Chapter 12
Can sperm be produced *in vitro*?

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Spermatogenesis, the production of male gametes, is initiated at puberty and through the self-renewal and differentiation of spermatogonial stem cells (SSCs) enables lifelong male fertility. The testicular microenvironment is critical to this delicate balance, as SSC maintenance and differentiation is controlled by various factors within the somatic niche. The SSC population is extremely limited within the testis and these cells are particularly susceptible to gonadotoxic agents such as radio- and chemotherapy. This means that the SSC pool may be decimated during cancer treatment, rendering patients infertile. As such, fertility preservation should be undertaken prior to treatment. For adult men this is a relatively simple process as semen samples can be collected and cryopreserved for future use with assisted reproductive technologies (ARTs) such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Chapter 35). Semen cryopreservation is reliable and inexpensive. However, fertility preservation of prepubertal boys poses a challenge (Chapter 24). How can we preserve their fertility when they do not yet produce sperm? The answer may lie at the bottom of the SSC pool.

One alternative male fertility preservation strategy could be the cryopreservation of testicular biopsies, taken prior to treatment, as whole tissue or as testicular cells isolated after enzymatic digestion of the testis biopsy. These samples can be stored until patients reach adulthood. However, testicular biopsies require much greater *in vitro* intervention than processing cryopreserved sperm for ARTs. As the testicular tissue is removed from a patient diagnosed with cancer, it is inadvisable to re-transplant that tissue later in adulthood due to the risk of reintroducing malignant cells harboured in the biopsied testicular tissue. This necessitates the need for *in vitro* culture methods to propagate SSCs and to produce haploid male gametes (Fig. 1). As of yet, this has not been achieved in species other than rodents and so is not practicable for therapeutic use. Thus, the recapitulation of spermatogenesis *in vitro* represents a major challenge in reproductive medicine of the 21st

century and is essential in achieving functional fertility preservation for prepubertal boys.

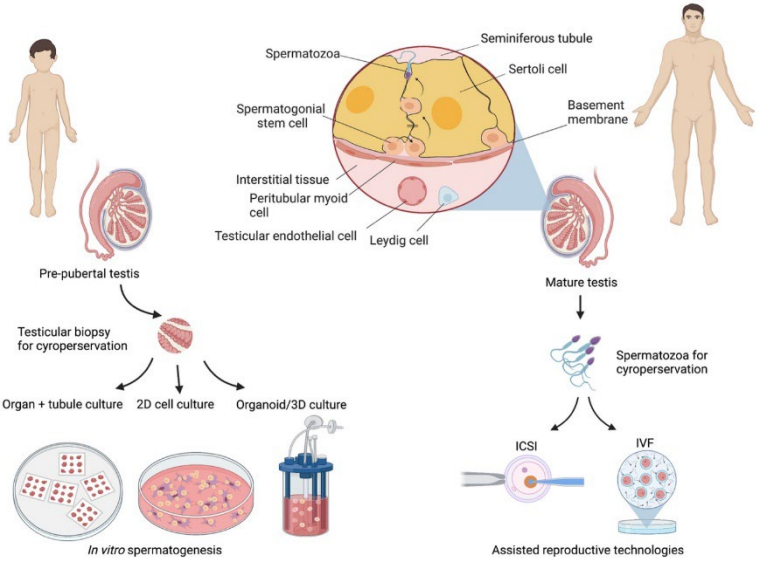

Figure 1. Schematic of the fertility preservation process for prepubertal boys and sexually mature men. The left side of the figure shows the *in vitro* methods that have been developed in a bid to recapitulate spermatogenesis *in vitro*. The right side shows the relatively simple fertility preservation process of sexually mature men, who can cryopreserve spermatozoa for later use in assisted reproductive technologies. If *in vitro* spermatogenesis is successful, the produced spermatozoa would also require assisted reproductive technologies in order to produce a viable embryo.

The effort to produce sperm *in vitro*, spanning over a century, aims to recapitulate the niche environment to initiate and sustain the successive steps of spermatogenesis through mitosis and meiosis to produce haploid male germ cells. The purpose of *in vitro* spermatogenesis (IVS) is two-fold; to enable the production of fertile sperm from biopsied tissue from patients with impaired spermatogenesis and to better understand the basic biology of spermatogenesis. In pursuit of IVS researchers have tried various

methods including different culture strategies, media supplementation and working within a variety of species. Nonetheless, the recapitulation of spermatogenesis *in vitro* has remained challenging.

Primarily three methods have been used for IVS including organ/tubule culture, cell culture and 3-D/organoid culture. Organ culture, using tissue pieces *in vitro*, is the longest established IVS method dating back to the 1920s. Here the first reports were made of *in vitro* differentiation of germ cells using pieces of rabbit testis cultured in rabbit plasma allowing the survival of undifferentiated germ cells and somatic cells for a brief period. Organ culture is particularly useful as germ cells remain spatially arranged as they are in vivo, somatic cells are maintained and cells are protected from the deleterious effects of enzymatic digestion. In the 1960s, organ culture was improved with the development of the gas-liquid interphase method, allowing tissue fragments to survive for longer periods facilitating spermatogenesis from zygotene to the pachytene stage. Additionally, developments in electron microscopy and flow cytometry enabled more precise discrimination of stage specific spermatogonia. However, a key challenge of this era was the support and maintenance of tissue viability which curtailed long term culture in organ culture systems.

In a bid to avoid whole testicular fragments, researchers in the 1980s focused their attention to 2D cell culture of enzymatically digested testicular cells. Dissociated testicular cells are of particular value in studying the role that different niche factors play in germ cell differentiation as the challenge of tissue degradation is avoided. Much of cell culture within IVS has relied on the use of feeder cell populations to sustain SSCs. Many different cell types have been trialled as feeder cells with OP9 bp stroma or fibroblastic cells having been identified as best suited to supporting mouse germ cell maintenance. Porcine Sertoli cells have been suggested as critical feeder cells for human spermatids, limiting apoptosis. This inter-species variation in response to different feeders highlights the challenge in translating these systems. This is further illustrated by the failure to translate results from the first culture system to support the growth and proliferation of murine spermatogonia to other species. This system used mouse fibroblasts as feeder cells supplemented with GNDF (glial cell line derived neurotrophic factor), FGF2 (fibroblast growth factor 2), EGF (epidermal growth factor) and LIF (leukaemia inhibitory factor). However, these conditions did not support SSC maintenance from larger non-rodent animals and humans. This translational barrier may be due to

differences in the metabolic requirements during the extended prepubertal stage of larger mammals compared to rodents.

However, it was by returning to organ culture that *in vitro* spermatogenesis succeeded. In a landmark paper by Sato et al., 2011, *in vitro* production of functional sperm in cultured neonatal mouse testes was finally achieved. Using neonatal mouse testis containing gonocytes or primitive spermatogonia, spermatids and sperm were produced *in vitro* using serum free media. This system maintained spermatogenesis for over two months in tissue fragments at the gas liquid interphase and produced healthy offspring via ICSI. This has since been replicated in rodents by a number of groups; however, successful culture in non-rodent species has remained intractable.

The modern era has seen the adoption of a number of new IVS systems in an attempt to develop a more translatable system. Microfluidic technology has been integrated into organ culture to replicate the circulatory system of the body. In this adapted system, cultured testis tissue is separated from flowing medium by a thin, porous membrane to allow for nutrient and waste exchange via diffusion mimicking the in vivo niche. This novel organ culture system facilitated testosterone production, which was supported by addition of luteinizing hormone, and maintained spermatogenesis in mouse samples for more than 6 months producing healthy offspring via ICSI. The most recently developed IVS system is 3D/organoid culture. This system has advantages over previous static 2D culture systems as the SSCs and somatic cells may self-organise to reassemble the microarchitecture of the niche and allow greater cell interaction. The first 3D system was designed in 2006 and used testicular cells from juvenile rats cultured on collagen gels to mimic the basal membrane and supported limited germ cell differentiation. Recent developments have seen researchers utilise testicular cells in microwells to form aggregates and in turn organoids consisting of germ cells, Sertoli cells, Leydig cells, peritubular myoid cells with distinct seminiferous epithelium and interstitial compartment separated by a basal membrane. Importantly these organoids have been developed in a number of larger mammals including pigs, macaques, and humans which in the future may facilitate sustained and successive SSC differentiation.

Nonetheless, despite a century of research investigating the recapitulation of spermatogenesis *in vitro*, much remains unknown. In order to develop suitable culture conditions, the repertoire of signalling factors required by SSCs including growth factors,

hormones, signals, and interactions between SSCs and the constituent somatic cells of the niche must be fully elucidated. Spermatogenesis is a highly regulated process occurring over a long period of time requiring orchestrated events from germ and somatic cells within the testes composed of 3 distinct phases – proliferation of spermatogonia, meiotic division of spermatocytes, distinct changes in shape and nuclear contents of haploid spermatids (spermiogenesis). Several of these factors required for SSC self-renewal have been identified. Sertoli cells secrete GDNF and FGF2, while CSF1 (colony stimulating factor 1) is secreted by the Leydig and peritubular myoid cells. However, these do not represent the entirety of the signalling molecules regulating the SSC niche microenvironment, and it is highly likely that a number of additional niche factors such as specific miRNAs remain to be identified. Another challenge is the lack of a definitive marker for SSCs. To achieve sufficient numbers of cells required for effective experimentation and therapy development, the spermatogonial population, which contains SSCs, must be increased *in vitro*. Finally, each species has a unique SSC niche which poses a challenge in translating murine culture systems. The full complement of signalling factors within more translational models such as the pig and primates must be uncovered in order to develop more attuned culture systems that may support human *in vitro* spermatogenesis.

At present, to answer the question whether sperm can be produced *in vitro* depends on which species you are interested in. The successful recapitulation of spermatogenesis in rodent species is a significant achievement towards this goal; however, to do so in non-rodent models, especially humans, remains challenging. Recent developments in organotypic culture coupled with the continuing identification of the full repertoire of signalling factors within the niche, as well as further refinements to media supplementation may allow *in vitro* spermatogenesis from larger animals in the near future. Achieving *in vitro* spermatogenesis in non-rodent species is the next hurdle in developing functional fertility restoration for prepubertal patients, enabling pharmacological and toxicological study of new drugs on the testis and the genetic manipulation of germ cells which is not possible *in vivo*.

Suggested reading

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