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Cryostorage of testicular tissue and retransplantation of spermatogonial stem cells in the infertile male

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Transplantation of own cryostored spermatogonial stem cells (SSCs) is a promising technique for fertility restoration when the SSC pool has been depleted.
In this regard, cryopreservation of pre-pubertal testicular tissue or SSCs suspensions before gonadotoxic therapies is ethically accepted and increasingly proposed.
SSC transplantation has also been considered to treat other causes of infertility relying on the possibility of propagating SSCs retrieved in the testes of infertile men before autologous re-transplantation.
Although encouraging results were achieved in animals and in preclinical experiments, clinical perspectives are still limited by a number of unresolved technical and safety issues, such as the risk of cancer cell contamination of cells intended for transplantation and the genetic and epigenetic stability of SCCs when cultured before re-transplantation. Moreover, while genome editing techniques raise the hope of modifying the SSCs genome before

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re-transplantation, their application for reproductive purposes might be a step too far for the moment.

Introduction

Cryostorage of testicular tissue has initially been considered to preserve Spermatogonial Stem cells (SSCs) aiming at fertility preservation for pre-pubertal boys before undergoing fertility threatening therapies leading to SSCs depletion.

Depletion of the SSC pool is often a consequence of cytotoxic therapies. By contrast with adult men where sperm cryopreservation may be proposed to preserve fertility before gonadotoxic therapies, for pre-pubertal boys about to undergo such treatments, the only promising fertility preservation technique is cryopreservation of either immature testicular tissue (ITT) containing SSCs or of isolated SSC suspensions [1–5].

The successful re-transplantation of cryopreserved testicular cells in mice [6], together with the need for fertility preservation options for pre-pubertal boys [4,7,8], stressed on the urgency of developing cryopreservation methods for ITT banking.

Even though still considered as experimental, cryostorage of ITT is increasingly being offered worldwide [4,9,10] and is now considered ethically acceptable. Current research is ongoing for determining optimal protocols for cryopreservation and for developing fertility restoration methods with cryostored ITT i.e. SSC transplantation, ITT transplantation and in vitro maturation [11].

Adult males affected by non-obstructive azoospermia (NOA) after gonadotoxic therapies [9] or other acquired or congenital conditions [12] can undergo surgical testicular retrieval of mature sperm in about half of the cases. However, when no spermatozoa are retrieved, no alternative to father a child with the patient's own gametes is available so far. Therefore, use of own SSCs, if still present in the testicular tissue, has recently been proposed to restore fertility in these patients [13]. Indeed, on the one hand, SSCs recovery and transplantation back to the patient's testis after being expanded in culture could theoretically be performed if there are remaining functional SSC niches in order to improve the spermatogenic efficiency. On the other hand, use of new tools for genome editing like crispr-cas9 that allow better understanding of genes involved in male infertility [14] could prove helpful to correct some SSC anomalies responsible for infertility and achieve SSC renewal and differentiation following their re-transplantation.

This review will focus on the current state of the art on cryopreservation of testicular tissue and perspectives with re-transplantation of adult or pre-pubertal SSCs in the infertile male.

Methods

We used the PubMed electronic database to search for articles using the following query: (cryopreservation of mature or immature testicular tissue) OR (transplantation of spermatogonial stem cells) OR (propagation of spermatogonial stem cells) AND (human), representing the main topic of interest. Studies published in English or in French until 01 March 2018 were included. Any additional references found in original articles or review papers that were found relevant and missing from the primary search were added.

In Fig. 1, a flow chart describes the article selection process.

Cryostorage of SSCs

Current cryopreservation protocols vary depending on tissue and cell characteristics [15].

Slow-freezing and vitrification protocols are mainly applied and include cryoprotective agents (CPAs) in concentrations that must make the balance between avoiding CPAs toxicity and protecting the cells or tissues from damage while freezing and thawing or vitrifying and warming [16].

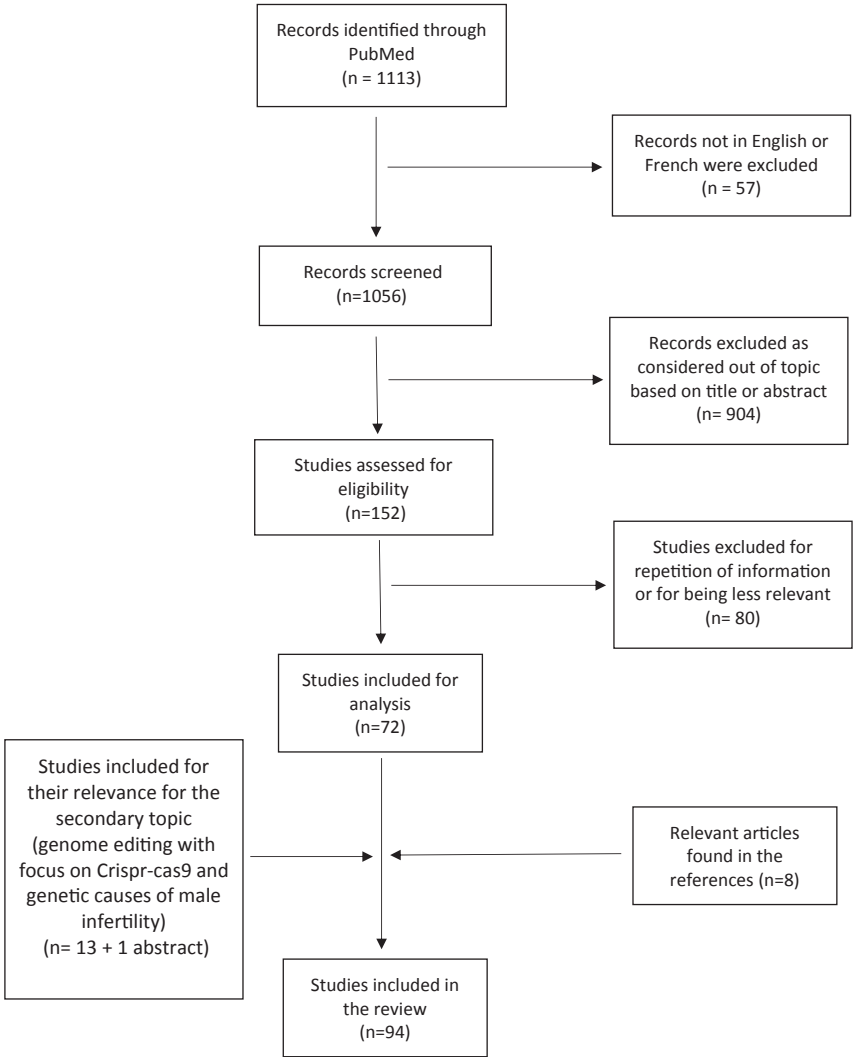

Fig. 1. Flow chart describing the selection process of articles included in this review.

Cryopreservation of SSCs can be done as whole with tissue fragments [1,2,17–20] or as germ cell suspensions [5,17–19,21]. The debate on which of both options should preferably be used is still open. Indeed, while whole testicular tissue cryopreservation is more challenging since different cell types have their own characteristics and requirements, cryopreserving SSCs as cell suspensions is easier from a technical point of view. However, because of tissue dissociation, it does not allow all options for fertility restoration [4]. Moreover, dissociation of cells can impair cell viability and the proper function of the SSCs niche, which includes supporting somatic cells i.e. Sertoli cells (SCs), Leydig cells (LCs), myoid cells, and which provide the physical and molecular microenvironment needed for germ cell survival and self-renewal, proliferation and differentiation to haploid germ cells [22,23].

Cryopreservation techniques and protocols for human testicular tissue and testicular cell suspensions

In order to optimize the cryopreservation method, different techniques and protocols have been studied using testicular tissue or cells of animal origin [24]. Briefly, maintenance of tissue integrity and viability, spermatogenesis recovery, and the production of fertile healthy offspring were obtained from cryostored murine testicular tissue and cell suspensions after transplantation [25,26]. Moreover, no occurrence of major chromosomal anomalies or DNA methylation differences were observed in offspring after long-term storage of cryopreserved testicular cells for 14 years [26]. Eventually, complete spermatogenesis, oocyte fertilization and embryo development using cryopreserved testicular cells of non-human primates when transplanted back into their testes was also achieved [27].

With regards to humans, successful cryopreservation of testicular tissue was achieved for both mature [5,17–19,21,28–30] and immature testicular tissue [1–3,20,31–34]. Based on the success reported for cryopreservation of animal pre-pubertal testicular tissue and on freezing requirements for survival of adult spermatogonia [28], controlled slow-freezing (CSF) protocols using Dimethyl-Sulfoxyde (DMSO) at a concentration of 0.7M supplemented with 10% sucrose [2,3,33], or not [1] were developed and reported as being suitable for human pre-pubertal tissue. Indeed, preservation of the niche architecture and cell viability after thawing and short-term culture [1] and a good cell and tissue integrity at the histological and ultrastructural levels, as well as the ability of cryopreserved-thawed SSCs to survive, proliferate and differentiate up to the pachytene spermatocyte stage after long-term tissue grafting using a xenotransplantation assay in nude mice were observed [3].

In search of finding an optimal cryopreservation technique, vitrification and uncontrolled slow freezing (USF) appeared to be promising techniques that might replace CSF. Vitrification, an ultra-rapid ice-free cryopreservation technique, is faster, cheaper and technically easier than CSF as it does not require expensive equipment and can be performed even outside the laboratory setting [35]. With regards to pre-pubertal testicular tissue, vitrification using open pulled straws (OPS) was applied to mouse [31], non-human primate [36] and human tissue [31,32]. Pre-pubertal human spermatogonial recovery rates proved to be similar for fresh, slow-frozen and vitrified-warmed tissue after short-term culture [31] and after 6 months xenografting in nude mice [32]. Because of the absence of a superiority of the vitrification technique applied in the sole study comparing vitrification and slow-freezing of human ITT [32], CSF remained the main protocol applied in clinical practice [7,8,37–39].

It was also suggested that solid-surface vitrification (SSV) might be preferred to vitrification with OPS because of the lower risk of infectious agent transmission, and for the lower dose of CPAs required compared to OPS [24]. Hence, vitrification protocols were studied using mice ITT. Recovery of spermatogenesis within seminiferous tubules after orthotopic tissue transplantation was similar when comparing SSV to fresh controls [40]. Similarly, Dumont et al. demonstrated the superiority of their SSV protocol when compared to CSF, OPS and single drop vitrification, showing a higher yield of spermatozoa after in vitro maturation [41]. By contrast, when SSV was applied to human adult testicular tissue, cryoinjury rates were high, with ruptured seminiferous tubules and decreased SSCs numbers [29] pointing to the need of further optimization of SSV protocols and evaluation of the impact of the technique on human ITT.

The potential of USF, which compared to CSF does not require the use of expensive bio-freezers and takes half the time for processing, was also investigated [29]. Preservation of adult human testicular tissue using USF with DMSO 1.5M + 0.15M sucrose (s) showed a good efficiency in terms of spermatogonial survival when compared to CSF 0.7M DSMO + s, CSF 0.7M DMSO - s, CSF 1.5M DMSO + s, SSV and Direct Cover Vitrification although no evaluation was made regarding cell function by culturing or transplanting the tissue [29]. Furthermore, testicular tissue after USF was shown to behave in a similar way as fresh tissue when cultured after dissociation but as the cell culture system did not allow maintenance of the different testicular cell types over the culture period and as spermatogonial survival was very poor (7.4% ± 7.8% and 0.8% ± 0.4% of all cells, after 1 and 2 months respectively) [30] the results are so far inconclusive.

Cryopreservation of human testicular cell suspensions (TCSs) has been almost exclusively done using mature testicular tissue [5,17,18,21] with one study using both adult and foetal testicular cells (22 weeks gestation) [19].

Table 1
Outcomes after thawing/warming of tissue pieces vs. cell suspensions cryopreserved with DMSO based protocols.

Testicular tissue donor	Cryopreservation method and CPAs	Av. % cell viability after thawing/warming of:		Av. % viable cell recovery per fresh tissue weight	
		CS	TP	CS	TP
11 adults with normal spermatogenesis [5]	CSF & 1.5M DMSO + 4% FCS	54	–	–	–
5 adults undergoing orchidectomy for prostate carcinoma [18]	CSF & 0–2.5M DMSO + 20%FBS, 0.1% ITS	73 ± 2.3/80[a]	75 ± 2.8/50[a]	–	–
5 adults undergoing sexual reassignment (hormone treated) [17]	CSF & 10% DMSO + 10% HSA 1% Dextran	52.4	64	33	37.4
4 adults with normal spermatogenesis [19]	CSF & 1.28M DMSO+ 25% FCS	67 ± 4	80 ± 3	33	20
3 (22-23w) foetuses [19]		75 ± 4	83 ± 1	38	48 & 66
5 adults undergoing TESE for OA (n = 4) and for severe oligozoospermia (n = 1) [21]	CSF & 1.4M DMSO + 2 mg/mL HSA	45.6 ± 20	–	–	–
	CSF & 0.7M DMSO + 2 mg/mL HSA	29.1 ± 14.2	–	–	–
	OPS V & 2 mg/mL HSA + 0.67M s + 2.3M DMSO + 3M EG	55.6 ± 23.8	–	–	–

CS (cell suspensions), TP (Tissue Pieces), DMSO (Dimethyl-Sulfoxyde), FCS (Fetal Calf Serum), ITS (Insulin-Transferrin-Selenium), HSA (Human Serum Albumin), EG (Ethylene Glycol), OA (Obstructive Azoospermia), V (Vitrification), w (weeks), Av. (Average).
[a] Cell viability 48 h after culture.

Table 1 summarizes the studies that compared cryopreservation of TCSs and whole tissue in terms of post thaw/warming cellular viability and cellular recovery per tissue weight. Different outcomes for mature and foetal tissue between the two approaches suggest that the maturity of the tissue may have an influence [19].

Vitrification of TCS with different protocols was also compared to CSF with better results achieved with vitrification although cell survival after CSF in this study was astonishingly low [21]. Tissue and TCS evaluation was mainly done immediately after thawing/warming without functional assay except in one study where it was performed after 48h culture [18]. More studies are thus awaited to better determine the ideal cryopreservation procedure. Moreover, final validation of the SSC function and reproductive potential will only be achieved through pilot clinical trials as there is no valid preclinical model available so far.

Transplantation of SSCs

Auto-transplantation of SSCs is a fertility restoration option that has already been described in animals with encouraging results. The successful transplantation of testicular cells including SSCs with development of mature sperm was first described in mice by Brinster and Zimmerman in 1994 [42]. In 1996, Brinster's team also demonstrated completion of spermatogenesis with cryopreserved SSCs that colonized the recipient's empty niches after transplantation [6]. In 2003, live offspring was obtained with cultured SSCs from neonatal mice after transplantation into the seminiferous tubules of congenitally infertile recipient mice [43].

A significant milestone for considering clinical application of this technique was the demonstration of functional spermatogenesis and embryo formation after allogeneic transplantation of SSCs in Busulfan treated non-human primates [27].

In humans, a clinical trial including re-transplantation of cell suspensions cryostored from 12 non-Hodgkin lymphoma patients before chemotherapy was reported but no follow-up results were published so far [44].

Despite progress made towards clinical application, many challenges still need to be overcome like the scarcity of SSCs within testis tissue, the risk of neoplastic contamination of cryostored tissue with

subsequent possibility of re-inducing the disease in a cured patient, the standardization of an efficient cell injection technique and the need of a receptive and functional SSC niche.

First, the success of SSC transplantation (SSCT) appeared to be greatly dependent on the number of transplanted SSCs [45] emphasizing the need to develop in vitro cell culture allowing SSC propagation to increase their number prior to transplantation and improve the efficiency of the technique. Indeed, in mice SSCs only constitute 0.02–0.03% of the testicular germ cell population [46] and, in non-human primates, SSCs are thought to represent around 3.5% of testicular cells [47].

Culture and propagation of human adult spermatogonia were successfully accomplished with achievement of a 18 000-fold increase in SSC number [48] and demonstration of the absence of chromosomal anomalies at aneuploidy screening after long-term culture although methylation patterns were modified [49].

The technique was further applied to propagate human pre-pubertal SSCs [50], where a 6.2-fold increase in SSCs within 21 days from the 6.5-year-old boy and a 5.6-fold increase within 14 days from the 8-year-old boy were obtained.

Cell sorting after culture to enrich human adult germ cell suspensions in SSCs also proved useful to improve the efficiency of the transplantation procedure showing an enhancement of the SSC niche colonisation [51].

Furthermore, the absence of occurrence of benign or malignant lesions during the whole lifespan of mice transplanted with cultured SSCs, was also demonstrated [52].

The second important challenge is the risk of re-inducing the disease following the transplantation of SSC suspensions contaminated by cancer cells. While initial studies on animal cell suspensions showed cell sorting techniques to be safe [53–55], these techniques did not allow complete removal of cancer cells when cells of human origin were sorted [54,56,57]. However, culturing the testicular cells to propagate SSCs led to elimination of all contaminating acute lymphoblastic leukaemia cells in three contaminated samples after 26 days of culture [58]. The question of the possibility of a different behaviour of cancer cells that were simply added to a cell suspension for the purpose of the experiment or cancer cells that invade a tissue needs to be investigated.

Third, the site of injection and the injection technique have not been standardized so far. The rete-testis ultrasound-guided injection was established as the best approach for large testes with 70% of the tubules filled after an average of 30 min in the monkey testis [59]. This rate was 87.5% when infusions driven by gravity were used [60]. More recently, an infusion pump was used to inject SSCs in human cadaver testes, showing less variability between subjects if compared to the injection under hydrostatic pressure [61]. However, leakage in the testicular interstitium was observed and further studies are warranted to improve the injection technique [60,61].

Eventually, as SSCs after being re-transplanted have to migrate to colonize the host testicular stem cell niche which must provide both the physical and molecular support to the germ cells for their proliferation and differentiation, an undamaged niche is of paramount importance for a successful SSCs transplantation.

Who are the candidates for SSC cryostorage and re-transplantation?

Pre-pubertal boys facing gonadotoxic treatments

Pre-pubertal boys facing gonadotoxic therapies were the first candidates to benefit from cryostorage of testicular tissue with a view to preserve their fertility, and a significant number of European and US hospitals currently offer this option [7–9,37–39].

Chemotherapy and radiotherapy may lead to the destruction of SSCs, resulting in azoospermia in up to 25% of adult survivors of childhood cancer [62]. Preconditioning chemotherapy before bone marrow transplantation or total body irradiation carry an even higher risk of azoospermia, affecting 85.4% of patients 5.6 years after treatment completion [63] and 3 out of 5 pre-pubertal boys who underwent Hematopoietic-Stem-Cell transplantation for sickle cell anaemia after 15,16 and 19 years respectively [64].

As the pre-pubertal state does not protect against cytotoxic damage to the testes carrying thus the risk of permanent infertility [65], and as fertility is considered an important quality of life issue [66], counselling about cryopreservation of ITT as the only potential fertility preservation option is strongly advised but also expected by the patient and his parents [7]. Indeed, in a large pilot study, 93.5% of referred patients accepted ITT cryobanking [8] and in a recent multi-centric trial, an infertility risk of 25–30% was regarded as the threshold for acceptance [67]. Furthermore, the sampling procedure appeared safe with few and infrequent short-term post-surgical complications, like bleeding or infections [8,68].

Besides successful SSCT in non-human primates [27] several preclinical studies now provide encouraging results that carry the prospect of a successful re-transplantation of cryostored SSCs to restore the patients' fertility [50,52,58,60,61,69].

One of the challenges is the integrity of the niche that can be damaged by gonadotoxic treatments, with the consequent risk of impairment of germ cell differentiation as suggested by Bar-Shira Maymon et al. [70] Transplantation of niche cells could be an option for reversing the damage endured by the niche after gonadotoxic treatment [71]. However, the limited knowledge about the niche functionality after gonadotoxic drug exposure calls for the necessity of further preclinical studies on the presence of a receptive microenvironment for SSCs recolonization and development.

Can males with acquired or congenital disorders involving SSC depletion over time be candidates?

Theoretically, if cryostorage of ITT and SSCT proves to be successful in pre-pubertal boys, it might be considered for a broader panel of candidates where SSC depletion due to mostly unknown mechanisms occurs or when after puberty no spermatozoa are available before gonadotoxic treatment due to inhibition of germ cell differentiation as in some testicular cancers or Hodgkin lymphomas [72,73]. For the latter, because of a high incidence of azoospermia after gonadotoxic treatment [62] with only 44% of cases where sperm is retrieved after TESE [9], no fertility option from the patient's own genetic material is possible unless SSC cryostorage had been performed before gonadotoxic treatments for later expansion and re-transplantation.

For patients presenting with NOA, the procedure aims at increasing the spermatogenic efficiency by supplying own SSCs able to self-renew and differentiate after transplantation [13]. SSC expansion in culture was already reported [74] and combination with genome modification when SSCs are genetically abnormal could prove useful [75].

However, a main requirement that mediates the fertility restoration procedure is the integrity status of the SSC niche. Prediction of true niche functionality is nevertheless challenging as there is no clinical specific marker besides abnormal hormonal profiles that are only suggestive of testicular deficiency.

While the damage to the SSC niche induced by gonadotoxic treatments is not predictable, when it comes to congenital or genetic anomalies responsible for SSC depletion over time, both the peculiar physical and molecular support of the niche as well as the specific SSC deficiencies may jeopardize the feasibility of such a fertility preservation strategy e.g. in KS patients where SSCs loss starts early in the pre-pubertal period [76] suggesting the need of an early intervention [77] but where spermatogenesis arrest attributed to the presence of a supernumerary X preventing aneuploid spermatogonia from meiosis [78], extensive fibrosis and hyalinization of the seminiferous tubules are major hurdles for SSCT [76]. Moreover, the actual timing of onset of SSC loss is not clear, with a recent hypothesis suggesting that this process has already started before birth [79].

For spermatogenic failure due to *AZF* deletions [80], the picture might be different. Indeed, for the *AZFc* deletion, being the most common deletion of the AZF locus, spermatogenesis can be present suggesting a supportive testicular niche [80] and when SSCs with *AZFc* deletion were cultured and propagated in vitro for 48 days, they showed to behave like SSCs from normal controls, paving the way for SSCT as a potential treatment option [81].

However, SSCT in these patients will not prevent the transmission of the mutation to male progeny but as the genetic content of the *AZFc* region is well known, correction of the deletion at the SSC level, together with SSC propagation before SSCT after germ cell depletion in the host testis, could lead to the production of disease free spermatozoa and healthy offspring.

Cryptorchidism has also been suggested as a potential candidate for SSC cryostorage as it is associated with a reduction in the total number of germ cells and a defective pre-pubertal germ cell maturation [82] with variable degrees of testicular dysfunction [83]. While freezing a tissue sample during the orchidopexy procedure for future fertility treatments in this group might be considered as reasonable [20], the impairment of germ cell maturation and Leydig cell functionality raises the question about the functionality of the niche after SSCs transplantation [84].

Potential candidates and pros and cons for SSC cryostorage with the perspective of re-transplantation for each patient category are presented in Table 2.

Perspectives with genome editing and SSC transplantation in the infertile male

SSCs play an important role in the maintenance of spermatogenesis and an abnormal genome of the SSC may impair the spermatogenic process, as aneuploid spermatogonia do not achieve meiosis [78].

The ability to culture SSCs for long periods of time without inducing genetic/epigenetic modifications [51,85] while preserving their spermatogenic ability [86] is of interest because this means that genome editing tools could be combined to modify the SSC genome in vitro prior to transplantation [75].

Indeed, back in 2002, Brinster had already shown that after transplantation of genetically modified SSCs, sperm and progeny carried the modified gene [87].

Nowadays, three types of nucleases can be used for genome editing, most specifically by making double stranded DNA breaks. Zinc Finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and crispr-cas9 [88] the latter presenting the advantages of simplicity and lower risk of off-target genetic or epigenetic changes [89]. Briefly, crispr-cas9 is constituted of a single guide RNA (sgRNA) sequence of interest, and a Cas9 protein. The sgRNA carries a 20bp recognition sequence that

Table 2
Pro and cons for the SSCs cryostorage and re-transplantation approach for genetic causes of infertility.

Candidates for SSCs cryostorage	Pros	Cons
Pre-pubertal patients facing gonadotoxic treatment [4]	High cancer survival rates Fatherhood desire Low complication rates of the procedure High acceptance rates	Safety issue after SSCT: Risk of cancer cell contamination Unknowns on genetic and epigenetic stability of SSCs Potential niche damage
Adolescent and adult patients before gonadotoxic treatment with cancer-inhibited spermatogenesis [72]	Alternative to sperm freezing	responsible for spermatogenesis failure
Pre-pubertal patients with Cryptorchidism [20]	Decreased SSCs numbers and risk of azoospermia regardless of timely repair or medical treatment	Unknown ability of the niche to support SSCs auto-renewal and differentiation due to Leydig cell hypoplasia Prolonged persistence of the foetal stem cell pool and delayed establishment of the adult SSCs pool
Yq microdeletions AZFc and partial AZFb [52,81]	Spermatogenesis foci in favour of some functional SSC niches	Possibility of SCOS Transmission to male progeny
Klinefelter syndrome [77]	Progressive depletion of GCs starting before puberty Reported loss of the extra X chromosome generating normal 46 XY SSCs in in vitro culture of 47 XXY SSCs [94] [a]	Inability of the niche to support SSCs colonization and function due to progressive hyalinization

SSCs (Spermatogonial Stem Cells), SSCT (Spermatogonial Stem Cell Transplantation), SCOS (Sertoli Cell Only Syndrome), GCs (Germ Cells).

[a] Only abstract available.

recognizes the targeted DNA and guides the cas9 nuclease which will create a double stranded break at a highly specific site (between the 3rd and 4th bases upstream from a protospacer-adjacent motif – NGG, N being any nucleotide). This break triggers endogenous cellular repair mechanisms, and correction is done by either Homology-directed repair (HDR) or Non-homologous end joining (NHEJ). NHEJ leads to insertions/deletions, that disrupt the gene function, and HDR leads to the insertion of specific exogenously supplied DNA sequences leading to gene correction or insertion [90,91]. Therefore crispr-cas9 allows the creation of knock-in, knock-out genes, and more recently elimination of whole chromosomes [92].

Crispr-cas9 has already been used to successfully repair mutations in genes that cause severe diseases like the dystrophin gene (*Dmd*), linked to Duchenne muscular dystrophy in mice [93]. Most interestingly, Wu et al in 2015, using the crispr-cas9 system on mice SSCs to correct a dominant mutation (1 base-pair deletion in exon 3) in the *Crygc* gene causing cataracts in mice, showed that SSCs and offspring after SSCT were disease-free [89].

Such study paves the way for potential application of genome editing tools to correct genetic abnormalities directly at the SSCs level to treat infertility and prevent disease transmission to the offspring such as *AZFc* Y chromosome microdeletions, single nucleotide polymorphism, point mutations or chromosomal translocations responsible for male infertility.

We are still far from a therapeutic genome editing in humans, especially because of the necessity of a further refinement of the crispr-cas9 model, to lower the incidence of off-target activity, together with huge ethical issues that will be raised for editing the human genome. Therefore, current application should be limited to the mapping of the human genome, and in the reproductive field should focus specifically on genes that mediate human spermatogenesis, because unravelling this process could improve our approach towards restoring male fertility.

Conclusion

Though we are still far from routine clinical application, substantial advances have been done in ITT cryopreservation offering the prospect of future transplantation of cryostored SSCs. Preclinical studies on the feasibility and risk assessment of transplantation of SSCs as well as achievements in non-human primates with SSC transplantation point to the potential of a near future clinical application to restore fertility after gonadotoxic treatments. However, broadening the applications with use of genome editing tools is tempting as it would radically change our approach to infertility treatment.

Identifying pathological conditions where niche support cells are intact and functional is a further challenge besides important ethical issues on genetic modification of expanded SSC and genetically modified offspring.

Practice points

- Counselling about cryopreservation of testicular tissue as a potential fertility preservation method is currently indicated in pre-pubertal boys about to undergo gonadotoxic treatments. A multi-collaborative care pathway is recommended for adequate support for patients and parents.
- Options for fertility restoration with cryostored SSCs are not yet implemented in clinical practice.
- SSCT can be a promising tool for fertility restoration. As encouraging results were obtained in non-human primates, IRB approved pilot clinical trials could be set up in a limited set of indications.
- Genome editing at the SSC level cannot be currently offered for obtaining normal sperm and preventing disease transmission to offspring.

Research agenda

- Development of an improved, cheap, reproducible and efficient cryopreservation method for testicular tissue or cell suspensions is awaited.
- More research is needed to elaborate an ideal clinical grade culture media in which SSCs can proliferate while maintaining their epigenetic and genetic stability, with complete elimination of malignant contaminant cells.
- Optimization of SSCs injection techniques and establishment of a standardized, clinical grade reproducible protocol for SSCT is required.
- Studies using crispr-cas9 should focus on the mapping of the genes implicated in spermatogenesis regulation although the technique might appear as a useful tool to correct some genetic abnormalities responsible for infertility in the future.
- Ethical issues of human genome modification in germ cells must be clarified before considering any application in human.

Summary

Significant advances have been made since banking of human immature testicular tissue for fertility preservation has been started. Teams around the world progressively developed clinical protocols based on what was done in rodents and higher order mammals. Eighteen years after the first case report on pre-pubertal testicular tissue cryostorage, fertility restoration from cryopreserved human ITT in pre-pubertal boys has not been achieved yet. This is mainly due to numerous challenges ranging from finding the optimal cryopreservation protocol to the safe isolation, propagation and transplantation of SSCs while questions are raised on the innocuity of the procedure for the recipient and on the consequences on the long-term health of children.

While successful production of embryos from allogeneic transplanted SSCs in non-human primates is one of the biggest milestones before human application of cryostored SSCs transplantation, long-term follow up studies for safety evaluation of both the host and progeny are missing in this model. Broadening the indications of cryostorage and transplantation of SSCs may be considered for fertility preservation when no spermatozoa are present in the testicular tissue before gonadotoxic treatment or for enhancing spermatogenesis in non-obstructive azoospermia. In addition, recent advances in genome editing tools, especially the crispr-cas9 system, could radically change the current therapeutic arsenal for men with infertility of genetic origin although huge safety and ethical issues should be addressed before considering such approach.

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