Germ cell transplantation into X-irradiated monkey testes

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BACKGROUND: An intense debate is ongoing regarding options for fertility protection in oncological patients. Germ cell transplantation has been applied to restore mouse spermatogenesis. Here, an attempt to apply autologous germ cell transplantation to a primate animal model is described.

METHODS: Five adult male cynomolgus monkeys were biopsied to retrieve and cryopreserve germ cells before both testes were irradiated (dose 2 Gy). Six weeks later, each monkey received an infusion of its own cell suspension into the right testis, while the left testes were infused with saline. Testis size, sperm counts and serum concentrations of inhibin, FSH and testosterone were analysed weekly for 9 months.

RESULTS: In four monkeys, the germ cell-infused right testes showed a slight to moderate increase in the rate of regrowth in comparison with the left testes. In two monkeys the right testis proceeded to recover more prominently, resulting in larger right testis volumes and better or full spermatogenic recovery at the study end. Restoration of spermatogenesis occurred as an all-or-nothing event. Inhibin B concentrations increased, while FSH and testosterone concentrations decreased with testicular regrowth. Sperm counts did not recover.

CONCLUSIONS: The present study demonstrates the immaturity and complexity of germ cell transplantation as a clinical approach.

Key words: cancer/fertility/irradiation/spermatogonia/testis

Introduction

Improved treatment regimens have led to high survival rates of oncological patients. However, as the rapidly dividing diploid spermatogonia are the testicular cells that are most sensitive to the cytotoxic effects of both radiotherapy (Meistrich, 1993) and chemotherapy (Meistrich et al., 1982), severe damage of the gonads often leads to temporary or permanent infertility as a side effect of therapy. In rats, inhibition of the pituitary-gonadal axis renders spermatogonia less susceptible to chemotherapy (Glode et al., 1981). However, in men all hormonal attempts to protect the seminiferous epithelium by reducing the rate of spermatogenesis have failed, and it appears necessary to develop new approaches either to protect or preserve fertility in oncological patients (Naysmith et al., 1998; Radford et al., 1999; Aslam et al., 2000). Young prepubertal patients—who cannot supply a semen sample for cryopreservation of sperm—have a particularly poor fertility prognosis. Autologous germ cell transplantation might become a clinically relevant technique by which germ cells can be removed from, and later reintroduced into, the male gonad (Aslam et al., 2000; Schlatt et al., 2000). The removal and storage of male germ cells from patients undergoing high-dose chemotherapy or radiotherapy might allow these patients to conserve their germ cells so that they may be retransplanted after recovery from treatment.

Microinjection of germ cell suspensions into seminiferous tubules of mice leads to the restoration of spermatogenesis from donor spermatogonial stem cells (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Further analysis of the recipient testes revealed that spermatogenesis is reinitiated focally, but most of the transplanted germ cells degenerate. Meiotic germ cells arising from transplanted stem cells first appeared after 1 month, and their number increased steadily thereafter (Parreira et al., 1998, 1999).

For germ cell transplantation into testes of larger species, modifications of the transfer technique were necessary. The easiest, least invasive and most efficient filling of the rete testis was obtained by injections into the rete testis of bull, monkey and man (Schlatt et al., 1999). This technique was also applied to surgically removed human testes (Schlatt et al., 1999) whereby ultrasonography was used to localize the rete testis, as well as to position the injection needle. While in rodent testes, immaturity or involution of the testes renders the injection of germ cells more difficult, it appeared to be a prerequisite for efficient filling of bovine and primate testes. The detection of transplanted B-spermatogonia 4 weeks after autologous germ cell transfer revealed that germ cell transplantation can, in principle, be applied in primates (Schlatt et al., 1999).
The present study outlines a preclinical approach using a non-human primate to study the feasibility, side effects and efficiency of restoration of autologous germ cell transplantation after X-ray-induced germ cell loss mimicking the testicular damage that occurs in patients during oncological treatment.

Materials and methods

Animals

Five adult male cynomolgus monkeys (Macaca fascicularis, aged 8–9 years and of body weight range 7.2–11.0 kg) were housed in individual cages under defined environmental conditions. Standardized monkey diet supplemented with fresh fruit was provided twice daily, and tap water was available ad libitum. Maintenance and handling of the monkeys were performed in accordance with the German Federal Law on the Care and Use of Laboratory Animals.

Body weight and testicular volumes of all animals were recorded at weekly intervals. The volume was determined as described previously by measuring the length and width of the testis using Vernier callipers (Weinbauer et al., 1998), the formula for an ellipsoid being used to estimate testicular volume. Blood samples were collected at weekly intervals by venipuncture of the saphenous vein under ketamine sedation (Ketavet®, 5–8 mg/kg, i.m., Parke Davis, Munich, Germany). Blood was allowed to coagulate overnight at 4°C, after which the serum was separated and stored at −20°C until analysed. Ejaculates were obtained by rectal probe electroejaculation, and the evaluation of sperm numbers was performed as described previously (Weinbauer et al., 1994). Sperm counts were expressed per total ejaculate (exudate plus coagulum). Azospermic samples as estimated under light microscopy on semen smears were centrifuged at 900 g for 5 min, and the sediment was subsequently examined for the presence of spermatozoa. Sperm morphology assessment was performed by Papanicolaou staining according to WHO criteria (World Health Organization, 1992).

Study design

At 3 weeks after the collection of baseline values, the monkeys were bilaterally biopsied for retrieval of testicular tissue as previously reported (Weinbauer et al., 1998). Three weeks later, all monkeys received a single local X-ray irradiation (2 Gy) of both testes as described previously (Foppiani et al., 1999). At 40 days after irradiation, an injection of germ cell preparations into the right testis was performed. The monkeys were bilaterally biopsied 13 weeks later (day 143 after germ cell transfer, 189 days after irradiation) and orchidectomized at the end of the study (day 254, 36 weeks after irradiation).

Preparation, culture and cryopreservation of testicular tissue

The fresh biopsy samples were carefully dissected into small fragments (<0.5 mm³) and transferred onto polycarbonate membranes (8.0 µm, tissue culture inserts; NUNC, Roskilde, Denmark) in 6-well culture plates (NUNC). All fragments of a single biopsy were randomly distributed and cultured separately on two membranes. The inserts were placed bottom to top in the wells. The culture medium [Dulbecco’s modified Eagle medium (DMEM), 1 mg/ml glucose; Gibco, Life Technologies, Paisley, UK] was adjusted to reach but not submerge the tissue (~11 ml medium/well). The medium contained penicillin–streptomycin–glutamine (Gibco, 2 vol.%) and non-essential amino acids (MEM; Gibco, 1 vol.%). Furthermore, bromodeoxyuridine (BrdU; 100 µmol/l) and gonadotrophins (Pergonal, 0.75 IE) were added to the medium. The fragments were cultured for 16 h at 35°C under an atmosphere of 5% CO₂.

After culture, the fragments of each individual biopsy were collected into a single tube. A two-step enzymatic digestion using collagenase I, DNase and hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) in DMEM F12 medium (Gibco) was performed to achieve a single-cell suspension. The cells were washed and transferred into Leibovitz L15 medium (Gibco) supplemented with glycerol (1.5 mol/l) and 4% serum of the individual monkey. A comparison of various cryoprotectants revealed no drastic differences in testicular cell survival after a freezing and thawing cycle (Brook et al., 2001). Glycerol was chosen as cryoprotectant in this study due to its low toxicity. Controlled freezing of the cell suspension was achieved using an automated freezing machine and a protocol used routinely for the cryopreservation of spermatozoa. The cell suspension was stored in liquid nitrogen until further use.

Germ cell transfer

The anaesthetized monkey was placed on a table, lying on its back, and ultrasound transmission gel applied to the scrotum. Systematic longitudinal and transversal scans (7.5 MHz sector scanner; Siemens Sonoline Versa Pro, Erlangen, Germany) were used to localize the rete testis due to its higher echogenicity. The injections were performed from the lower testicular pole under continuous sonographic monitoring. The testicular cells were washed and resuspended in a small volume (100–200 µl) of saline immediately before injection. The volume of saline containing the germ cells was the first fluid to be flushed into the right testis. Injection was continued under constant manually controlled pressure, and additional saline was infused until a thickening of the testis similar to the fully active, non-regressed testis was achieved. The left testis received an injection of saline only, using the same procedure. The procedure required 20–30 min for each monkey. All sonographies and injections were performed by the same scientist. The date of germ cell transfer was considered to be day 0 of the experiment.

Dissection of tissue and histological procedures

The testes were dissected and weighed. In order to obtain systematic sampling, the testes were transversely dissected into four slices, with the apex of the testis presenting slice 1. All four slices were dissected crosswise into four pieces of which the epididymis was located between the two apical slices. Tissue fragments from slices 2 and 4 were dissected and transferred into phosphate-buffered saline-buffer for flow-cytometric analysis. The procedure for quantification of germ cell fractions by flow cytometry has been published previously (Weinbauer et al., 1994). Pieces of tissue were immersion-fixed in Bouin’s solution and the fixed tissue was routinely embedded in paraffin and cut at a section thickness of 5 µm for light microscopy. Sections were stained with periodic acid–Schiff’s reagent (PAS) and haematoxylin.

A systematic randomized histological evaluation was performed. In total, 500 seminiferous tubules per testis were scored for the most advanced germ cell type, and 120 tubular cross-sections were analysed across all four regions of each testis. The results were expressed as the relative number of tubules showing Sertoli cell-only (SCO), spermatogonia, spermatocytes, round and elongated spermatids. The relative number of tubules showing Sertoli cell-only (SCO), spermatogonia, spermatocytes, round and elongated spermatids.

Hormone assays

FSH was assayed using a heterologous radioimmunoassay (Foppiani et al., 1999). The sensitivity was 0.156 ng/ml, and the intra- and interassay coefficients of variation (CV) were 6.2 and 7% respectively. Inhibin B was assayed using a two-site enzyme-linked immunosassay (Serotec, Oxford, UK); sensitivity was 7.8 pg/ml and intra- and interassay CV were 3.1 and 13.7% respectively. Testosterone was assayed by an established radioimmunoassay (Khurshid et al., 1991);
were expressed as mean data, the calculation was performed on log-transformed data. Data (Bonferroni). Because of the skewed distribution of the sperm count of variance (ANOVA), followed by multiple comparison test FSH, inhibin B and testosterone was performed by one-way analysis of variance (ANOVA)

Evaluation of data for body weight, testis volume, sperm counts, sensitivity was 0.068 nmol/l, and intra- and interassay CV were 5.8 and 15% respectively.

Statistical analysis
Evaluation of data for body weight, testis volume, sperm counts, FSH, inhibin B and testosterone was performed by one-way analysis of variance (ANOVA), followed by multiple comparison test (Bonferroni). Because of the skewed distribution of the sperm count data, the calculation was performed on log-transformed data. Data were expressed as mean ± SEM. Statistical significance was assumed at P < 0.05.

Results
Clinical observations and body weight
The histological and endocrine changes induced by testicular irradiation have been reported in detail previously (Foppiani et al., 1999). No serious side effects were observed. The body weights decreased after irradiation and did not change during the latter course of the study (data not shown).

Testicular parameters and sperm count
At day 0 of the study, all monkeys received a germ cell suspension through the rete testis, leading to an ~25% increase in the volume of both testes (left testes, before transfer 8.3 ± 1.6 ml; right testes, before transfer 8.6 ± 1.9 ml, after transfer 10.3 ± 1.6 ml; right testes, before transfer 8.6 ± 1.9 ml, after transfer 10.8 ± 2.3 ml) (Figure 1). As the infused volumes of saline differed due to infusion being continued until the increase in testis volume led to hardening of the organ, individual data for the volumes of saline infused into each testis are presented in Table I.

In three monkeys (6084, 6096 and 6090), testis volumes returned to normal or lower values during the 2 weeks following germ cell infusion, while in two monkeys (6105, 6102) the testis volumes increased during the same period (Figure 2). These effects were observed in both testes, independent of the presence of germ cells in the infused fluid.

Irradiation evoked a reduction of testis volume to ~40% of the pretreatment value (Foppiani et al., 1999). Recovery of the testes started from 50 days after germ cell transfer (Figure 1). While the left testes showed no significant regrowth compared with values estimated before germ cell transfer, a consistent significant recovery of testicular volumes was observed in the right testes from week 31 after germ cell transfer. Compared with the lowest value of testicular volumes at week 4 after germ cell transfer, significant regrowth of the right testes was observed from week 24 onwards, whilst significant enlargement of the left testes occurred 7 weeks later, from week 31 onwards.

When individual testis volumes are followed, three of the five monkeys (6105, 6102 and 6090) showed consistently higher right versus left testis volumes from weeks 6–20 after the infusion, while monkey 6084 showed a unilateral enlargement of the right testis from 18 weeks onwards after germ cell transfer. Both testes of monkey 6105 showed a late and slow start of recovery from 150 days onwards. While the recovery of the right testes proceeded, the left testis stopped growing from 180 days onwards, leading to a clear difference in final testis volume (Figure 2). Histological analysis of the spermatogenic activity supported a better recovery of the right testis (Figures 3 and 4a–d). The most striking recovery of all testes, and the most obvious difference between the left and right testis, was observed in monkey 6105 (Figures 2 and 4e,f). While the left testis recovered only marginally, the right testis showed a rapid regrowth from day 150 after transfer, with full recovery to the pre-irradiation volume being achieved 240 days after germ cell transfer. While the restoration of spermatogenesis was complete in 94% of the seminiferous tubules of the right testis, only half this value was recorded in the left testis (Figure 3). In monkey 6090, both testes began to recover slowly and steadily. The analysis of spermatogenic recovery in this monkey showed that in both testes 70% of the seminiferous tubules contained elongated spermatids (Figures 2 and 3). No bilateral difference between the regrowth of testes was observed in monkey 6096 (Figures 2 and 3). A slightly better recovery of the right compared with the left testis was seen in monkey 6084 (Figure 2), but this was no longer evident at the end of the study and therefore not confirmed by histological analysis (Figure 3).

Immunohistochemical detection of BrdU was negative in all testes, although control sections from rats and monkeys that received an injection of BrdU between 2 h and 3 weeks before fixation showed an intense staining of germ cells at various stages of development. Flow cytometric analysis of testicular tissue at the study end confirmed the histological scores. Whilst in monkeys 6096 and 6084, the ratio of elongated spermatids between the right and the left testis was 0.86 and 1.24 respectively, in monkeys 6090, 6102 and 6105 the number
Table I. Testicular volumes of individual monkeys before and after germ cell infusion representing the quantities of saline infused into the testes of each monkey at day 0 of the study

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Left testis volume (ml) Before saline infusion</th>
<th>Volume of saline infused (ml)</th>
<th>Right testis volume (ml) Before saline infusion</th>
<th>Volume of saline + germ cells infused (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6102</td>
<td>3.3</td>
<td>0.2</td>
<td>5.1</td>
<td>0.2</td>
</tr>
<tr>
<td>6096</td>
<td>13.2</td>
<td>1.9</td>
<td>12.5</td>
<td>13.7</td>
</tr>
<tr>
<td>6084</td>
<td>12.5</td>
<td>3.9</td>
<td>11.1</td>
<td>13.9</td>
</tr>
<tr>
<td>6090</td>
<td>5.4</td>
<td>3.4</td>
<td>4.3</td>
<td>8.2</td>
</tr>
<tr>
<td>6105</td>
<td>8.5</td>
<td>1.5</td>
<td>8.4</td>
<td>10.5</td>
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</table>

Measurement of testis volume was performed ultrasonographically immediately before and after the infusion procedure. Ultrasonographic measurements corresponded well with estimates of testis volume by calliper measurements and with records of the volume of saline dispensed from the injection syringe during infusion.

Figure 2. Individual changes in right and left testis volumes following germ cell transfer. Each curve represents a single testis. The right testes received an infusion of saline plus germ cells, and the left testes an infusion of saline only at time point 0 of the study. The first two data points of each graph located on the y-axis presents the pre-irradiation testis volume. The second pair of data points show testis volume before germ cell infusion. The first data point of the curve is the testis volume determined immediately after infusion.

of elongated spermatids was increased in the right testis compared with the left (ratios 1.55, 1.58 and 2.73 respectively).

Although recovery was variable between animals, histological analysis revealed an all- or-nothing pattern of the restoration with respect to individual seminiferous tubules

Figure 3. Histological analysis of the extent of spermatogenic recovery in irradiated saline-injected control testes (left) versus irradiated and germ cell-infused testes (right). Each value (mean ± SEM) represents the relative number (%) of seminiferous tubules containing the indicated cell type as the most advanced stage of spermatogenesis in 500 seminiferous tubules scored across four different regions of each testis (125 scores/region). The SEM (dark area of column) represents intra-testicular variation calculated from the differences between the four analysed regions. SCO = Sertoli cell-only tubules; SPG = spermatogonia; SPCT = spermatocytes; RD SPTD = round spermatids; EL SPTD = elongated spermatids.

(Figure 3). Analysis of the most advanced germ cell type in the seminiferous tubules showed that almost 90% of the seminiferous tubules belonged to the category SCO, or had
Germ cell transplantation in monkeys

Figure 4. Representative micrographs showing the testicular histology of monkey 6105 (a–d) and 6102 (e,f) at the end of the study. Low- (a,b) and high-power (c–f) magnification of the left (a,c,e) and right (b,d,f) testes reveal the bilateral differences as well as the all-or-nothing pattern of spermatogenic recovery. The right testes received an infusion of saline plus germ cells, and the left testes an infusion of saline only at time point 0 of the study. While the left testes display many seminiferous tubules without germ cells (Sertoli cell-only) and few tubules containing elongating spermatids, the right testes show full recovery up to elongating spermatids in most seminiferous tubules. Very few tubules are observed showing a partial recovery up to spermatocytes or round spermatids. Periodic acid–Schiff and haematoxylin staining; scale bars = 100 µm.

been restored to the level of elongating spermatids. Only about 10% of the seminiferous tubules showed recovery to the level of spermatogonia, spermatocytes or round spermatids.

Except for the last few time points when the ejaculates of two animals contained less than 10⁶ spermatozoa per ml, no recovery of sperm counts was observed during the observation period (Figure 5).

Endocrine parameters

Here, only additional data on the recovery of endocrine parameters that were not included among previously published data on irradiation-induced early endocrine changes (Foppiani et al., 1999) are reported (Figure 6). At the time of germ cell transfer, inhibin B concentrations reached the lowest values, but during the first weeks after germ cell transfer these increased slightly and plateaued for about 100 days to begin a slow, but permanent, recovery at >150 days after transfer. The recovery of inhibin concentrations reached significance compared with the time of germ cell transfer from week 25.
after germ cell transfer. Inhibin concentrations were no longer significantly different from pre-irradiation values from week 30 onwards. Significantly higher concentrations of FSH followed an almost inverse relationship to those of inhibin B, with highest concentrations occurring from days 0 to 100 and a decrease up to day 150, ultimately with return almost to pretreatment values. Testosterone concentrations were more variable, returning to pretreatment values by 150 days after germ cell transfer.

**Discussion**

In the present study, an attempt was made to mimic the damage of spermatogenesis occurring in oncological patients as a result of testicular X-irradiation, using a total dose of 2 Gy. This dose was sufficiently high to induce complete azoospermia for more than 200 days in all five monkeys. Although the X-ray treatment induced temporary azoospermia (similar to observations in oncological patients), this pretreatment did not block spontaneous regrowth of the testes and recovery of spermatogenesis. Assuming that the infusion process of saline itself did not have an effect, the regrowth and re-induction of spermatogenesis of the left testis serves as a measure for the degree of spontaneous recovery which was individually variable and extended from focal restoration of qualitative spermatogenesis (monkey 6105) up to a 75% restoration of testis volume and spermatogenesis (monkey 6084). The occurrence of spontaneous recovery made it difficult to define a beneficial effect of unilateral germ cell infusion, since an autologous transfer of germ cells was performed and no genetic marker allowed the germ cells restored from transplanted spermatogonia to be disentangled from those undergoing spontaneous recovery. The restoration of spermatogenesis by use of genetic markers has been demonstrated in mice, and provided assistance in developing the basic tools for germ cell transplantation (Brinster and Avarbock, 1994). However, such an approach cannot be used for a preclinical application of germ cell transplantation as transgenic primate models are not yet available for this type of study. Repetition of a previous successful approach to label transplanted cells by incorporating BrdU (Schlatt et al., 1999) was not feasible, indicating that this thymidine analogue has disappeared from the germ line stem cells with time. The most likely reason is that numerous mitotic divisions of the transplanted cells diluted the incorporated BrdU to an undetectable level. In view of these problems, the only valid parameter for any beneficial effect of germ cell transplantation in this study was the determination of testis growth, and histological analysis of spermatogenic recovery after termination of the study.

Four monkeys out of five responded with a better regrowth of the right compared with the left testis. Although the extent of regrowth as well as the timing differed among individuals, a significantly better and earlier regrowth of the right testis was observed. The initial changes of testis volume after infusion were variable. Three monkeys showed a rapid reduction in testis size after the infusion, while the testes of the other two monkeys showed an initial volume increase. The significance of this observation is unknown, but it might reflect the success of the infusion procedure, the degree of damage to the rete testis, or the amount of infused germ cells in one of the testes. Since these changes always occurred bilaterally, they are most likely induced by humoral stimuli, and this finding is supported by the initial increase in inhibin B concentration and the corresponding fall in FSH. It is known from mouse studies that during the first few weeks after germ cell transfer the transplanted germ cells attach to the seminiferous epithelium (Parreira et al., 1998). The testis weight increase seen at 1–2 days after infusion was unrelated to the volume of the injected fluid, but was provoked by an increase in the volume of the tubular lumen. It was suggested that the presence of injected germ cells increased secretion by the Sertoli cells (Parreira et al., 1999). The temporary increase in testis volume observed in two monkeys might reflect a response of the Sertoli cells to the infusion of germ cells. Interestingly, while the testis volumes returned to pre-infusion values after 4 weeks, inhibin B concentrations remained slightly elevated after the infusion of germ cells, indicating a response of Sertoli cells to the presence of transplanted spermatogonia. The short-term initial increase in testicular volume might indicate that, identically to the mouse, most transplanted germ cells disappear from the testis during the first few weeks after germ cell transfer. The fact that the recovery follows an all-or-nothing pattern indicates...
the importance of stem cells in this process. Indeed, if stem cells are present it is most likely that full spermatogenesis is achieved with time.

In three of the five monkeys, at exactly 6 weeks after germ cell transfer, a consistent increase of the right testis size was observed which remained constant over the next 14 weeks. This unilateral growth is most likely dependent on the presence of transplanted germ cells, since the intra-animal variability of testis size determination was extremely low, and two of these three monkeys responded with earlier and better recovery at later stages. It is known from studies in mice that it takes at least 1 month for repopulation of spermatogonia before the first differentiating germ cells enter meiosis (Parreira et al., 1998, 1999). Since little is known about testicular stem cells in monkeys, it might be speculated that the long period of slightly increased right testis size represents the period of spermatogonial expansion. No hormonal changes were observed during this period, indicating no change in the feedback mechanism, and further stimulation of Sertoli cells. Since spermatogonia present only a minor proportion of germ cells in the fully active testis, it is expected that the beneficial effect will become most obvious after the reappearance of meiotic and post-meiotic germ cells. The clearly better unilateral recovery of the right testis in three monkeys which occurred later than 150 days after germ cell transfer supports this hypothesis.

The data obtained in the present study do not show unequivocally a beneficial effect of germ cell transplantation on the recovery of spermatogenesis. The successful recovery of the infused right testis in monkeys 6105 and 6102 might also be due to a delayed recovery of the contralateral testis for reasons other than the depletion of germ cells. Although this is an unlikely event, it would be unwise to present too optimistic an approach of the study outcome. However, depending on the interpretation of the results, an effect of the treatment can be neither convincingly demonstrated, nor disproved. The complexity of such a study renders its results difficult to interpret, as not only the transfer procedure but also the experimental manipulations (including exposure to irradiation, organ culture, preparation of cell suspensions and the cryopreservation procedure) present sources of possible variation and individual differences. Although the results are marginally inconclusive, they do provide valuable hints for the further development of the technique as they suggest that further investigation into the development of efficient and safe strategies is required before germ cell transplantation can be applied clinically.

The approach used to cryopreserve the germ cell suspension for the period of extracorporeal storage appeared to be successful. It has been shown previously in mice that reconstitution of spermatogenesis by germ cell transplantation is possible using a cryopreserved germ cell preparation (Avarbock et al., 1996). A comparison of various cryoprotectant agents for the preservation of testicular cells showed that several of the commonly used agents are similarly effective in achieving good survival of germ cells after a freezing and thawing cycle (Brook et al., 2001).

As only a small tissue sample can be retrieved from oncological patients, germ cell transplantation might only become an attractive treatment option when the isolation and culture of spermatogonia allows the selection and expansion of the stem cell population. Although it has been shown that spermatogonia can be maintained in culture for several months prior to transfer (Nagano et al., 1998), no in-vitro expansion of spermatogonial stem cell populations prior to the transfer had been achieved. We and others have developed new strategies for the isolation of spermatogonia (Shinohara et al., 1999; von Schönfeldt et al., 1999). However, the absence of good markers for male germ line stem cells prevents the application of these techniques for the sorting of spermatogonia before germ cell transfer. A major concern is the safety of the procedure, as the testis is a likely organ for settlement of metastases. A study in rats has shown that testicular infusion of only a few malignant cells induces transmission of the disease (Jahnhuinen et al., 2001), and hence additional strategies combined to the germ cell transplantation procedure must ensure that the risk for transfer of malignant cells back to the testis of a patient is minimal. In addition to practical implications regarding safety and efficiency of the procedure, it appears noteworthy to consider ethical issues for further development of the technique. Ethical guidelines for the various steps of the procedures—especially with regard to the invasive collection of germ line stem cells and the culture and storage of the germ line cells—need to be designed and agreed upon.

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