important. To date, no studies of autologous testicular tissue transplantation using human tissues have been reported.

Whilst autologous transplantation of testicular tissue fragments may ultimately provide a simple and effective method for generating gametes under normal physiological conditions, recent attention has been given to the use of tissue engineering to optimise the cellular environment and promote cell development and function. This may involve providing structural support to tissues or cells by encapsulation in hydrogels or the use of 3D scaffolds (Del Vento et al. 2018). Furthermore these structures may be used to provide local and/or sustained delivery of molecules such as growth factors, hormones or pharmaceuticals in an attempt to promote germ cell survival and differentiation. A limited number of studies have investigated the use of such strategies specifically using testis tissue (Del Vento et al. 2018). To date, no studies have demonstrated the differentiation of germ cells following encapsulation and transplantation of immature testicular cells.

Use of sperm generated by testicular tissue transplantation

One often overlooked aspect of the potential clinical application of transplantation of cryopreserved prepubertal testicular tissues is the potential for the tissue to restore natural fertility. Whilst in females, ovarian tissue transplanted back to the ovary is capable of ovulating and restoring natural fertility (Donnez & Dolmans 2017), in males, a connection between the seminiferous tubules of the transplanted tissue and the rete testis is required to permit passage of sperm for natural fertility. This connection will have been disrupted and therefore transport of any generated sperm through the reproductive tract is unlikely to occur. As a result,

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sperm generated from re-transplanted testicular tissue will need to be extracted for use in ART, requiring a further surgical procedure to remove transplanted tissue.

**Spermatogonial stem cell transplantation**

SSC transplantation has been shown to have the potential to generate functional gametes in animal models, providing proof of principle for the development of a clinical option for humans. Isolated SSC from a neonatal mouse testis subsequently transplanted into seminiferous tubules of a recipient mouse, resulted in generation of sperm and donor-derived offspring (Brinster & Avarbock 1994). SSC transplantation has been applied successfully for generation of offspring in several species (Honaramooz et al. 2003, Herrid et al. 2009). Xenotransplantation of human SSC into the seminiferous tubules of immunocompromised mice has also been performed. Whilst not intended as a strategy for clinical application, xenotransplantation provides a model to determine whether sperm can be produced in transplanted human SSC. Whilst initial reports described no survival of donor cells (Reis et al. 2000), subsequent studies demonstrated colonisation of the seminiferous tubules and survival of SSC for up to 6 months (Nagano et al. 2002, Sadri-Ardekani et al. 2009). Transplanted spermatogonia were able to proliferate, but there was no evidence of germ cell differentiation or initiation of meiosis (Nagano et al. 2002). Whilst xenotransplantation has not proved successful for generating sperm from human SSC, this may relate to the lack of a compatible somatic environment in the host mouse testis. In such cases, autologous transplantation would be more likely to support germ cell development.

**Autologous SSC transplantation in primates**

Full spermatogenesis has been achieved in a primate model of SSC transplantation (Herrman et al. 2012). SSCs were isolated and autologously transplanted into germ cell depleted juvenile or adult recipient testes via the rete testis. Spermatogenesis subsequently occurred in the testes of 16/17 of the recipients and donor-derived sperm could be demonstrated in the ejaculate of the host. Crucially, sperm generated from allogenic SSC transplants were capable of fertilising rhesus oocytes by ICSI to produce blastocysts, demonstrating the functional capacity of the gametes (Herrman et al. 2012). To date, a single report of transplantation of a suspension of testicular cells in humans has been published (Radford et al. 1999). In this study of 11 men with Hodgkins lymphoma, testicular tissue was removed prior to treatment and cryopreserved as a single cell suspension. Cells were transplanted back into five men following completion of treatment. The outcome of this study in terms of potential restoration of fertility has not been reported (Radford et al. 1999).

**In vitro propagation of SSCs**

A potential limiting factor for development of autologous SSC transplantation is the number of SSCs that can be obtained for transplantation. The SSC population is relatively rare and recent studies using human prepubertal testis tissue have demonstrated the limited quantity of spermatogonia within the testis, which may also be negatively influenced by underlying disease or previous treatments (Poganitsch-Korhonen et al. 2017, Heckmann et al. 2018, Stukenborg et al. 2018) and the small size of biopsy material.

Whilst efficient isolation of SSC using specific cell surface markers is a focus for current research and may significantly increase the yield of human SSC (Zohni et al. 2012, Nickkhohgh et al. 2014a, Valli et al. 2014), it is likely that in vitro propagation of the SSC population will also be required in order to generate sufficient SSC to restore fertility in patients. Successful long-term culture of testicular cells from human adult testes resulted in >18,000-fold increase in spermatogonial number after 64 days in culture (Sadri-Ardekani et al. 2009). Furthermore, the cultured cells were able to colonise the seminiferous tubules of a host mouse. The authors calculated that the expansion of spermatogonial cell number in culture followed by transplantation would be sufficient for future clinical application using SSCs isolated from human prepubertal testicular biopsies (Sadri-Ardekani et al. 2009). A subsequent study using spermatogonia from human prepubertal testes produced similar results, and it was calculated that 83 days of culture would be sufficient to repopulate a recipient testis for clinical application (Sadri-Ardekani et al. 2011). However, these calculations are based on extrapolation from small numbers of spermatogonia following transplantation and further studies including a larger range of patient material would be required to validate these findings.

A summary of the progress, gaps in knowledge and future work for generation of post-meiotic germ cells in prepubertal testicular transplants is provided in Table 4.

**Safety issues for transplantation of immature testicular tissue and cells**

Whilst progress is being made towards the development of clinical strategies for fertility preservation, the priority must always focus on ensuring safety and protecting the best interests of the patient. Safety in relation to potential for re-introduction of tumour cells, contamination with infectious agents, genetic stability of the gametes produced and the health of resulting offspring and future generations are critical. The specific safety issues will depend on the underlying condition such as potential for malignant contamination of tissue obtained from patients with cancer. In addition, the strategy used for restoration of fertility might also have specific safety issues such as
potential for contamination with infectious agents in xenotransplantation or genetic/epigenetic stability of in vitro-derived gametes.

**Malignant contamination in transplanted material**

For testicular tissue obtained from patients undergoing gonadotoxic therapies for malignancy, infiltration of the tissue with malignant cells is a key consideration. Assessment of a portion of the biopsy material should be routinely performed, including histological analysis and specific immunohistochemical, RT-PCR and/or genetic analysis (Dolmans et al. 2010, Rosendahl et al. 2010, Sorensen et al. 2014). The assessment should be based on the type of malignancy and can be performed prior to cryopreservation of the material. For haematological malignancies (e.g. leukaemias), the risk of malignant contamination of the testis is high as a result of the testis acting as a sanctuary site for infiltrating leukaemic cells. For acute lymphoblastic leukaemia (ALL), focal leukaemic infiltrates have been reported in 7/33 (21%) prepubertal boys prior to treatment (Kim et al. 1986). Furthermore, late relapse may occur >10 years after treatment (Barredo et al. 2018).

Residual disease has been described in ovarian tissue cryopreserved for fertility preservation in patients with leukaemia (Rosendahl et al. 2010) and histological analysis alone may fail to identify the presence of malignant cells (Dolmans et al. 2010). Whilst the risk for solid tumours may be lower than for haematological malignancy, demonstration of a previously unidentified foci of Ewing’s Sarcoma in an ovarian tissue biopsy indicates the potential for malignant contamination, even in low-risk cases (Sorensen et al. 2014).

It has been shown in a rat model that as few as 20 leukaemic cells in a fresh or cryopreserved testicular cell suspension can result in relapse following testicular injection in a recipient host (Jahnukainen et al. 2001). Elimination of malignant cells from tissues may be achieved by dissociating into a single-cell suspension and removing malignant cells by fluorescent-activated cell sorting (FACS) prior to transplantation. Studies in leukaemic mice have demonstrated survival of mice and the generation of functional gametes in the host testis, providing proof of principle for this approach (Fujita et al. 2005). However, a subsequent study aiming to remove malignant cells from a mixed suspension of human testicular cells and an ALL cell line via FACS and/or MACS demonstrated evidence for persistent malignant contamination by PCR in 9/10 patients (Geens et al. 2007).

A study involving prepubertal non-human primate testis used FACS to isolate spermatogonia from a mixed suspension of testicular and leukaemic cells (Hermann et al. 2011). As expected, transplantation of the leukaemic cell (CD90 +/CD45 −) population resulted in the formation of tumours in host mice. However, transplantation of the putative SSC population (CD90 −/CD45 +) also resulted in the formation of tumours, and purity checks of the cell population revealed 0.1% contamination with leukaemic cells (CD90 −/CD45 +) (Hermann et al. 2011). Refinement of cell-sorting techniques, with post-sorting purity checks, improved the outcome with no tumours formed in samples shown to be devoid of malignant cells (Hermann et al. 2011), with similar results reported in a subsequent study involving isolation of spermatogonia using the EpCAM cell-surface marker (Dovey et al. 2013).
Xenografting of cryopreserved testicular tissue has been proposed as an alternative method of detecting malignant contamination within the tissue (Hou et al. 2007). Transplantation of testicular tissues from rats with terminal leukaemia resulted in the development of leukaemia in the host animals (Hou et al. 2007). However, whether this approach can be used to detect contamination in tissues containing very few malignant cells or whether this also can be applied to human tissue is unknown. Furthermore, the duration of xenografting used for such studies may not be sufficient to determine the potential for late relapse from leukaemia as has been described in humans many years after completion of treatment (Barredo et al. 2018).

In vitro propagation of testicular cell suspensions has been proposed as a method to simultaneously develop spermatogonia whilst removing potential contamination with malignant cells (Sadri-Ardekani et al. 2014). In this pilot study, the maximum survival of ALL cells in culture was 14 days and for co-culture with spermatogonia, the ALL cells did not survive beyond 26 days (Sadri-Ardekani et al. 2014). These results suggest that in vitro propagation of SSCs for future transplantation may also be used to decontaminate the suspension of malignant cells prior to transplant. Elimination of malignant cells using nanoparticles delivering chemotherapy has recently been described for neonatal mouse spermatogonial cell co-cultures (Shabani et al. 2018). Further validation of methods to eliminate malignant cells from testicular tissues or cultures is required before clinical application of transplantation can be attempted.

**Contamination of testicular tissue or cells with infectious agents**

Harvesting, cryopreservation, propagation and transplantation of human prepubertal testicular tissues should be conducted according to good clinical practice guidelines to ensure a sterile environment at all times (Pacchiarotti et al. 2013). Sterility testing should also be performed to identify potential tissue infection before it is transplanted. For xenografts, additional considerations relating to transmission of zoonosis must be considered (Mohiuddin 2007). Tissues xenografted into a host animal of a different species may result in the transmission of viruses or incorporation of retroviruses (Chapman & Bloom 2001), as has been demonstrated in the case of porcine endogenous retrovirus (PERV) (Patience et al. 1997). Previously unidentified infectious agents should be considered as has been shown historically for example prion disease in Creutzfeldt-Jakob disease (Chapman & Bloom 2001). The risks of infection, rejection and the need for immunosuppression make re-transplantation of xenografts back into the patients unfeasible for clinical application. Therefore, use of xenografted testicular tissues from prepubertal patients would be limited to extraction of sperm generated in the xenograft for use in ART, although further studies would be required to ensure the safety of such an application.

**Genetic/epigenetic stability of gametes from transplanted testicular tissues and cells**

For gametes generated following transplantation of testicular tissue or cells, ensuring genetic and epigenetic stability of cryopreserved or in vitro cultured prepubertal human testicular cells

### Table 5  Summary of the progress, gaps in knowledge and future work ensuring safety of prepubertal testicular transplantation.

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Progress</th>
<th>Current gaps in knowledge and limitations</th>
<th>Future work</th>
</tr>
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<tbody>
<tr>
<td>Malignant contamination</td>
<td>(1) Studies demonstrate risk of contamination in human tissues for transplant (2) Animal studies demonstrate risk with transplant of &lt;20 leukaemic cells</td>
<td>• No studies have demonstrated a reliable method for ensuring no risk of re-introduction of tumour • Limited studies involving human testicular tissues</td>
<td>• Refinement of methods to eradicate malignant cells from tissues or cell suspension prior to transplantation • Increase access to testicular tissues through clinical research programmes</td>
</tr>
<tr>
<td>Infection and sterility</td>
<td>(1) Animal studies demonstrating risk of transmission of infectious agents in xenotransplanted tissues</td>
<td>• Limited data on infection risk of xeno- or autologously transplanted testes (including human) cells/tissues</td>
<td>• Develop and test sterility of tissues following biopsy, cryopreservation and transplantation • Assess the risk of transmission of infectious agents after xenotransplantation</td>
</tr>
<tr>
<td>Genetic and epigenetic stability</td>
<td>(1) Genetic and epigenetic stability of offspring generated from cryopreserved mouse SSC after transplant and ICSI (2) Evidence for change in methylation status of human adult SSC after in vitro culture</td>
<td>• No data on genetic/epigenetic stability of cryopreserved or in vitro cultured prepubertal human testicular cells</td>
<td>• Determine genetic/epigenetic stability in human germ cells following cryopreservation and transplantation • Long-term follow-up of offspring from gametes generated by transplantation of human testicular tissues and cells</td>
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stability of these cells is important. Theoretically such changes may occur as a result of manipulation involved in cryopreservation, dissociation, culture or transplantation. Evidence indicating genetic/epigenetic stability in mouse SSC has been shown following cryopreservation (for 14 years), transplant and ICSI (Wu et al. 2012). No abnormalities were identified in the offspring in terms of chromosomal deletions/duplications. Furthermore, global DNA methylation was unchanged, as was methylation at two imprinted loci (Wu et al. 2012). Similarly, chromosome number was normal in epididymal sperm obtained following transplantation of SSC from immature mouse testis into a germ cell-depleted host (Goossens et al. 2010). A study comparing epigenetic modifications in mouse germ cells after transplantation of tissue or cells demonstrated a similar DNA methylation profile in both groups and in fertile controls; however, expression of histone modifications were altered in elongated spermatids from the SSC transplant group (Goossens et al. 2011). Evaluation of first- and second-generation offspring following transplantation of immature mouse SSC found no effect on growth of the offspring or on DNA methylation of selected imprinted and non-imprinted loci (Goossens et al. 2009).

Genetic and epigenetic stability of sorted and cultured human SSCs from two adults with prostate cancer has also been reported (Nickkholgh et al. 2014b). Chromosomal analysis revealed no aneuploidy following culture; however, there were changes in the methylation status of several imprinted genes (Nickkholgh et al. 2014b). The significance of this remains to be determined, but could reflect changes in methylation in the SSCs. Alternatively, it may reflect changes in somatic cell proportions within the cultures, as has been demonstrated in testicular cultures from marmoset tissues (Langenstroth-Rower et al. 2017). Further studies of genetic/epigenetic stability of SSCs will require analysis of cells obtained from prepubertal testes throughout the process from isolation through to transplantation with assessment of offspring and subsequent generations. This is likely to require extensive use of animal studies in addition to long-term follow-up in human populations.

Table 5 includes a summary of the progress, gaps in knowledge and future work ensuring safety of prepubertal testicular transplantation.

Conclusion

Significant progress has been made towards the development of clinical options for fertility preservation in humans using transplantation of testicular tissues and cells. Animal models provide proof of principle for autologous transplantation of SSC or testicular tissues to generate gametes for the production of healthy offspring. Current research is aimed at translating this knowledge into clinical applications, particularly for patients due to receive gonadotoxic therapies. A number of challenges remain before this could be applied clinically, the most significant of which include post-meiotic differentiation of germ cells and ensuring the safety of transplantation approaches for the patient and resulting offspring. The recent interest in tissue engineering of artificial gonads in which the cellular composition and structure can be designed to support spermatogenesis may provide additional options, particularly for those with underlying testicular abnormality. Experience and knowledge gained from previous studies, coupled with the successful development of ovarian transplantation for fertility preservation in females, provide optimism that fertility preservation using transplantation of cryopreserved testicular tissue and cells may soon be a viable clinical option for prepubertal boys.

Declaration of interest

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

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