Chapter 46
Can we preserve testicular germ cells of prepubertal patients for later fertility?
Next generation therapies to preserve fertility and treat infertility

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Chemotherapy and radiation treatments for cancer or other conditions can cause infertility. Similar treatments used for myeloablative conditioning prior to bone marrow transplantation for non-malignant conditions (e.g., sickle cell disease, β-thalassemia) also can cause infertility. Another potential cause of infertility is gender affirming treatments for gender dysphoria. Adult survivors of childhood cancers and transgender or gender non-conforming teenagers express a desire to have biologically related children. Therefore, all patients should be educated about the impact of their diseases or medical treatments on future fertility and about options to preserve fertility. Adolescent and adult people with testes have the option to cryopreserve a semen sample with sperm prior to gonadotoxic treatment; sperm can be thawed in the future and used to achieve pregnancy using standard assisted reproductive technologies such as in vitro fertilization (IVF) (Chapters 24, 35). That option is not available to prepubertal patients who are not yet producing sperm or for those with gender dysphoria who do not want to discontinue their gender affirming treatments to produce sperm. The only fertility preservation option for those patients is testicular tissue cryopreservation (TTC) (Chapter 12). We will review several techniques that are currently in the research pipeline and may be available in the future to mature frozen/thawed testicular tissues and produce sperm that can be used to alleviate infertility.

Spermatogonial Stem Cells and Spermatogenesis

Spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis, the process that produces sperm in the testes

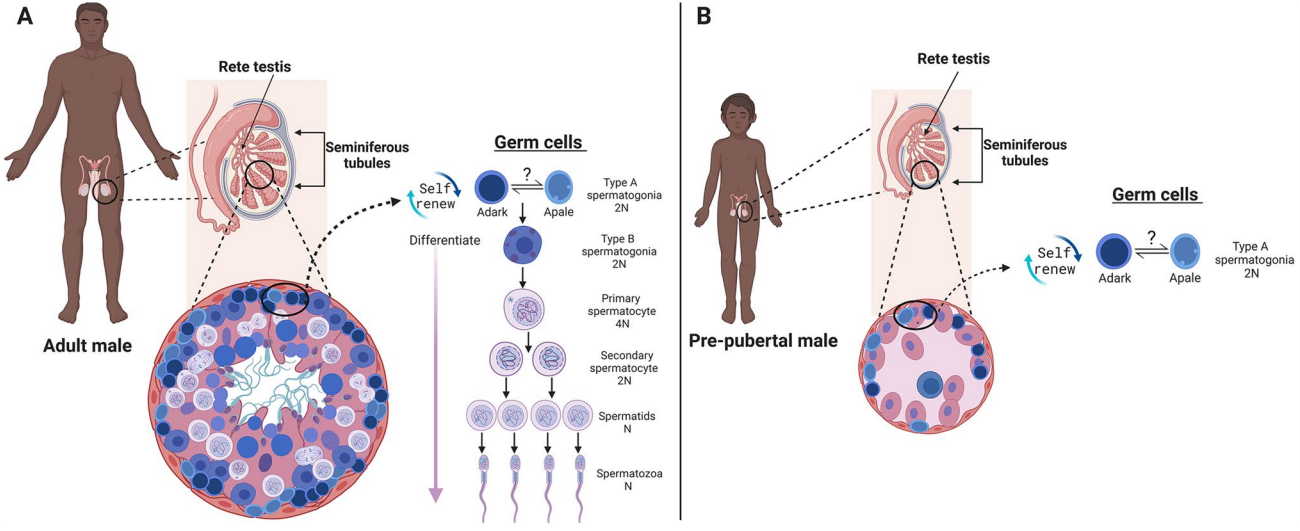

Figure 1. Stem cells, spermatogenic lineage development and testis anatomy in adult and prepubertal males. A) Spermatogenesis occurs in the seminiferous tubules of the testis. The spermatogonial stem cells (SSCs) that are at the foundation of spermatogenesis are thought to be among the pool of A-dark and A-pale spermatogonia. **B)** Prepubertal boys do not have spermatogenesis in their seminiferous tubules, but they do have A-dark and A-pale spermatogonia that have the potential to produce sperm. Created with BioRender.com.

(Chapter 8). SSCs or their precursor prospermatogonia (gonocytes) are present in the testis before birth. At the time of puberty, under the influence of gonadotropic hormones from the pituitary, SSCs initiate spermatogenesis and maintain continuous sperm production throughout postpubertal life. This is possible because SSCs precisely balance self-renewing divisions that maintain the stem cell pool with differentiating divisions that give rise to spermatogenesis. In humans, SSCs are described as A-dark and A-pale spermatogonia based on the intensity of their nuclear staining with hematoxylin. A-dark and A-pale spermatogonia are located on the basement membrane of seminiferous tubules and may undergo one or two transit amplifying mitotic divisions before giving rise to differentiating type B spermatogonia, that divide one more time to produce primary spermatocytes. Two meiotic divisions give rise sequentially to secondary spermatocytes and then haploid spermatids, which undergo spermiogenesis to produce terminally differentiated sperm (Fig. 1A) (Chapter 9). Chemotherapy and radiation treatments are toxic to the spermatogenic germ cells in the testis and can cause temporary or permanent infertility. Depending on the treatment, up to 70 percent of cancer patients will recover spermatogenesis after treatment because at least a portion of the stem cell pool survived treatment and could regenerate spermatogenesis. The speed of spermatogenic recovery is likely related to the size of the surviving pool of SSCs. The remaining 30 percent of cancer patients are rendered permanently infertile by their treatment, and this is likely caused by complete depletion of the stem cell pool and/or damage to testicular somatic cells that are necessary to support spermatogenesis.

Testicular Tissue Cryopreservation

Prepubertal boys are not producing sperm, but they do have A-dark and A-pale spermatogonia in their testes that have the potential to produce sperm (Fig. 1B). Clinics around the world are actively cryopreserving testicular tissues from young patients in anticipation that those tissues can be thawed in the future and matured to produce sperm. Immature testicular tissues are typically collected by wedge biopsy of one or both testes and tissues have been cryopreserved for patients ranging in age from a few months old to >18 years old. All reporting centers use slow freezing and most use DMSO as the cryoprotectant (Fig. 2A). Over 2,500 testicular tissues have already been frozen for patients worldwide (unpublished,

informal discussions among a network of international providers) and many are already approaching their reproductive years. It is necessary to develop next generation reproductive technologies that will allow patient-survivors to use their tissues for reproduction, and responsibly translate them into clinical practice. There are several testicular cell-based and tissue-based technologies in the research pipeline that are summarized in Fig. 2.

Using Cryopreserved Testicular Tissues to Produce Sperm

Autologous Spermatogonial stem cell transplantation. SSC transplantation was first described in mice by Brinster and colleagues in 1994 and has now been translated to numerous animal models. Transplanted stem cells regenerate spermatogenesis in the seminiferous tubules of the testis with the production of fertilization competent sperm that have produced embryos or offspring in mice, rats, goats, sheep and monkeys. Under some circumstances, transplanted stem cells can restore natural fertility. However, small biopsies obtained from prepubertal boys contain only a small number of SSCs, which are likely to regenerate small pockets of spermatogenesis in the testis but not restore natural fertility. Nonetheless, testicular sperm can be retrieved surgically and used to fertilize oocytes by IVF with intracytoplasmic sperm injection (ICSI, Fig. 2B). Testicular sperm do not swim, so cannot fertilize by intrauterine insemination (IUI) or conventional IVF. Methods to expand spermatogonial stem cell numbers in culture are well-established in rodents, producing stem cell numbers sufficient to restore natural fertility. Methods to maintain and expand SSCs in culture have not been translated to any higher animal models or humans, although this is a very active area of research.

Autologous testicular tissue grafting. Testicular tissue grafting involves placing intact pieces of immature testicular tissue under the skin where it matures to produce fertilization competent sperm and offspring. In this approach, SSCs are maintained in their cognate seminiferous tubule niches in intact pieces of testicular tissue. The goal of testicular tissue grafting is not to restore natural fertility since the grafted tissue is not connected to the excurrent duct system. The goal is to mature grafted tissue to produce sperm that can be recovered for fertilization by ICSI to produce offspring

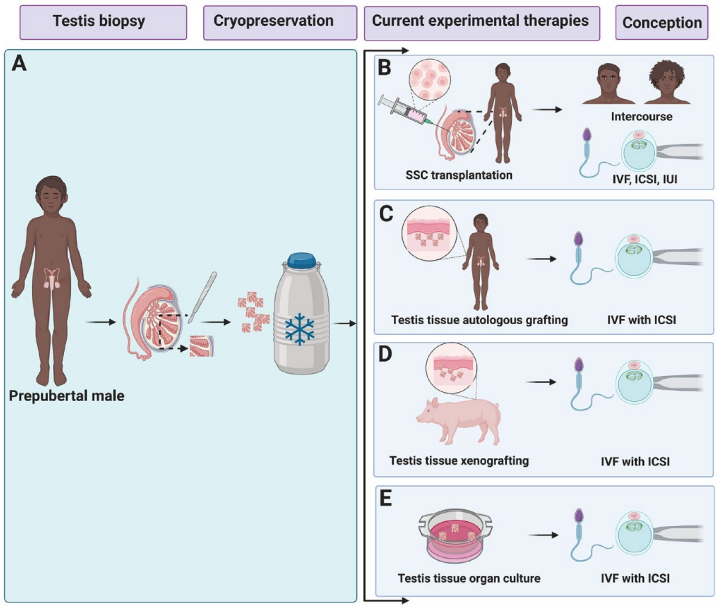

Figure 2. Fertility preservation and fertility restoration for people with testes. A) Testicular tissue cryopreservation is the only fertility preservation option available to prepubertal boys who are not making sperm. Testicular tissues are cut into small pieces (2-5 mm diameter) and cryopreserved for future use. Freezing intact pieces of testicular tissue preserves the option for cell- or tissue-based therapies in the future. **B)** Spermatogonial stem cell (SSC) transplantation. Frozen and thawed testicular tissues are digested with enzymes to produce a suspension of cells that is injected back into the seminiferous tubules of the testis. This method can lead to regeneration of spermatogenesis in the testis and possibly restore fertility. If sperm counts are low, sperm can be used to achieve pregnancy using standard of care assisted reproductive technologies (IVF, ICSI, IUI). If there are no sperm in the ejaculate, regenerated sperm can be retrieved surgically from the testis and used to fertilize by IVF with ICSI. **C)** Testicular tissue grafting. Frozen and thawed testicular tissues are grafted under skin of the patient where they can mature to produce sperm. Graft-derived sperm can used to fertilize by IVF with ICSI. **D)** Testicular tissue xenografting. Frozen and thawed testicular tissues are grafted under the skin of an animal host where they can be matured to produce sperm. Graft-derived sperm can be used to fertilize by IVF with ICSI. **E)** Testis tissue organ culture. Frozen and thawed testicular tissues are placed in tissue culture and matured to produce sperm that can be used to fertilize by IVF with ICSI. Created with BioRender.com

(Fig. 2C). Homologous species testicular tissue grafting was first reported in mice by Dobrinski, Schlatt and colleagues and was more recently translated to nonhuman primates with the production of fertilization-competent sperm and a baby monkey.

SSC transplantation and testicular tissue grafting are mature technologies that have been replicated in numerous mammalian species with production of fertilization competent sperm and live offspring. These technologies may be ready for translation to the human clinic for patients who have cryopreserved their testicular tissues or cells. The autologous transplantation approaches described above may not be appropriate for all patients. It may not be safe to transplant testicular tissues or cells from leukemia patients or patients with testicular cancer due to the potential risk of reintroducing malignant cells to the survivor. Transgender subjects may not want to experience the male puberty with testosterone production that would be required to mature their testicular cells or tissues inside their own bodies. Therefore, outside-the-body approaches are needed to mature the cryopreserved immature testicular tissues to allow production of sperm for reproduction.

Testicular tissue xenografting. A potential alternative to autologous testicular tissue grafting is to graft tissues into an animal host (Fig. 2D). Immature testicular tissues from pigs, goats, rabbits, hamsters, dogs, cats, horses, cattle and monkeys have been grafted under the back skin of immune-deficient nude mice and matured to produce spermatids or sperm. This approach is effective with immature or prepubertal tissues but not with adult tissues, so it may be an ideal option for prepubertal patients who have cryopreserved testicular tissues. Xenograft-derived sperm have been used to fertilize and produce offspring in rabbits, pigs and monkeys. Prepubertal human testicular tissues survive in mouse hosts and initiate spermatogenesis up to the spermatocyte stage, but production of sperm from those tissues has not been reported. Future directions may include treating mouse hosts with hormones or growth factors to promote complete spermatogenesis from xenografted human tissues or to test alternative animal hosts. Using xenograft-derived sperm in reproduction may raise concerns about xenobiotics that could be harmful to human health, but the path forward is already being paved by initial reports transplanting pig organs into human patients.

Testicular tissue organ culture. Another alternative to autologous transplantation approaches is to mature prepubertal testicular tissues in vitro (Fig. 2E). Ogawa and colleagues performed ground-breaking work on the method of testicular tissue organ

culture at the air-gas interface in mice. Briefly, small pieces of testicular tissue from neonatal mice were placed on an island of agar that was half soaked in tissue culture medium. Tissue was in direct contact with the air on the upper surface and in direct contact with media-soaked agar on the bottom surface. Spermatids or sperm collected from the tissues between 20-42 days were competent to fertilize and produce offspring. Supplementing culture medium with knockout serum replacement (KSR) instead of or in addition to fetal bovine serum (FBS) was critical to achieving complete spermatogenesis. The production of haploid spermatids or sperm started to decline after 30-40 days in culture but persisted up to two months. Also, the tissues mounded up on top of the agar island. Complete spermatogenesis was observed only in tubules on the outer surface exposed to the air interface while tubules in the center of the mound were empty or necrotic. The same group later developed different variations of microfluidics devices to maintain testicular tissues in a monolayer of seminiferous tubules for 4-6 months with continuous production of haploid cells. If this approach can be replicated by other groups in mice and translated to higher animal models, including humans, it may have important implications for cancer patients or transgender subjects where it would be unsafe or inappropriate to transplant cryopreserved testicular tissues or cells back into their bodies.

Concluding Thoughts and Future Directions

In this chapter, we reviewed next generation technologies that may allow patients who cryopreserved their immature testicular tissues when they were young to use those tissues to produce sperm and have children. The autologous methods of spermatogonial stem cell transplantation and testicular tissue grafting are mature technologies that have been replicated in several animal models and may be ready for translation to the clinic. When those methods are deployed in the clinic, it is important to manage expectations of the patient and of the medical/research community. Experiences with ovarian tissue cryopreservation and ovarian tissue transplantation in human patients may be instructive. The first reports of ovarian tissue cryopreservation were in 1994 and the first reports of autologous re-transplantation of those tissues with return of hormone production, menstrual cyclicity and offspring were in 2004 and 2005. There are now over 130 reported live births from transplanted ovarian tissues. Those outcomes led the American Society for Reproductive Medicine

to advise that the experimental label could be removed from ovarian tissue freezing, which may help reduce barriers in access to care. However, it is important to note that it was not always possible to determine whether pregnancies were from transplanted tissues or surviving endogenous follicles; most of those live birth outcomes were from women who were already adults when they cryopreserved their tissues, many of whom returned to used their tissues within a decade of their cryopreservation. There is, thus far, only one documented live birth from ovarian tissue that was prepubertal at the time of cryopreservation.

To our knowledge, the first reports of testicular tissue cryopreservation for prepubertal boys were in 2005 and there are no reports of transplanting those testicular tissues or cells back into patients. As indicated above, in the absence of methods to expand SSC numbers in culture, SSC transplantation is likely to regenerate focal areas of spermatogenesis in the testis but unlikely to restore natural fertility. Also, because these are autologous transplants back into the seminiferous tubules of the testis, it will not be possible to know whether sperm are from transplanted SSCs or from endogenous SSCs that survived the gonadotoxic treatment. Finally, since most patients were children at the time that they cryopreserved testicular tissues, it may be many years before the first patients attempt to use their tissues for family building. Will it be necessary to document 130 live births to remove the experimental label from testicular tissue cryopreservation? Perhaps it would be adequate to show that transplanted testicular tissues can be matured to produce "normal" sperm. Since grafted tissues will be placed under the skin and separate from the endogenous testis, the origin of sperm derived from those grafts will be unequivocal. If graft development occurs in a time course similar to what was observed in nonhuman primates, sperm outcomes could be known within a year of transplantation. Graft derived sperm can be used immediately or cryopreserved for the patient's future use, providing assurance that their future fertility using standard assisted reproductive technologies is secured. For the medical/research community, this will provide evidence of the reproductive potential of cryopreserved, prepubertal testicular tissues that may justify removing the experimental label from testicular tissue cryopreservation.

Autologous transplantation will not be appropriate or safe for all patients. We reviewed testicular tissue xenografting and testicular tissue organ culture as methods to mature prepubertal testicular tissues and produce sperm outside the body. More work is needed to

demonstrate that those methods are robust, reproducible across species and safe before they can be translated to the human clinic. Other methods that are in the research pipeline but were not described in this chapter including de novo testicular morphogenesis, in vitro gametogenesis from testicular cell organoids and in vitro gametogenesis from patient-derived pluripotent stem cells. Those methods are early stage but may expand options to remove malignant cells before transplanting patient testicular cells back into their bodies or for producing sperm outside the body. Assisted reproductive technologies that produced the world's first "test tube" baby, Louise Brown (born July 25, 1978), have now produce over 6 million babies worldwide. Louise was possible because her dad was able to produce sperm and her mom was able to produce eggs that were combined in the laboratory to achieve in vitro fertilization by the team of Patrick Steptoe (Physician) Bob Edwards (Researcher, Nobel Prize winner) and Jean Purdy (Research nurse). We may be at the dawn of a new era in reproductive medicine where it will be possible to help patients who are not able to produce mature eggs or mature sperm to have biologically related children.

Suggested reading

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