Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination

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BACKGROUND: Fertility protection is an urgent clinical problem for prepubertal male oncology patients who undergo either chemotherapy or radiotherapy. As these patients do not have mature sperm to be frozen, there is as yet no effective method to preserve their fertility. METHODS AND RESULTS: Single pieces of immature mouse (1.5×1.5×1.5 mm) or rabbit (2.0×2.0×3.0 mm) testis were cryopreserved, thawed and transplanted into mouse testes. Histological techniques were used to determine the presence of spermatogenesis, which was restored in both mouse and rabbit testicular pieces, and led to the production of mature sperm after both cryopreservation and syngeneic or xenogeneic transplantation into mouse testes. Using sperm developed in the frozen–thawed transplants, mouse offspring were born after in-vitro microinsemination. Furthermore, rabbit offspring were obtained using rabbit sperm that developed in fresh transplants in a xenogeneic surrogate mouse. CONCLUSIONS: This approach of ‘testicular tissue banking’ is a promising technique for the preservation of fertility in prepubertal male oncology patients. Xenogeneic transplantation into immunodeficient mice may provide a system for studying spermatogenic failure in infertile men.

Keywords: cancer/infertility/in-vitro microinsemination/testis/transplantation

Introduction

Recent advances in chemotherapy and radiotherapy have significantly improved remission and complete recovery rates in cancer patients. However, because germ cells are highly susceptible to cytotoxic treatments, iatrogenic loss of fertility has emerged as a major side effect of successful treatment (Aslam et al., 2000). In males, sperm freezing is an established method to preserve germine cells (Fossa et al., 1989; Royère et al., 1996; Lass et al., 2001), and is performed routinely in clinics for those patients who wish to preserve their fertility before undergoing treatment for malignancy (Lass et al., 2001). Despite the small amount and poor quality of semen specimens from oncology patients (Fossa et al., 1989; Hovatta, 2001), pregnancies have been reported using frozen–thawed sperm (Royère et al., 1996; Lass et al., 2001). Unfortunately, the technique cannot be applied to prepubertal patients, who do not have sperm, although their testes contain gonocytes or immature spermatogonia. Approximately 1 in 650 children develop malignancies during childhood and it is estimated that, by 2010, one in 250 young adults (aged 20–29 years) will be long-term survivors of childhood cancer (Aslam et al., 2000). Thus, the preservation of male germline cells in prepubertal boys is an urgent clinical problem (Aslam et al., 2000; Hovatta, 2001).

In females, transplantation of frozen–thawed ovarian pieces has been successful in both laboratory and domestic animals. Live births or pregnancies have been reported after orthotopic transplantation of frozen–thawed ovarian tissues or whole ovary in the mouse (Parrot, 1960), rat (Wang et al., 2002) and sheep (Gosden et al., 1994). Based on the success in animal experiments, the first ovarian transplantation trial was recently initiated in humans, and the results demonstrated the promise of possibly ‘frozen banking’ of an ovarian tissue piece for female cancer patients (Oktay, 2001; Radford et al., 2001).

Given the successful outcome of tissue cryopreservation in females, a valuable strategy to preserve male fertility would be to develop spermatogenesis from immature spermatogonia in pieces of frozen–thawed testicle. Early attempts to freeze testicular pieces met with limited success (Nugent et al., 1997);
Figure 1. Transplantation of a frozen–thawed immature mouse testicular piece. (a) Microscopic appearance of the testis of a donor Green mouse. Note the absence of differentiated germ cells and the presence of immature spermatogonia (arrows). (b) Development of the frozen–thawed donor testis after transplantation into a busulfan-treated testis. Left: a donor testicular piece immediately after freeze–thawing; middle: a donor testicular piece 2 months after transplantation; right: a B6 recipient testis that contains a donor testicular piece, 2 months after transplantation. Note the significant growth of the transplant. (c, d) The histological appearance of spermatogenesis in the frozen–thawed testicular piece. Note the normal organization of spermatogenesis and the production of sperm (arrows, d). (e) Pups born after microinsemination with sperm that developed in the testicular piece. The presence of the donor cell haplotype was confirmed by the green fluorescence seen under GFP excitation light (f). Haematoxylin and eosin staining. Scale bars: (a) 50 µm; (b) 2 mm; (c) 200 µm; (d) 25 µm.

however, a few germ cells that survived in immature, frozen–thawed grafts matured in one study (Deanesly, 1954), albeit without a donor graft marker. In the present study, an investigation was made as to whether spermatogenesis could be restored in immature testicular pieces in syngeneic and xeno-
genic recipients after cryopreservation. If it were possible to develop sperm from a frozen–thawed biopsy specimen after autotransplantation, these could be used to fertilize oocytes when the patient reached adulthood. Spermatogenesis in xeno-
genic surrogates might also provide a useful system to check for contaminating cancer cells or to study spermatogenic failure in infertile animals and men.

Materials and methods

Donor animals
Donor transgenic mice, C57BL/6 Tg14(act-EGFP)OsbY01 (designated Green mice) (Okabe et al., 1997), were originally provided by Dr M. Okabe (Osaka University), and maintained in the present authors’ animal facility. Donor rabbits (Dutch belted) were purchased from Kitayama Labes (Nagano, Japan).

Freezing and thawing of testicular pieces
The testes were dissected into small pieces measuring ~1.5×1.5×1.5 mm (mouse) or 2×2×0.4–4.0 mm (rabbit) and placed into Dulbecco’s modified medium supplemented with 10% fetal calf serum (DMEM/FCS). Two pieces were transferred into a cryogenic vial (Sumitomo Bakelite, Tokyo, Japan) containing 500 µl of cryopreservation solution containing dimethyl sulphoxide and FCS (Cellbanker; DIA-IATRON, Tokyo, Japan) and held at 4°C for 10 min. Using a programmable freezer (ET-1N, Fujihira, Tokyo, Japan), the vials were cooled at a rate of 2°C/min to −7°C, and then held for 10 min to allow ice crystal formation. After cooling at 0.3°C/min to −30°C, the vials were transferred into liquid nitrogen.

For transplantation, the vials were incubated at 37°C, transferred to 2 ml of DMEM/FCS, and washed in three changes of medium before transplantation.

Transplantation of donor testicular pieces
In the first experiments, C57BL/6 (B6) mice (Japan SLC, Shizuoka, Japan) were used as syngeneic recipients. To avoid immunological rejection, ICR nude mice (Charles River, Yokohama, Japan) were used in the second experiments. At 6 weeks of age, recipient mice received a single injection of busulfan (44 mg/kg, i.p.) to destroy
Cryopreservation of immature testis

Figure 2. Transplantation of a frozen–thawed immature rabbit testicular piece. (a) Microscopic appearance of the testis of a 4-week-old donor rabbit. Note the absence of differentiated germ cells and the presence of immature spermatogonia (arrows). (b) Development of the frozen–thawed donor testicular piece after transplantation into busulfan-treated nude mouse testis. Left: a donor testicular piece immediately after freeze–thawing; middle: a donor testicular piece 2 months after transplantation; right: an ICR nude mouse recipient testis that contains a donor testicular piece, 2 months after transplantation. Note the growth of the transplant and vascularization (arrow). (c) Histological appearance of the recipient testis. Rabbit testicular pieces (arrows) transplanted in a recipient mouse testis. Endogenous mouse spermatogenesis was observed in some tubules (arrowhead). (d, e) Histological appearance of spermatogenesis in the frozen–thawed testicular piece. Note the normal organization of spermatogenesis and the production of sperm (arrows, e). (f) A pup born after microinsemination with sperm that developed in the fresh rabbit testicular piece (arrow). The black coat indicates the donor origin (Dutch belted). Haematoxylin and eosin staining. Scale bars: (a) 50 µm; (b) 2 mm; (c) 0.5 mm; (d) 200 µm; (e) 25 µm.

endogenous spermatogenesis (Bucci and Meistrich, 1987) and thus mimic the damage to spermatogenesis that occurs in oncology patients. The mean (± SEM) recovery of endogenous spermatogenesis was 23.7 ± 6.7% (n = 5) and 6.1 ± 2.2% (n = 8) respectively for B6 and ICR nude mice. As such treatment is toxic to the haematopoietic system of nude mice, these animals received bone marrow transplantation from untreated mice at 5–7 days after busulfan treatment. The mice were used as recipients at least 4 weeks after the busulfan treatment.

For transplantation, the testis was exteriorized through a midline abdominal incision after anaesthetizing the recipient mouse. Using fine forceps, a small cut was made in the tunica albuginea, and a single graft inserted 2–3 mm into the testicular parenchyma.

Analysis of recipient testes

The donor testis piece was identified by expression of the enhanced green fluorescent protein (EGFP) gene under a stereomicroscope equipped with an ultra-violet light (Nikon) (first experiment) or by the pale white colour and distinct morphology of the rabbit seminiferous tubules (second experiment). Four histological sections were taken at 12-µm intervals from each testis piece and stained with haematoxylin and eosin. Each slide was viewed at ×400 magnification to determine the level of spermatogenesis. The number of tubule cross-sections with or without spermatogenesis (defined as the presence of multiple layers of germ cells in the seminiferous tubule) was recorded for one section for each sample. Statistical analysis was performed using Student’s t-test.

Microinsemination

Microinsemination was performed using ICSI (Kimura and Yanagimachi, 1995) into C57BL/6×DBA/2 F1 (B6D2F1) (first experiment) or Japanese white oocytes (second experiment) collected from superovulated females. In the first experiment, the donor testis cell suspension was refrozen before microinsemination, as previously described (Ogura et al., 1996b). In the second experiment, fresh rabbit sperm were used.

Results

Transplantation of frozen–thawed mouse testicular pieces into syngeneic recipient testes

To examine whether spermatogenesis occurred in frozen–thawed pieces of immature testes, donor testes were collected
from 5- to 7-day-old Green mice, and frozen in liquid nitrogen. As the mice express the EGFP gene ubiquitously, including in the testis, this allows specific identification of a donor testis piece after transplantation into a recipient testis. The seminiferous tubules of the donor testis contained only immature spermatogonia and lacked differentiating germ cells (Figure 1a). The pieces were frozen for 10–35 days, thawed, and then transplanted beneath the tunica albuginea of a busulfan-treated B6 recipient testis. Four experiments were performed, and 23 testis pieces were transplanted in total.

At 2 months after transplantation, the mean weight of the recipient testis (58.1 ± 8.2 mg) was increased 2.2-fold compared with untransplanted control testis (26.8 ± 0.8 mg, P = 0.03). Growth of the donor testicular piece was evident after transplantation (Figure 1b). Next, the level of spermatogenesis was evaluated in histological sections. All 23 testicular pieces showed complete spermatogenesis after transplantation (Table I). The structure and pattern of spermatogenesis appeared morphologically normal (Figure 1c and d). On average, spermatogenesis occurred in 54% of the donor seminiferous tubules, and 80 of 100 (80%) seminiferous tubules contained mature sperm in the most successful case. These results indicate that the donor testicular pieces had survived the freeze–thaw procedure and differentiated to produce sperm after transplantation into chemically castrated recipient testes.

To test whether offspring could be produced from the sperm generated in the frozen–thawed testis piece, in-vitro microinsemination—a technique commonly used to produce offspring from infertile animals and humans—was used (Palermo et al., 1992; Kimura and Yanagimachi, 1995; Ogura et al., 1996a). Five testis pieces were collected from prepubertal pups, frozen for 10 days, and transplanted into busulfan-treated B6 testes. At 2 months after transplantation, live spermatogenic cells were recovered by repeatedly pipetting the GFP-positive testicular piece. After refreezing for 3 days, mature sperm from the frozen testicular piece were injected into B6D2F1 oocytes.

Of the 248 embryos thus constructed, 230 (93%) progressed to the 4-cell stage by 48 h in culture. After embryo transfer to pseudopregnant ICR females, 123 embryos (53%) implanted in the uterus, and the twelve recipients gave birth to a total of 62 pups (25%) (Figure 1e). Donor origin was confirmed by fluorescence under ultra violet light (Figure 1f).

**Table I. Spermatogenesis in fresh and frozen–thawed testicular pieces after transplantation**

<table>
<thead>
<tr>
<th>Donor animal</th>
<th>Type of transplant</th>
<th>No. of testes transplanted</th>
<th>Testis weight (mg)</th>
<th>No. (%) testes with spermatogenesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tubules (%) with spermatogenesis&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Frozen</td>
<td>23</td>
<td>58.1 ± 8.2</td>
<td>23 (100)</td>
<td>54.3 ± 5.4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Fresh</td>
<td>27</td>
<td>42.4 ± 3.3</td>
<td>20 (74)</td>
<td>32.2 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Frozen</td>
<td>20</td>
<td>39.0 ± 5.1</td>
<td>18 (90)</td>
<td>24.0 ± 5.0</td>
</tr>
</tbody>
</table>

Combined results from four separate experiments for each type of transplant. Values are mean ± SEM. The recipients were analysed at 2–3 months after donor testis transplantation.

<sup>a</sup>Seminiferous tubule cross-sections with multiple layers of germ cells were considered to be positive for spermatogenesis.

<sup>b</sup>Percentage of the tubule cross-sections in the recipient testis with spermatogenesis.

**Transplantation of fresh and frozen–thawed rabbit testicular pieces to xenogeneic nude mouse recipient testes**

In the second set of experiments, the feasibility of generating rabbit spermatogenesis in fresh or frozen–thawed testicular pieces transplanted into xenogeneic mouse recipients was examined. As puberty occurs at 3–4 months of age in rabbits (Hafez, 1993a), donor testes were collected from 1- to 6-week-old prepubertal rabbits. The testis at this stage contains prespermatogonia or immature spermatogonia in the seminiferous tubules (Figure 2a). Some testicular pieces were frozen for 4 to 53 days in liquid nitrogen, and either fresh or frozen–thawed pieces were transplanted into busulfan-treated nude mouse recipients.

Four experiments using 27 fresh and 20 frozen–thawed testicular pieces were performed, and the recipient testes were analysed at 2–3 months after transplantation. Although the average weight of both types of recipient testes (fresh, 42.4 ± 3.3 mg; frozen, 39.0 ± 5.1 mg) increased slightly compared with the untransplanted controls (30.3 ± 1.5 mg), the difference was not significant (Table I). However, some growth of the transplants was apparent when the donor testis piece was dissected out (Figure 2b). The grafts appeared healthy and were vascularized (Figure 2b). Histological analysis of the donor testes revealed the presence of spermatogenesis in the transplants (Figure 2c–e; Table I). In total, spermatogenesis occurred in 20 of 27 (74%) fresh and 18 of 20 (90%) frozen transplants, and mature rabbit sperm were found in both types of transplants. These results demonstrated that both fresh and frozen–thawed rabbit testicular pieces can restore spermatogenesis after transplantation into immunodeficient mouse recipients.

In order to examine the developmental potential of rabbit sperm generated in mice, a microinsemination experiment was performed using sperm that developed in fresh rabbit transplants. At 3 months after transplantation, the testes were mechanically dissociated, and rabbit (Dutch belted) sperm (identified by their unique sperm head shape; Yanagimachi, 1994) were injected into rabbit (Japanese white) oocytes. Of the 49 embryos thus constructed, 29 (59%) progressed to the 4-cell stage by 24 h in culture. After embryo transfer to two pseudopregnant Japanese white females, one of the recipients became pregnant and gave birth to one male offspring (Figure 2f). The donor origin (Dutch belted) was identified by...
the coat colour, demonstrating that the offspring was derived from donor rabbit sperm transplanted into nude mice. The offspring was proved to be fertile.

Discussion
This study extended previous observations and demonstrated that: (i) spermatogenesis deriving from spermatogonia/gonocytes can occur in the developing tissues after cryopreservation and transplantation into syngeneic or xenogeneic hosts; and (ii) offspring can be derived from these sperm after microinsemination. In contrast to the difficulties associated with freezing sperm in many species (Glenister and Thornton, 2000), this technique is remarkably simple, and may provide a new approach to protecting fertility in prepubertal male patients who do not have the option of freezing sperm.

Microinsemination has revolutionized conventional assisted reproduction techniques and is now a widely applied means for overcoming male infertility in animals and humans (Palermo et al., 1992; Kimura and Yanagimachi, 1995; Silber, 1995; Ogura et al., 1996a). Although it is possible to perform microinsemination using sperm from frozen–thawed biopsy tissue from adult patients (Hovatta et al., 1996; Salzbrunn et al., 1996), the technique cannot be applied to prepubertal patients who have only gonocytes or immature spermatogonia in their testes. The only possible method to obtain offspring from such tissue is to induce the primitive cells to differentiate into mature sperm, which can then be used for fertilization either in vivo or in vitro.

At present, two potentially useful approaches exist to protect fertility in young oncology patients. The first method is spermatogonial transplantation, in which stem cell spermatogonia are transplanted into the seminiferous tubules of sterile recipients (Brinster and Zimmerman, 1994). Transplantation of fresh stem cells was shown to restore fertility in mice (Ogawa et al., 2000), and the cell transfer technique is now being established in several other species (Schlatt et al., 1999, 2002), including humans (Schlatt et al., 1999; Brook et al., 2001). Using stem cells recovered from the testes of immature patients, autologous transplantation of cryopreserved stem cells would restore fertility, in a manner analogous to bone marrow transplantation. Although the restoration of spermatogenesis has been demonstrated in both rodents (Avarbock et al., 1996) and monkeys (Schlatt et al., 2002), no offspring have yet been obtained from frozen stem cells in any animal species. One reason for this lack of success in some species might be that the cytotoxic treatment of the recipients disturbs the balance between germ cells and an endocrine compartment essential for successful spermatogenesis (Meistrich, 1998; Ogawa et al., 1999). The second method is to administer a GnRH analogue to prevent endogenous germ cell damage, which suppresses intratesticular testosterone levels, and relieves the block of stem spermatogonia (Meistrich, 1998). However, the clinical benefit of this approach has not been demonstrated (Howell and Shalet, 2001), and it is clearly safer to remove the germ cells from the zone of genetic harm.

The results of the present study indicate that cryopreservation and transplantation of prepubertal testicular pieces might be a useful approach for protecting fertility. As shown in the first experiments, both immature spermatogonia and Sertoli cells survived in the testicular piece and matured after cryopreservation. As the cryopreservation of tissues generally requires greater permeation of the cryoprotectant compared with a cell suspension, and different cell types have different optima (Nugent et al., 1997), it is surprising that both cell types survived the simple cryopreservation procedure. In ovarian cryopreservation, between 10 and 80% of follicles are damaged during the preservation (Nugent et al., 1997), and the number of oocytes does not recover because they are not self-renewing. Whereas in males, continuous division of spermatogonial stem cells provides an unlimited supply of sperm at any desired time for fertilization (Russell et al., 1990), a single rat spermatogonial stem cell is theoretically capable of producing 4096 sperm (Russell et al., 1990). Hence, compared with the female the male germline has a strong advantage in cryopreservation.

The most striking result from the present study was the generation of xenogeneic offspring using rabbit sperm developed in mouse recipients. Spermatogenesis is governed by complex regulatory signals involving several organs (Orth, 1993), including the pituitary, thyroid (Palermo et al., 1989) and salivary glands (Tutsumi et al., 1986). The fact that the rabbit and mouse are believed to have diverged evolutionarily over 60 million years ago (Dobrinski et al., 1999), indicates a striking conservation of a complex process. Since rabbit spermatogonia can only proliferate, but not differentiate, in the mouse seminiferous tubule after spermatogonial transplantation (Dobrinski et al., 1999), the difference between the two studies clearly shows that the germ cell–Sertoli cell interaction in the intratubular environment requires more species-specific factors than the extratubular interactions. In this context, the present approach is advantageous to spermatogonial transplantation, because somatic cells in the donor piece can supply essential nutrients to induce the differentiation of spermatogonia cells. Therefore, it may be possible to obtain sperm from animals separated by wider phylogenetic distances, which is not possible with spermatogonial transplantation (Dobrinski et al., 1999; Nagano et al., 2001).

On the basis of the present results, it might be anticipated that a frozen–thawed testicular biopsy of a human oncological patient could undergo spermatogenesis and produce sperm, which can then be used for microinsemination when the patient reaches adulthood and plans to have a baby. The freezing of testicular pieces has several advantages over spermatogonial transplantation or GnRH analogue administration. The first advantage is its simplicity; unlike spermatogonial transplantation, the freezing technique does not require either extreme enzymatic digestion (Brook et al., 2001) to collect spermatogonia, or ultrasound guidance of the microinjection (Schlatt et al., 1999, 2002; Brook et al., 2001). The second advantage is that the co-transplantation of somatic cells may alleviate the hormonal imbalance caused by cytotoxic therapy (Meistrich, 1998; Howell et al., 2001). Third, the method is effective with the small number of stem cells that are present in a small piece of biopsy specimen. As only 5–10% of stem cells can colonize the seminiferous tubule after spermatogonial
transplantation (Ogawa et al., 2000; Nagano et al., 2001), increasing the number of stem cells in a small biopsy would be a prerequisite for its clinical application. Therefore, the successful production of offspring in this study has important implications for human clinics, and testicular piece freezing or ‘testicular tissue banking’ represents a promising approach to protect fertility.

An additional benefit is that xenogeneic transplantation may also provide a system to check the risk of retransplanting contaminating cancer cells into a cured patient. The re-seeding of cancer cells is a serious concern for therapeutic transplantation, and a previous study has shown that as few as 20 leukaemic cells could cause a cancer relapse when injected into the testis (Jahnukainen et al., 2001). In view of such an apparent risk, it would be useful to use xenogeneic immunodeficient host mice to circumvent the possibility before attempting autologous transplantation, as recently applied in human female patients (Kim et al., 2001). If mature human sperm can develop in mice, it would provide an assay to examine the developmental potential of frozen–thawed tissue before autologous transplantation, or to examine the effect of various treatments for studying the mechanism of spermatogenic failures in infertile men. Although fertilization using human sperm generated in mice would raise ethical concerns, and also involve the biological risk of infection due to endogenous retroviruses (Patience et al., 1997), the use of xenogeneic surrogates would be clinically beneficial.

Several potential problems need to be examined before the technique can be applied clinically. The human testis is more fibrous and has a different architecture and hormone requirements; therefore, it may not be possible simply to extrapolate results obtained in small animals to humans, as has been encountered in ovarian transplantation (Oktay, 2001). Indeed, the lower success rate in xenogeneic transplants suggests that a xenogeneic transplantation of human tissues may not be successful using the same procedure. Furthermore, it is important to establish ethical guidelines for developing the procedure, because children cannot always provide their informed consent and an intrusive testicular biopsy may cause future side effects in the developing testis (Bahadur et al., 2001). Nonetheless, these results demonstrate a promising way to overcome this serious clinical problem. Future studies using a wider range of animals are required to reveal the limitations and potential benefits of the current approach in clinical medicine.

Acknowledgements

The authors thank Ms Y. Doi for her technical assistance, and Mr S. Takei and Ms Y. Yamamoto for animal care. Financial support was provided by the Kanae Foundation for Life & Socio-Medical Science and the Ministry of Education, Science, Sports, and Culture of Japan. M.K.-S. was supported by the Japan Society for Promotion of Science.

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Submitted on May 24, 2002; accepted on July 20, 2002