Spermatogonial stem cell autotransplantation: towards clinical application
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Summary, conclusions and implications for future research

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SUMMARY

Fertility preservation has become a prominent area of interest in reproductive medicine and oncology\(^1\). Effective treatments have increased survival rates of patients with cancer, especially in childhood cancer where the 5-year event-free survival rates are currently around 80\%\(^2,3\). Gonadal damage is a relatively common consequence of these treatments and many cancer survivors suffer from permanent sterility\(^4,5\). In prepubertal or adult women with cancer, cryopreservation of ovarian cortical strips or oocytes prior to start of treatment can be performed\(^6\). In adult men with cancer, cryopreservation of semen is a longstanding routine practice. As spermatogenesis does not commence until the initiation of puberty\(^7\), cryopreservation of semen prior to the start of cancer treatment is not possible in prepubertal boys and hence fertility preservation is currently not available for them.

Spermatogonial stem cells (SSCs) are responsible for the continuous production of spermatozoa throughout adult life\(^8\), and these SSCs are very sensitive to chemo- or radiotherapy\(^9\). The theoretical approach to achieve fertility preservation in prepubertal boys is to store testicular tissue before the initiation of gonadotoxic therapy and to propagate and autotransplant SSCs from this tissue after cancer survival\(^10\). Since 1994, when Brinster and colleagues developed the first animal model of SSC transplantation\(^11,12\), advances in isolation\(^13-15\), cryopreservation\(^16-19\) and culturing\(^20-25\) of SSCs in several animal species have opened an avenue to preserve fertility in prepubertal boys with cancer. Currently, several groups all over the world are working on translating these preclinical achievements to human clinical application.

As outlined in chapter 1, the goal of this thesis was to investigate several important steps towards clinical application of SSC autotransplantation.

In chapter 2, we systematically review the literature for original reports on semen quality in men before and after gonadotoxic treatments and on reports on pregnancy rates after ART using spermatozoa obtained before or after gonadotoxic therapy.

We reviewed and analyzed 93 included studies. Data from 1296 (pre-treatment) and 1210 (post-treatment) adult men showed that the overall prevalence of azoospermia increased from 12\% before gonadotoxic therapy to 41\% at follow-up. In 549 adult men who were treated before puberty or during adolescence, the overall prevalence of azoospermia was 42\%. There was no indication for a difference in prevalence of azoospermia between boys that had been treated with...
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chemotherapy alone and boys treated with chemotherapy and radiotherapy. In couples who were referred for ART, the mean ongoing pregnancy rates per couple after ART using cryopreserved spermatozoa (781 couples) or spermatozoa obtained after gonadotoxic therapy (127 couples) were 50% and 38%, respectively.

These data reveal the importance of spermatozoa or testicular cryopreservation before gonadotoxic therapy in both adult men and adolescent/prepubertal boys, respectively. Furthermore, ART using either fresh or cryopreserved spermatozoa is an effective method to help these men to achieve paternity.

Chapter 3 shows the knowledge and attitudes of parents of boys with cancer towards fertility preservation in the context of different risk levels of infertility and success rates of fertility restoration.

In this study we sent a questionnaire to 465 families whose son was treated for cancer. Seventy-eight percent of all parents responded. We found that at the time of cancer treatment only 15% of parents were aware of the effect of this treatment on fertility. Sixty-four percent of parents of boys ≥12 years would agree to store spermatozoa obtained by masturbation and/or electro-ejaculation, while 54% of parents of boys <12 years would agree to store a testicular biopsy. If the risk of infertility or the success rate of fertility restoration would be 20% at most, more than one-fourth of parents would still opt for fertility preservation.

These data show that parents of boys with cancer have limited knowledge on the risks of infertility due to cancer treatment, but the majority would want some sort of fertility preservation once informed about these risks. All parents should be counseled about this issue, as many parents want to preserve their son’s fertility, even if the risks of becoming infertile or the chances on successful fertility restoration are low.

Chapter 4 describes the first in vitro propagation system of human SSCs from small testicular biopsies.

In this study we isolated and cultured human testicular cells from six men who underwent orchiectomy as part of treatment for prostate cancer. Testicular cells were cultured in supplemented StemPro medium. Germline stem cell clusters that arose were subcultured on human placental laminin coated dishes in the same medium. The presence of spermatogonia was determined by reverse transcriptase polymerase chain reaction for markers ITGA6, ITGB1 and ZBTB16 and by immunofluorescence for ZBTB16. To test for the presence of SSCs in culture, xenotransplantation to the testes of busulfan treated immunodeficient mice was performed, and migrated human SSCs after transplantation were detected by COT-1 Fluores-
cence In Situ Hybridization (FISH). The number of colonized SSCs transplanted at early and later time points during culture was counted to determine propagation. We showed that testicular cells could be cultured and propagated up to 15 weeks. Germline stem cell (GSC) clusters arose in the testicular cell cultures from all six men and could be subcultured and propagated up to 28 weeks. Expression of spermatogonial markers on both the RNA and protein level was maintained throughout the entire culture period. In 4 of 6 men, xenotransplantation to mice demonstrated the presence of SSCs, even after prolonged culture. SSC numbers increased 53-fold within 19 days in the testicular cell culture and increased 18450-fold within 64 days in the germline stem cell subculture.

For future clinical application, with a biopsy of 0.2 mL from a prepubertal testis, the number of SSCs within this small sample needs to be increased 65-fold to colonize an adult testis of approximately 13 mL. Assuming that the efficiency of autotransplantation in future clinical application will be approximately 5%, a 1300-fold increase in SSC number is needed for efficient transplantation. Our established in vitro human SSCs propagation system is therefore efficient enough to provide adequate numbers of SSCs for autotransplantation.

Chapter 5 demonstrates that in vitro propagation of human prepubertal SSCs using the culture system for adult human testicular cells described in chapter 4, can be achieved. In this study, we used cryopreserved testicular biopsies of two boys, 6.5 and 8 years of age, diagnosed with Hodgkin lymphoma that were referred to the Avicenna Research Institute (Tehran, Iran) for fertility preservation. The main part of each biopsy specimen was stored for possible future clinical use, and with written informed consent from both parents, a small part was donated for research and transferred in liquid nitrogen to the Academic Medical Centre (Amsterdam, the Netherlands). Isolated testicular cells were cultured using our previously described protocol (chapter 4). The presence of spermatogonia was demonstrated by RT-PCR (ITGA6, ITGB1, ZBTB16, CD9, GPR125, GFRA1 and UCHL1) and immunohistochemistry (ZBTB16 and UCHL1). Their stemness was ascertained by xenotransplantation to the testes of busulfan treated immunodeficient mice. Testicular cell cultures from the biopsies of both boys could be cultured for 15.5 and 20 weeks, from the 8 and 6.5 year old boy, respectively. Germline stem cell clusters formed in the cultures of both patients and could be subcultured on laminin for at least 20 and 29 weeks from the 8 and 6.5 year old boy, respectively. Spermatogonial markers were expressed throughout the entire culture period. Xenotransplantation confirmed the presence and propagation of SSCs in culture. Trans-
plantation of cultured cells from early and later passages from the 8 year old boy showed a 9.6-fold increase in the number of SSCs in 11 days of culture. Similarly, subcultured GSC clusters from the 8 year old boy showed a 5.6-fold increase within 14 days and a 6.2-fold increase in SSCs within 21 days was seen in the subcultured GSC clusters from the 6.5 year old boy.

Assuming SSCs grow exponentially, 35 days of testicular cell culture or 58 to 83 days of GSC clusters subculture would be necessary to achieve the 1300-fold increase in SSC number that we previously estimated as necessary for repopulation of adult human testes after autotransplantation. As we succeeded in culturing SSCs from cryopreserved prepubertal testis biopsies for at least 15 weeks (=105 days), our culture system appears efficient enough to propagate sufficient SSCs for autotransplantation in sterile survivors of childhood cancer.

Chapter 6 describes the elimination of malignant cells from testicular cells using the testicular cell culture conditions as described in chapters 4 and 5. In this study, we cultured acute lymphoblastic leukemia (ALL) cells separate and mixed with human testicular cells. As demonstrated by flow-cytometry analysis, ALL cells cultured separately did not survive beyond 14 days of culture. We also demonstrated that irrespective of the initial concentrations (0.04%, 0.4%, 4% and 40%), ALL cells mixed with testicular cells were undetectable beyond 18-26 days of culture as measured by a highly-sensitive (depending on the patient 1:10,000 to 1:100,000 cells) Minimal Residual Disease-patient specific PCR (MRD-PCR).

These results demonstrate that our culture system not only allows for efficient propagation of SSCs but also eliminates contaminating malignant ALL cells.

**CONCLUSION**

- Both adult men and prepubertal boys who are treated with gonadotoxic agents are at high risk of long term or permanent sterility.
- Cryopreservation of spermatozoa should be offered to all adult and adolescent men ≥12 years who undergo gonadotoxic therapy.
- Cryopreservation of testicular biopsies should be offered to all prepubertal boys who undergo gonadotoxic therapy.
- The use of ART allows cancer survivors to achieve paternity. The use of spermatozoa cryopreserved prior to gonadotoxic treatment in ART cycles appears more successful compared to the use of spermatozoa obtained after gonadotoxic therapy.
• Parents of boys with cancer have limited knowledge on the risks of infertility due to treatment, but the majority would want some sort of fertility preservation once informed about these risks.

• Many parents want to preserve their son’s fertility even if the risk of becoming infertile or the chances on fertility restoration are low.

• Long-term culture and propagation of both adult and prepubertal human SSCs in vitro is now possible.

• Our recently established culture system not only allows for efficient propagation of SSCs but also eliminates contaminating malignant ALL cells.

Taken together, these results are important steps forward in our knowledge on how to preserve fertility in prepubertal boys and adolescent men diagnosed with cancer and therefore all parents of these boys with cancer should be counseled and offered the possibility to store testicular tissue or -if possible- semen for future clinical use.

**IMPLICATION FOR FUTURE RESEARCH**

Long term culture and in vitro propagation of human SSCs from cryopreserved mature (adult) and immature (prepubertal) testes biopsies has been established. These results are a crucial step forward in enabling the restoration of fertility in sterile male childhood cancer survivors by autotransplantation of in vitro propagated SSCs derived from a small testis biopsy of the patient himself.

Before clinical application can be considered, some important issues need to be addressed. First, genetic and epigenetic integrity of long term cultured human SSCs needs to be demonstrated. Second, the health of offspring derived from autotransplanted cultured SSCs needs to be carefully evaluated in a mouse model system.

We and other researchers in this field are now in a shuttle towards clinical application of SSC autotransplantation. *Have a nice and safe trip!*
REFERENCES


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