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Cryopreservation of testicular tissue in young cancer patients

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Cryopreservation of testicular tissue might benefit prepubertal boys who must undergo chemotherapy or radiotherapy. Cryopreservation of testicular tissue and testicular cells for intracytoplasmic sperm injection (ICSI) is feasible and widely applied. Testicular tissue from prepubertal boys can also be frozen, by applying techniques used with other tissues and with testicular tissue from adult men before ICSI. Good results have been obtained when propanediol is used as a cryoprotectant, but glycerol has also been used when freezing testicular tissue. Spermatogonia might also be isolated and cryopreserved as a cell suspension, though practical experience in humans is lacking. Transplantation of the frozen–thawed cells back to the testes after cancer treatment might result in restoration of spermatogenesis. Live offspring have been born to mice after transplantation of fresh, but not cryopreserved, testicular cells. Transplantation is technically feasible also in larger species, but to date no offspring have been born. Spermatogenesis *in vitro* would be an excellent option for boys with haematological malignancies who carry a risk of relapse after transplantation; however, at present the method is feasible only for the late stages of spermatogenesis.

Keywords: cancer/cryopreservation/infertility/testis/transplantation

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Introduction

Improved survival rates after chemotherapy and radiotherapy for many malignant diseases have resulted in a new group of infertile patients. Approximately 1 in 650 children develop malignancies during childhood, and at least 60% of these are cured. As gonadal damage is a relatively common consequence of these life-saving treatments (Siimes *et al.*, 1992; Howell and Shalet, 1998), maintenance of fertility and a normal sexual lifestyle would be extremely important with regard to the quality of life of these survivors.

Cryopreservation of spermatozoa before chemotherapy or radiotherapy is an established option for 'storing' the fertility of postpubertal men (Royère *et al.*, 1996). By using intracytoplasmic sperm injection (ICSI), pregnancies can be achieved in spite of the often poor semen quality found in these subjects. In the case of prepubertal boys, however, another strategy must be used, and this is not without problems (Bahadur and Ralph, 1999; Radford *et al.*, 1999). Techniques for the cryopreservation of both testicular and ovarian tissues of prepubertal individuals are already feasible, but methods of how to use these tissues for infertility treatments in the future have still to be developed. Azoospermic men who undergo testicular biopsy for diagnostic or treatment purposes also benefit from the cryopreservation of testicular tissue (Tuuri *et al.*, 1999).

Who would benefit?

Cryopreservation of testicular tissue would be especially important for prepubertal boys who are not yet able to produce spermatozoa, but it might also be valuable in older boys and young adults who might achieve sperm production and hence avoid the need for assisted reproduction in later life. Because cryopreservation of spermatozoa has been well established, it should be carried out routinely in postpubertal individuals who are at risk of severe testicular damage.

The degree of testicular damage depends on the type of chemotherapy used. Alkylating agents (cyclophosphamide, ifosfamide, mustine, busulphan, chlorambucil, melphalan and carmustine) are gonadotoxic during childhood and adolescence, as well as during adulthood (Meirow and Schenker, 1995; Forman *et*

al., 1996; Howell and Shalet, 1998). Men who have received a total dose in excess of 18 g are usually azoospermic. However, when alkylating agents are used in combination with other drugs, as smaller doses, the effects are less severe. Cyclophosphamide is responsible for the smaller size of testes seen in survivors of childhood cancers (Siimes and Rautonen, 1990); cisplatinum is probably less deleterious, but damage after its administration has also been reported (Forman *et al*., 1996). Follow-up data are available after administration of various combinations (Meirow and Schenker, 1995; Forman *et al*., 1996; Howell and Shalet, 1998), and although the effects are milder, severely impaired sperm production has been described after treatment with most of these. Over the past few years, the doses used have been higher, though the survival of young men has been better. At the onset of treatment it is not clear how much chemotherapy will be required. Chemotherapy used before stem cell transplantation is certainly an indication for tissue cryopreservation, and especially in leukaemia, where it is not known even by the time that treatment is started who will require stem cell transplantation at a later date. This is why cryopreservation of spermatozoa is recommended before any chemotherapy is carried out. Similarly, the cryopreservation of testicular tissue might be indicated for all prepubertal boys, and this is important in all types of malignancies. Radiotherapy, and especially that applied directly to the testes in testicular cancer and leukaemia, or total body irradiation before stem cell transplantation, is often used in combination with chemotherapy, and may cause severe testicular damage.

Repeated testicular biopsies can be avoided if testicular tissue or testicular spermatozoa from adult men is cryopreserved whenever a biopsy is carried out. These spermatozoa can be later used in ICSI.

Methods of cryopreservation

Cryopreservation of various cell suspensions is widely practised in science and medicine, and rapid freezing, using either dimethyl sulphoxide (DMSO) or glycerol as a cryoprotectant, provides satisfactory results for most purposes. The cryopreservation of tissues is technically more demanding because many different cell types will be present, and the cryoprotectant must penetrate greater distances into the tissues. Nonetheless, successful programmes for tissue cryopreservation have been developed.

Ovarian tissue

Human ovarian tissue, including ovarian follicles containing oocytes, has been cryopreserved by using propanediol–sucrose, DMSO, ethylene glycol or glycerol as a cryoprotectant (Hovatta *et al*., 1996a; Newton *et al*., 1996; Gook *et al*., 1999). The viability of the ovarian tissue after freezing and thawing has been proved by achieving live offspring in both mice (Carroll *et al*., 1990) and sheep (Gosden *et al*., 1994). In human tissue, viability has been demonstrated in organ culture (Hovatta *et al*., 1997), by transplantation to immunodeficient mice (Newton *et al*., 1996), and by viability assay of isolated follicles (Oktay *et al*., 1997).

Testicular tissue of adult men

Testicular tissue from infertile men has been cryopreserved, using glycerol as a cryoprotectant, either as a cell suspension (Gil-Salom *et al*., 1996; Podsiadly *et al*., 1996; Friedler *et al*., 1997; Khalifeh *et al*., 1997; Ben-Yosef *et al*., 1999; Gianaroli *et al*., 1999) or as pieces of tissue (Hovatta *et al*., 1996b; Salzbrunn *et al*., 1996; Allan and Cotman, 1997; Oates *et al*., 1997; Perraguin-Jayot *et al*., 1997; Tuuri *et al*., 1999). Healthy children have been born as a result of ICSI carried out using spermatozoa after both freezing methods. Either gradual manual cooling or programmable freezers have been successfully used in the above-mentioned studies.

When testicular tissue from infertile men is cryopreserved for possible ICSI, the purpose is to cryopreserve mature spermatids and spermatozoa. Freezing spermatozoa—which are very small cells—either in semen or as washed preparations, by using glycerol as a cryoprotectant has been widely used since the early 1950s (Royère *et al*., 1996). Glycerol does not penetrate tissues very well, but is likely to function as a cryoprotectant for spermatids and spermatozoa, as well as for suspensions of testicular cells. The motility of spermatozoa after cryopreservation of cell suspensions prepared from human testicular biopsies was compared with that of spermatozoa cryopreserved within a piece of testicular tissue when glycerol was used as cryoprotectant (Crabbe *et al*., 1999). The spermatozoa frozen as a testicular cell suspension showed better motility after thawing, but if the main purpose is to cryopreserve larger cells (e.g. spermatogonia, spermatocytes, Sertoli cells and Leydig cells), then another cryoprotectant might function better. Nonetheless, relatively good morphology was maintained within testicular tissue frozen using glycerol (Salzbrunn *et al*., 1996).

Recently, a protocol was applied to mouse and human testicular tissue (M.Huhtanen, M.Siimes, L.C.Andersson and O.Hovatta, unpublished results) which was similar to that used in the freezing of human ovarian tissue (Hovatta *et al*., 1996a), and which used propanediol–sucrose as a cryoprotectant in a medium containing human serum. This protocol appeared to provide good tissue morphology after thawing; examples of frozen–thawed human and mouse testicular tissue are shown in Figures 1 and 2. In the mouse testis, the seminiferous tubules were almost intact, while in the human testis all cell types identified within the tissue appeared to have survived. The structure of the seminiferous epithelium was slightly disrupted, but this may also occur when the original testicular tissue is abnormal; alternatively, it may have been caused by the fixation process, as this is often problematic in human testicular tissue.

Testicular cell suspension in animals and man

One group (Avarbock *et al*., 1996) were able to establish spermatogenesis in recipient animals by transplanting a frozen–thawed suspension of mouse testicular cells having used DMSO as a cryoprotectant, but no offspring were produced. The cell suspension was cooled rapidly to –70°C, maintained at this temperature for 12 h, and then transferred to liquid nitrogen. About one-third of the cells survived the freezing and thawing procedure. Using the same method for cryopreservation, the same group (Ogawa *et al*., 1999) cryopreserved hamster testicular cells and showed 43% viability after thawing, as revealed by the trypan blue exclusion test. These cells were integrated to seminiferous tubules of recipient mice similarly to non-frozen cells, but no mature, normal spermatozoa were seen.

Recently, 87% and 66% survival of mouse and human testicular cell suspensions were reported respectively after

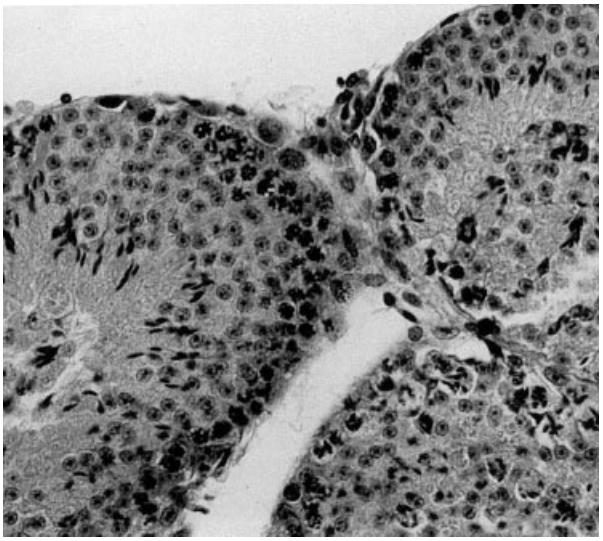

Figure 1. Mouse testicular tissue which has been frozen using propanediol–-sucrose as a cryoprotectant, slow-programme frozen, and rapidly thawed. Seminiferous tubules with intact spermatogenesis and cells with histologically normal appearance are seen. Original magnification, ×200.

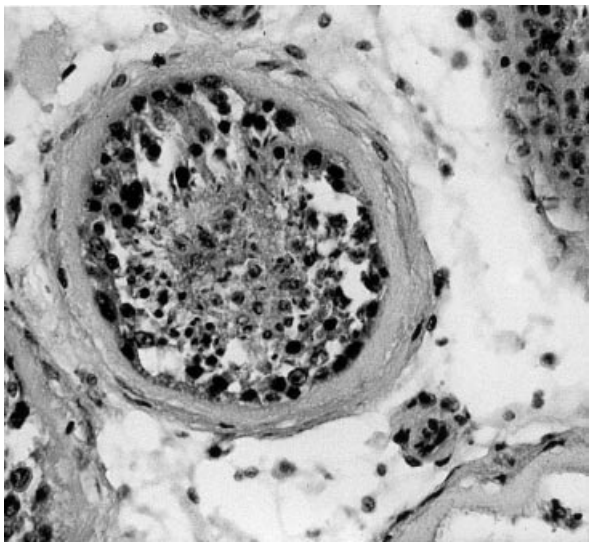

Figure 2. Human testicular tissue after cryopreservation using slow-programme freezing with propanediol–sucrose as a cryoprotectant. All cell types of the seminiferous epithelium are seen. The inner structure of the tubuli is slightly disrupted; this may be due either to an original disturbance in the structure, or to fixation problems. Original magnification, ×200.

enzymatic disaggregation of testicular tissue with collagenase Ia (1 mg/ml) to isolate tubules from the interstitium, followed by bovine pancreatic trypsin (6 µg/ml), ovine hyaluronidase (16 µg/ml) and DNase I (0.4 µg/ml) (Brook *et al*., 2001). A trypan blue exclusion test was used as a marker of survival. After slow programmed cooling using four different cryoprotectants (glycerol, DMSO, 1,2-propanediol and ethylene glycol; all 1.5 mol/l) in Leibovitz medium containing 4% fetal calf serum, the mean viability of the cells, as shown by trypan blue exclusion, was 52–58%.

Six different freezing and thawing protocols have also been compared for mouse and human testicular cell suspensions (M.Huhtanen, M.Siimes, L.C.Andersson and O.Hovatta, unpublished results). Propanediol–sucrose, DMSO and glycerol were used as cryoprotectants, and rapid and slow freezing programmes were applied. Human serum was used in the freezing medium. The best cell survival, as revealed by a viability assay (esterase activity), was obtained by using propanediol–sucrose and slow programmed freezing. Survival rates of the mouse and human testicular cells were 65% and 60% respectively. When DMSO was used, the survival rate for mouse cells was 32%, as reported previously for mouse cells with rapid DMSO freezing (Avarbock *et al.*, 1996). Only 10% of human testicular cells survived the same programme, however.

Freezing tissue from prepubertal boys

With regard to prepubertal boys, freezing by using cryoprotectants other than glycerol might provide some advantages, since the purpose is to cryopreserve spermatogonia. Spermatogonia, which are much larger than either spermatids or spermatozoa, are located at the basal region of the seminiferous epithelium. Unfortunately, the ease with which relatively dense human testicular tissue can be dispersed to create a cell suspension is unclear. Extreme enzymatic digestion before cryopreservation may cause the cells to be damaged, as shown by the reduced viability of suspensions after dispersion (Brook *et al*., 2001). On the other hand, the cells may be even more vulnerable during digestion after freezing and thawing. Whether it is better to produce the suspension before or after cryopreservation remains to be shown; however, for re-implantation, a cell suspension is needed for injection into the testes.

Isolation of cell types before or after freezing

Leydig cells

The isolation of interstitial cells from seminiferous tubules has been performed in several species, both before re-implantation (Brinster and Zimmermann, 1994) and before cryopreservation (M.Huhtanen, M.Siimes, L.C.Andersson and O.Hovatta, unpublished results). Testicular tissue is placed in a culture medium containing collagenase, and incubated for 10 min at 34°C, with vigorous shaking. The tubules are collected, and the remaining interstitial cells can be separately washed. In this way a cell suspension containing Leydig cells is obtained for cryopreservation. These cells can be injected into the interstitial space of the testis to correct possible Leydig cell dysfunction caused by cancer treatment, and especially that preceding bone marrow transplantation (Siimes *et al.*, 1992; Howell *et al.*, 1999). The possibility of re-injecting malignant cells simultaneously must be borne in mind in such cases, and the success of this method of re-implanting cryopreserved Leydig cells remains to be shown.

Spermatogonia

The isolation of spermatogonia from other testicular cells is also feasible (Bellvé *et al.*, 1977; De Felici and McLaren, 1982; Bucci *et al.*, 1986). In the successful experiments, as regards transplantation (Brinster and Zimmermann, 1994), cryopreservation and transplantation (Avarbock *et al.*, 1996) or culture of testicular cell suspension and transplantation after long-term culture (Brinster and Nagano, 1998), no isolation was carried out.

Magnetic cell sorting has recently been used to enrich viable spermatogonia in several animal species (von Schönfeldt *et al.*, 1999). The number of spermatogonial stem cells is relatively low in the testes (de Rooij and Grootegoed, 1998), and their identification is not easy. However, others (Shinohara *et al.*, 1999), showed that β_1 and α_6 integrins are specific surface markers for mouse spermatogonial stem cells. After preparing a testicular cell suspension, the cells that were positive for these markers were selected using a magnetic bead procedure. After transplantation, there was a higher number of colonies of spermatogenic cells originating from donor cells in the recipient seminiferous tubuli, after enhancement of the concentration of the stem cell spermatogonia. Others (Brook *et al*., 2001) attempted to demonstrate the presence of spermatogonia in the cell suspension by using immunohistochemical staining for c-kit. Fewer than 1% of the cells were positive in the suspensions prepared, suggesting that the isolation method was not sufficiently complete. C-kit is not positive in stem cell spermatogonia (Dym *et al*., 1995), and integrins (Shinohara *et al*., 1999) would have been better prognostic markers than c-kit. Cryopreservation of a testicular cell suspension with an enhanced concentration of spermatogonial stem cells might also offer the most effective means of storing transplantable spermatogonia for future use in prepubertal boys.

How to use cryopreserved cells

Re-implantation

Following recent progress in animal experiments, the re-implantation of cryopreserved testicular cells into the donor's testes has become an important possible option for the use of cell suspensions. One group (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994) succeeded in initiating spermatogenesis in infertile mice by transplanting donor testicular cells into the testes of mice previously made infertile by an intraperitoneal injection of busulphan. Initiation of spermatogenesis resulted also from the transplantation of rat testicular cells into the seminiferous tubules of infertile mice (Clouthier *et al.*, 1996). Donor testicular cells taken from prepubertal and adult mice and frozen and stored in liquid nitrogen, were also capable of generating spermatogenesis in recipient seminiferous tubules, although no offspring were reported. Xeno-transplantation in other species also proved successful, but there appeared to be problems in the regulation of normal sperm production (Dobrinski *et al.*, 1999). Testicular cells from hamsters and mice have been used in establishing spermatogenesis in recipient mice, also after cryopreservation (Ogawa *et al*., 1999, 2000; Russell and Griswold, 2000). In these animals, problems were seen during spermiogenesis, and mature normal spermatozoa were not seen. Spermatogenesis of infertile mice could be restored by transplanting a cell suspension from their testes to recipient mice (Ogawa *et al.*, 2000). Spermatogenesis in rats has also been established after transplantation (Jiang and Short, 1995).

Re-injection of non-frozen testicular cells of rat, bovine, monkey and human testes, using injection through the rete testis under ultrasound control, has also been successful (Schlatt *et al.*, 1999). Others (Brook *et al*., 2001) injected blue dye into human testes which had been removed because of prostatic cancer, and filled about 50% of the tubules by intratubular injection. Ultrasound-guided injection through the rete testis, as described previously (Schlatt *et al*., 1999), might be more practicable in humans.

In Manchester, testicular tissue from boys suffering from Hodgkin's disease or non-Hodgkin's lymphoma has been cryopreserved, and five of them have undergone re-injection of cell suspension into their testes (Radford *et al.*, 1999); the outcome of this study will generate much excitement as to the possible success of these treatments.

Spermatogenesis in vitro

Spermatogenesis *in vitro*, starting from spermatogonia, would be an excellent option for boys with haematological malignancies that are at high risk of being transmitted with the cell suspension. However, this approach has not yet been successful, despite several promising attempts in experimental animals (Miura *et al*., 1991; Boitani *et al*., 1993; Abe and Ji, 1994; Zhou and Hutson, 1995). Nonetheless, this remains a challenging area for further research.

Spermatogenesis is known to occur to some extent in the testes of boys at very early stages of pubertal development (Muller and Skakkebaek, 1983; Janczewski and Bablok, 1985; Nielsen *et al.*, 1986). Indeed, spermatozoa has been shown to be present in the urine of boys at a mean age of 13 years, though many may be azoospermic as regards their ejaculate. Hence, it may be possible to obtain spermatids and spermatozoa for ICSI from cryopreserved testicular biopsy samples obtained from boys who are aged only 10–13 years and do not yet have spermatozoa in their semen. Methods also exist to mature testicular spermatids and spermatozoa *in vitro* for ICSI (Tesarik *et al.*, 2000). At present, this is possible only during the later stages of spermatogenesis, but such an approach might be applicable to boys of this age.

Risks

The risk of re-implanting malignant cells together with the cryopreserved cell suspension is real, at least in haematological malignancies (Söder, 1997; Radford *et al.*, 1999; Jahnukainen *et al.*, 2001), though in other malignancies the risk is lower. In future, it will be important to identify the presence of even small numbers of malignant cells in the suspensions before re-implantation.

In haematological malignancies the risk of re-implanting malignant cells might be avoided by culturing spermatogenic cells *in vitro*, but this alternative seems remote at present. Xenogeneic transplantation of rat and hamster testicular cells to the testes of mice, also after cryopreservation, has resulted in spermatogenesis, albeit defective spermiogenesis (Ogawa *et al*., 1999, 2000; Russell and Griswold, 2000). In theory, cryopreserved human testicular suspension might be injected into immunodeficient mice or rats, and the resulting spermatids or spermatozoa used for ICSI in cases of malignancies with a high risk of relapse. However, growing human spermatozoa in rodents would raise many concerns that were not only ethical but also biological, for example the risk of infection(s). Nonetheless, such an animal model might be used in testing the cell suspensions with regard to their malignant potential. If the rodent develops malignancy, then the suspension cannot be injected into the man. In-vitro cultures bear the lowest risks.

Ethical aspects

Ethical concern has been expressed when tissue from young boys is frozen (Bahadur and Ralph, 1999), as although cryopreservation of testicular cells may be applied in clinical practice, there is no guarantee that fertility will be restored. Moreover, a young child cannot give his consent for the procedure, and the technique must be performed on the granting of parental consent. Pubertal boys may give their own consent, but they often already have at least some spermatozoa in their semen (Janczewski and Bablok, 1985), and cryopreservation of spermatozoa is a good solution in such cases. Testicular biopsy is an invasive procedure for a young boy, and hence it must be well motivated. In practice, the biopsy can be performed under the same general anaesthesia that is used to insert a central line for chemotherapy, but the risk of bleeding must be taken into account. If the boy does not yet have mature spermatozoa in his semen, there may be some spermatozoa (or at least spermatids) in his testicular tissue, and these may later be used in ICSI, either directly or after in-vitro culture. For boys in early puberty, cryopreservation of testicular tissue might provide a beneficial option at an early stage, and as they understand the procedure they should be able to provide their (informed) consent.

Even if a needle biopsy (Tuuri *et al.*, 1999) can be easily performed, it is necessary to consider the optimal timing in relation to the chemotherapy. In cases of leukaemia, a biopsy should probably not be taken before the first treatment, but rather during the first remission. These experimental procedures must be offered with great caution until more data are available regarding both their risks and benefits, however.

Conclusions

The cryopreservation of semen can be used to store germ cells in young cancer patients who can already ejaculate. However, for ICSI, mature spermatozoa or spermatids might be obtained from testicular biopsies of boys aged 10–13 years, while in younger boys testicular tissue or testicular cell suspensions—both enriched as regards Leydig cells and spermatogonia—can be cryopreserved, the future aim being to re-implant the Leydig cells into the testicular interstitial space, and the spermatogonia into the seminiferous tubuli. The latter approach has been found successful in many animal species, but experience in humans is still lacking. Re-implantation also carries a risk of transferring malignant cells back to a man once cured from the original condition; hence, it may be important to develop in-vitro maturation methods. It is important to bear in mind that these techniques are still at a highly experimental stage, and careful counselling and consent is essential before such procedures may be performed in the clinical setting.

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