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Reproductive capacity of sperm obtained after germ cell transplantation in a mouse model

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BACKGROUND: The testicular stem cell transplantation technique has become an established research model in the mouse. This technique may also become useful for clinical applications. Therefore, it is necessary to investigate whether sperm obtained after testicular stem cell transplantation retain their full functional capacity and whether they are able to produce normally-developing embryos. This study aimed at evaluating the fertilizing and developmental abilities of sperm obtained after stem cell transplantation. METHODS: First, transplanted male mice were mated with females in order to evaluate in-vivo conception. Subsequently, functionality of sperm obtained after testicular germ cell transplantation was investigated by performing both IVF and ICSI. RESULTS: After in-vivo conception we found that in the control group 90% of the mice with a copulating plug became pregnant. In the experimental group only 35% of the mice with a copulating plug became pregnant ($P = 0.006$). After IVF, fertilization and blastocyst developmental rates were significantly lower in the transplanted group ($P < 0.0001$). Fertilization and blastocyst developmental rates after ICSI were comparable with control sperm. CONCLUSIONS: Our study showed that in the mouse, sperm obtained after stem cell transplantation are able to fertilize oocytes on the basis of assisted reproduction. It is recommended to further investigate this method in the human, as well as to investigate the post-implantation development of the embryo.

Key words: early development/fertilization/spermatogenesis/testis/transplantation

Introduction

Nowadays, many cancers can be cured thanks to advances in chemo- and radiotherapy. However, loss of rapidly dividing testicular spermatogonial stem cells is one of the important side-effects of these treatments. As a result, spermatogenic failure and infertility may occur (Meistrich, 1993). For an adult, sperm banking before treatment may circumvent this adverse effect. However, pre-pubertal patients cannot benefit from this option because of the lack of complete spermatogenesis in their testes. In female patients, preventive cryobanking of gonadal tissue has been introduced recently (Gosden *et al*., 1994). In analogy to this strategy, male germ cells could be cryobanked and reintroduced into the adult testis of a cured cancer patient (Aslam *et al*., 2000; Schlatt *et al*., 2000; Brook *et al*., 2001).

In the mouse, it has been shown that injection of germ cell suspensions from a fertile donor mouse into the seminiferous tubules of an infertile recipient mouse can restore spermatogenesis from donor spermatogonial stem cells (Brinster and Avarbock, 1994; Brinster and Zimmerman, 1994). The first meiotic germ cells appear after 1 month and their number gradually increases thereafter (Parreira *et al*., 1998). Successful spermatogonial transplantation has also been described in rats (Ogawa *et al*., 1999a), in the cynomolgus monkey (Schlatt *et al*., 1999), in pigs (Honaramooz *et al*., 2002) and in goats (Honaramooz *et al*., 2003). Recolonization of mouse testes was also observed with stem cells obtained from rabbits, dogs, pigs, bulls, stallions and baboons, but without further differentiation taking place (Ogawa *et al*., 1999b; Dobrinski *et al*., 1999a; 2000; Nagano *et al*., 2001). Recently, Nagano *et al*. (2002) even reported survival and proliferation of human stem cells in the mouse testes until 6 months after transplantation.

Before considering stem cell transplantation for preserving the fertility of pre-pubertal boys, there are concerns that need careful evaluation (Aslam *et al*., 2000). The testis biopsy taken from the cancer patient may contain malignant cells. These cells should be removed from the cell suspension since it has been shown in rats that one single malignant cell may reintroduce the disease (Jahnukainen *et al*., 2001). Furthermore, the cell suspension consists of all testicular cells. The proportion of spermatogonial stem cells is very low (estimated at 1/5000) (Meistrich and van Beek, 1993). Use of

an in-vitro culture system to enrich the stem cell population may provide a solution to this problem. Purification of stem cells may also increase the efficiency of transplantation. Shinohara *et al*. (1999) used β1- and α6-integrin as markers for selecting mouse spermatogonial stem cells, but unfortunately these markers were also present in other progenitor cells. Furthermore, there is the need to develop a safe and efficient cryopreservation protocol. Avarbock *et al*. (1996) has cryopreserved murine spermatogonial stem cells with dimethylsulphoxide (DMSO) as a cryoprotectant. This cryoprotectant may not be suitable for human use, because of its carcinogenic properties. On the other hand, DMSO does not differ from other cryoprotectants with respect to survival rates after freezing and thawing (Brook *et al*., 2001).

Finally, it remains unknown whether meiosis occurs normally after transplantation and whether the fertilizing and developmental potential of sperm obtained after transplantation is normally preserved. Only a few live offspring have been reported after testicular germ cell transplantation since the first report on this technique (Brinster and Avarbock, 1994). At present, it remains unclear whether a human application will be feasible in the future and whether techniques of assisted reproduction would be required for reproduction after testicular germ cell transplantation.

This study therefore aimed at evaluating the fertilizing and developmental abilities of sperm obtained after stem cell transplantation. Transplanted animals were mated with females in order to evaluate in-vivo conception. The functionality of sperm obtained after testicular germ cell transplantation was further investigated by assisted reproductive techniques. We performed both IVF and ICSI in order to determine whether such sperm have normal fertilizing capacity and whether they can support normal preimplantation embryonic development *in vitro*.

Materials and methods

Transplantation

Preparation of pipettes

The transplantation, holding and injection pipettes were made of borosilicate glass (Drummond Scientific Co., USA) using a puller (P-97; Sutter Instrument Company, USA), a microgrinder (EG-4; Narishige, Japan) and a microforge (MF-9; Narishige) (Ogawa *et al*., 1997; Joris *et al*., 1998). The transplantation pipette had an inner diameter of 60 μm and a bevelled angle of 30°. The outer and inner diameters of the holding pipette were 80 and 10 μm respectively. The injection pipette had an outer diameter of 7 μm, was bevelled to an angle of 30° and heat-polished to obtain a sharp spike at the prominent edge of the bevelled tip.

Transplantation procedure

Donor cells were obtained from C57Bl ×Cba F1 mice (Iffa Credo, France) that were made cryptorchid at the age of 6 weeks, 3 months before transplantation. Cryptorchid testes contain a higher percentage of spermatogonia, including stem cells. As a result, the suspensions used for transplantation contain more stem cells (1/200), so increasing the chances of inducing donor spermatogenesis (Dobrinski *et al*., 1999b). The testes were decapsulated and the testicular tissue was digested according to a modified technique as described by Brinster and Avarbock (1994). First, the tissue was digested with 380 IU/ml collagenase Ia (Sigma, Belgium) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Belgium) for 1 h in a 37°C water bath. The digested tissue was then centrifuged at 400 *g* for 6 min. The supernatant was discarded, the cells were resuspended in DMEM with 2.5% trypsin (Sigma) and 10 μg/ml DNase (Sigma) and put in a water bath at 37°C for 15 min. Afterwards, 4% fetal calf serum (FCS; Life Technologies) was added and the cell suspension was again centrifuged at 400 *g* for 10 min. Finally, the cells were resuspended in DMEM supplemented with 5% penicillin (Sigma), 5% streptomycin (Sigma) and 10% FCS, so as to obtain a concentration of 10^7 cells/ml. Trypan Blue dye, used to visualize the entry of the injected cells in the seminiferous tubules, was not added to the injection medium, but was aspirated only in the tip of the pipette to minimize its possible toxic effects.

As recipient animals, 4–6 week old W/W^v mice (Jackson Labs, USA) were used, because these mice lack c-kit receptor and do not present endogenous spermatogenesis (Handel and Eppig, 1979). Transplantation was performed under a stereomicroscope. The transplantation pipette was introduced into the rete testis via the efferent duct (Ogawa *et al*., 1997). A volume of 3–10 μl, equivalent to ~3–10×10^4 cells, was injected into the tubules.

Immediately after transplantation the mice were injected s.c. with 100 μl of antibiotics [1900 μl phosphate-buffered saline + 100 μl Baytril® 2.5% (Bayer, Belgium)].

All experimental procedures were approved by the Animal Care and Use Committee at the Brussels Free University.

Histology

In order to analyse the histological appearance of the tubules after transplantation; the testes were fixed overnight in 2.5% paraformaldehyde (Sigma) at 4°C and embedded in paraffin. Sections 5 μm thick were cut and stained with eosin and haematoxylin. The percentage of tubule cross-sections with complete spermatogenesis was recorded. One hundred cross-sections were counted per testis. The slides were analysed under an inverted microscope with a magnification of ×200. An adult W/W^v mouse was used as a negative control.

In-vivo conception

Four months after transplantation, animals were mated with 6 week old C57Bl×Cba F1 hybrid females to evaluate in-vivo conception. Control experiments were also performed using fertile males. The animals were housed together at 15:00 and evaluated for the presence of a vaginal plug on the next morning. Females with a positive plug were selected and killed on day 17 of gestation. The uterine horns were dissected and examined for the presence of fetuses.

IVF

Preparation of sperm

Recipient males were maintained between 120 and 150 days before IVF was performed. The animals were killed by cervical dislocation. The epididymides were removed and transferred into one well of a 4-well plate containing 400 μl glucose-supplemented sodium-enriched synthetic oviductal medium [(modified potassium simplex optimised medium) mKSOM] (Summers *et al*., 1995) supplemented with 3% bovine serum albumin (BSA; Sigma) covered with oil. Three to four incisions were made to allow sperm to move into the medium. After 5 min of incubation at 37°C, the epididymides were discarded and 100 μl of the sperm suspension was transferred to another well with 400 μl mKSOM supplemented with 3% BSA under oil. Twenty minutes later, cells were counted and, if necessary, diluted to obtain a concentration of 10×10^6 cells per ml. Capacitation was allowed to proceed for 2 h at 37°C, in a 5% CO_2 and 5% O_2 incubator.

Preparation of oocytes

Six to ten week old C57Bl×Cba F1 hybrid female mice (Iffa Credo, France) were induced to superovulate by i.p. injection of 5 IU pregnant mare's serum gonadotrophin (PMSG; Intervet, Belgium) followed by 5 IU hCG (Intervet) 48 h later. Animals were killed by cervical dislocation ~16 h after hCG injection. Oviducts were collected in a test tube with 2 ml HEPES–DMEM supplemented with 0.5% BSA. Afterwards, they were transferred to a Petri dish where the oocytes were recovered from the oviducts with a fine pincet. Cumulus–oocyte complexes (COC) were washed twice in 120 µl mKSOM supplemented with 3% BSA under mineral oil and transferred to a 10 µl droplet of the same medium. One COC (± 10 oocytes) was placed per droplet.

IVF procedure

Ten µl of the sperm suspension were added to each of the three droplets with the COC and placed in the incubator for 3.5 h. Oocytes were then washed in 120 µl mKSOM supplemented with 0.5% BSA under oil and finally transferred to 30 µl droplets of the same medium. Ten oocytes were placed per droplet.

Incubation and examination of the oocytes

The dishes were incubated at 37°C in 5% CO_2 and 5% O_2 in a humidified incubator. Fertilization was assessed by recording the number of 2-cell embryos 24 h after fertilization. The dishes were further kept in culture until day 5 after fertilization. Embryos were observed at ×200 magnification on the warmed stage (37°C) of an inverted microscope.

IVF experiments with either sperm obtained after transplantation or control sperm were performed in the same time period according to an alternating schedule. Experiments were repeated five times for each condition.

ICSI

Preparation of sperm

The transplantation procedure was similar to that used for IVF. Epididymides were removed and placed in a 1 ml droplet of mKSOM supplemented with 3% BSA under mineral oil. Three cuts were made in the epididymides with a fine needle to allow the sperm cells to be released into the medium. After 20 min of incubation at 37°C, 50 µl of the sperm suspension was transferred into a droplet of 450 µl of the same medium under mineral oil and incubated for up to 2 h at 37°C.

For the control experiments, epididymal sperm from adult C57Bl×Cba F1 hybrid mice were used.

Preparation of oocytes

Oocytes were obtained as for IVF. The cumulus cells were removed by treating COC with 0.025% hyaluronidase (Sigma) in HEPES–DMEM for 1 min. The oocytes were rinsed thoroughly and kept in mKSOM supplemented with 0.5% crystalline BSA (Sigma) for 2 h on 37°C in a 5% CO_2 and 5% O_2 incubator.

ICSI procedure

For micromanipulation, nine drops were prepared under mineral oil in a 50×9 mm Petri dish. The central drop contained 1 µl of sperm suspension in 4 µl of DMEM containing 2.5% polyvinylpyrrolidone (PVP; Sigma). The eight surrounding drops contained 5 µl DMEM supplemented with 0.5% BSA and 20% FCS. A high concentration of FCS was used in order to increase the survival and fertilization rates (Suzuki and Yanagimachi, 1997). In each of the eight drops, one oocyte was placed. Fifteen minutes before injection the dish was put on the cooling stage (17°C) of an inverted microscope (Zeiss Axiovert 200, Germany).

Micromanipulation was performed with motor-driven micromanipulators (Eppendorf, Germany). A motile spermatozoon with normal morphology was immobilized by touching its tail and it was then aspirated tail-first into the injection pipette. The oocyte was held by the holding pipette with the polar body in the 6 or 12 o'clock position. The injection pipette was introduced through the zona pellucida and the oolemma until it almost reached the opposite side. If the membrane did not break immediately, it was sucked into the injection pipette until it broke and was then released. At the same time, the spermatozoon was injected into the oocyte (Palermo *et al*., 1992; Suzuki and Yanagimachi, 1997).

Incubation and examination of the oocytes

After injection, the oocytes were kept on the cooling stage (17°C) for 15 min. They were then put at room temperature for 10 min, and finally the oocytes were transferred into 20 µl droplets of mKSOM supplemented with 0.5% crystalline BSA under mineral oil and incubated at 37°C under 5% CO_2 and 5% O_2. Twenty-four hours later, the oocytes were examined for survival and fertilization under an inverted microscope at ×200 magnification. Two-cell embryos were considered fertilized. Embryos were examined every 24 h for up to 120 h.

The experiments with either sperm obtained after transplantation or control sperm were performed in the same time period according to an alternating schedule. Experiments were repeated five times for each condition.

Statistical analysis

Student's *t*-test was used for statistical analysis of concentration and motility of sperm. Fisher's exact test was used for statistical analysis of fertilization and developmental rates. $P < 0.05$ was considered significant.

Results

Transplantation

A testicular cell suspension was successfully transplanted in 33 testes of 18 W/W^v mice. A transplantation was called 'successful' when blue dye, injected in the rete, flowed into the tubules. Four to 5 months later, sperm were observed in 19 epididymides (19/33 or 58%) of 12 mice. However, spermatogenesis was observed in 21 testes of 14 mice (21/33 or 64%).

Fertile mice showed spermatogenesis in 55% (interquartile range of 48–57%) of the testicular cross-sections. Mature sperm were seen in the lumen of the seminiferous tubules (Figure 1A). However, 60% of the cross-sections showed stage C: VI–VII; or stage D: VIII–IX; an observation also reported by Ventelä *et al*. (2002). The testes of a negative control W/W^v mouse did not show any spermatogenesis (Figure 1B).

In-vivo conception

Twenty-three females were mated with nine transplanted W/W^v mice. Although 17 revealed a vaginal plug, only six of them became pregnant (35%). The testes of the transplanted males were analysed afterwards and donor spermatogenesis was observed in at least one testis per animal. Sperm were also found in their testes and epididymides. In the control group, 15 females were mated with eight control males and ten females

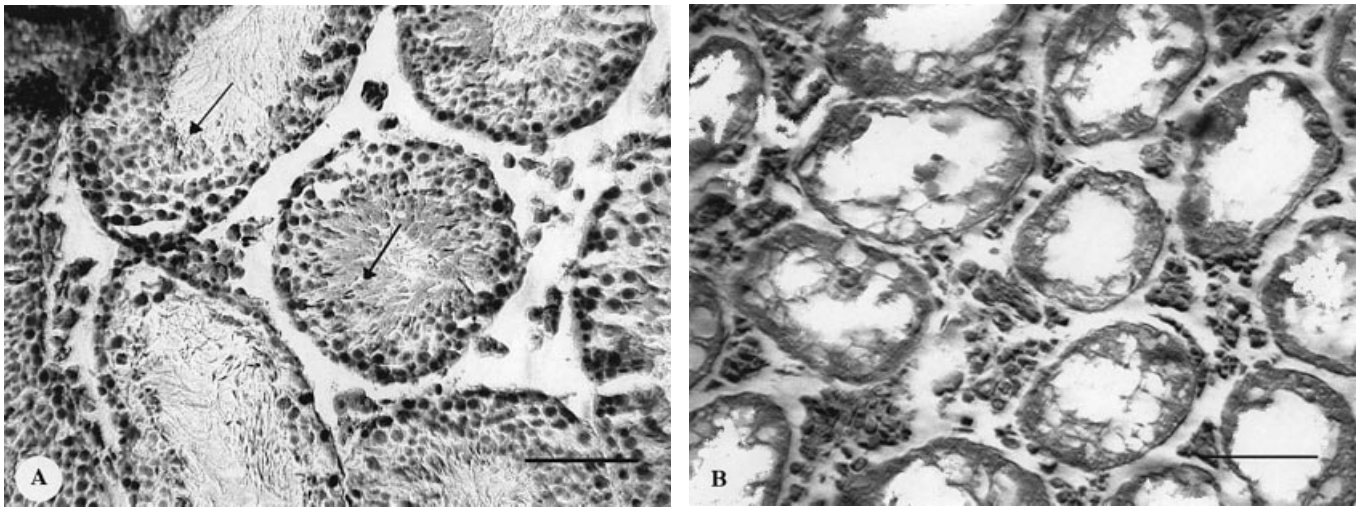

Figure 1. (**A**) Seminiferous tubules of a W/W^v mouse, 142 days after transplantation. The tubules show all stages of spermatogenesis, including mature sperm (arrows). (**B**) Negative control: seminiferous tubules of an adult W/W^v mouse with absence of any spermatogenesis. Scale bars: (**A**) 100 μm; (**B**) 50 μm.

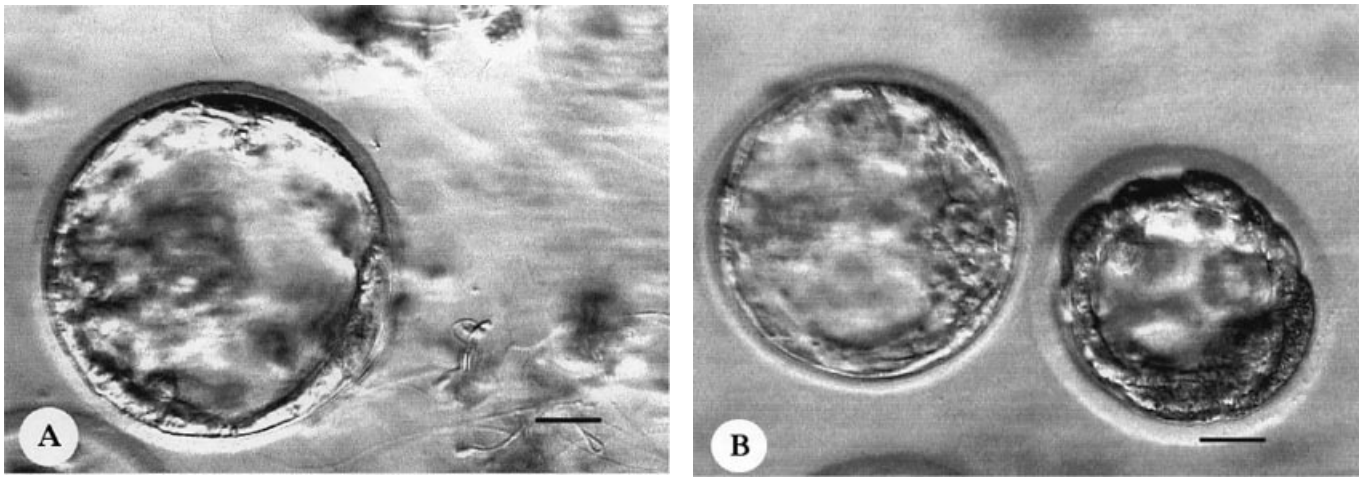

Figure 2. Blastocysts, obtained on day 5 by (**A**) IVF and (**B**) ICSI with sperm from a transplanted mouse. Scale bars: 25 μm.

had a vaginal plug. Of the latter, nine mice became pregnant (90%). This difference is statistically significant ($P = 0.006$) (Table I).

IVF

The median of the concentration of sperm obtained from epididymides from transplanted mice was 9.5×10^6 per ml, with an interquartile range of $9.2–10.4\times10^6$ per ml. The sperm showed poor motility: an interquartile range of 9–39%, with a median of 23%. IVF was performed with 154 oocytes using sperm obtained after transplantation. Of these, 88 became fertilized (57%). Twenty-four 2-cell embryos eventually developed into blastocysts (27%) (Table II) (Figure 2A).

In the control group, the median of the concentration of sperm was 9.9×10^6 per ml, with an interquartile range of $8.3–13\times10^6$ per ml. Motility showed an interquartile range of 39–45%, with a median of 40%. The concentration of sperm was not significantly different between the two groups; however, the motility of sperm from transplanted animals was significantly lower than that of fertile males ($P = 0.039$). A total of 155 oocytes out of 195 were fertilized (79%). Eighty-eight 2-cell embryos developed into blastocysts (57%).

The fertilization rate and the blastocyst development rate were significantly lower in the transplanted group (both $P < 0.00001$).

ICSI

Motile epididymal sperm obtained after transplantation were used to microinject a total of 187 oocytes. Eighty-three oocytes survived the injection procedure (44%) and 57 of these became fertilized (69%). Seventeen 2-cell embryos became blastocysts (30%) (Table III) (Figure 2B).

In the control mice, 112 oocytes were injected and 62 survived injection (55%). Of these 62, 38 became fertilized

Table I. Natural mating with transplanted and control mice

Origin of sperm	No. of females	No. of plugs	Pregnancy rate (%)[a]	No. of fetuses	No. of aborted fetuses
Transplanted mice	23	17	6 (35)[b]	17	1
Control mice	15	10	9 (90)[b]	76	6

[a]Pregnancy rate = (no. of pregnancies/no. of plugs)×100.
[b]$P = 0.006$.

Table II. IVF with epididymal sperm obtained from transplanted and control mice

Origin of sperm	No. of exp.	No. of oocytes	Fertilization rate[a] (%)	Developmental rate[b] (%)
Transplanted mice	5	154	88 (57)[c]	24 (27)[c]
Control mice	5	195	155 (79)[c]	88 (57)[c]

[a]Fertilization rate = (no. of 2-cell embryos/no. of survived oocytes)×100.
[b]Developmental rate = (no. of blastocysts on day 5/no. of 2-cell embryos)×100.
[c]$P < 0.00001$ by Fisher's exact test.

Table III. ICSI with epididymal sperm obtained from transplanted and control mice

Origin of sperm	No. of exp.	No. of oocytes	Survival rate (%)	Fertilization rate[a] (%)	Developmental rate[b] (%)
Transplanted mice	5	187	83 (44)	57 (69)	17 (30)
Control mice	5	112	62 (55)	38 (61)	14 (37)

[a]Fertilization rate = (no. of 2-cell embryos/no. of survived oocytes)×100.
[b]Developmental rate = (no. of blastocysts/no. of 2-cell embryos)×100.

(61%). Fourteen 2-cell embryos developed into blastocyst (37%). There was no significant difference in fertilization rate or developmental rate between the two groups.

Discussion

The model of testicular stem cell transplantation, as introduced recently by Brinster and Zimmerman (1994), may provide a means by which to circumvent the sterility of young male cancer patients surviving a sterilizing chemotherapy. In this study we aimed to evaluate the reproductive capacity of sperm obtained after germ cell transplantation in a mouse model. Extrapolating the findings of a mouse model to the human situation is certainly not without bias. Some preliminary results on testicular stem cell transplantation in primates have been reported (Schlatt *et al*., 1999; 2002), but because of the cost and the technical difficulties of this model together with the lack of transgenic donor animals, the mouse model remains the most studied model for testicular stem cell transplantation (Brinster, 2002).

In our study we used sperm from animals that were recovered after an average of 5 months (⩾4 months) post-transplantation. This time period covers more than three spermatogenic cycles (Brinster and Avarbock, 1994), i.e. long enough to omit the first waves of spermatogenesis which may include more apoptotic sperm or sperm with a limited reproductive capacity. However, in a human situation, transplantation may be performed at puberty leaving ⩾10 years for spermatogenic recovery. In the case of human application, autologous transplantation of cryopreserved testicular cells will be performed to a testicular environment that has been exposed to chemotherapy. This exposure may induce functional changes to the Leydig cell population (Howell and Shalet, 2002) and may eventually interfere with the outcome after transplantation. In our study we used W/W^v mice as recipient animals rather than mice treated with busulphan because W/W^v mice do not show endogenous spermatogenesis, but have the potential to support donor spermatogenesis (Ogawa *et al*., 2000). Russell *et al*. (1996) observed some morphological abnormalities in germ cells obtained after stem cell transplantation. Whether this implies that after transplantation in a recipient animal spermatogonial stem cells do not differentiate correctly and may generate sperm with limited functional capacities remains unclear. In order to explore this functional capacity, we examined both in-vivo and in-vitro reproduction with sperm obtained after transplantation.

First, we evaluated in-vivo conception by mating transplanted animals with females. Whereas in the control group 90% of mice with a copulating plug were impregnated, only 35% of mice became pregnant in the experimental group. Furthermore an average of 7.6 fetuses was observed in control mice with a copulatory plug versus only 1.0 in the experimental mice having a copulatory plug. This difference in fecundity may be due to the lower motility observed in the epididymal sperm of transplanted animals. Although it cannot be excluded that this difference is caused by deficiencies in spermatogenesis after transplantation as described by Russell *et al*. (1996), an insufficient recovery period post-transplantation may also

be at the origin of the observed reduction in motility. The lower motility may also be the result of local reactions in the testes. Although all animals had an antibiotic treatment after transplantation, an non-specific inflammatory reaction to the transplanted cell suspensions or the minute concentrations of Trypan Blue used, may have caused this response.

The testis is assumed to be an immunologically privileged organ (Barker and Billingham, 1977), yet an adverse immune response may also be involved. In the human, where autotransplantation instead of allogeneic transplantation would be performed, an adverse immune reaction is less likely.

In order to further examine the functional capacities of sperm obtained after transplantation, we also performed assisted reproductive techniques. We observed that sperm obtained after transplantation are able to fertilize oocytes both after IVF and ICSI and that fertilized oocytes can develop into blastocysts *in vitro*.

The results of IVF showed that fertilization rates were lower in the experimental group (27 versus 79%, $P < 0.00001$). In contrast, fertilization after ICSI was not different between controls and transplanted animals (69 versus 68%). The lower fertilization rate after IVF may again be due to the lower motility of sperm obtained from transplanted animals, although functional deficiencies cannot be excluded. Russel *et al*. (1996) reported more elongation abnormalities after transplantation. ICSI may correct for both motility and morphological deficiencies that may interfere with gamete interaction during fertilization. Once fertilization was achieved, the development of fertilized oocytes was comparable between experimental and control oocytes when ICSI was applied, but not when IVF was applied. After IVF, a lower developmental rate was observed in the experimental group. Because the results after ICSI are not different from controls, we may assume that the meiotic process is undisturbed in transplanted germ cells. The lower fertility rates after IVF and after in-vivo mating are probably related to the lower number of sperm observed after transplantation in our experiments.

Our study shows that in-vivo conception after testicular stem cell transplantation is realistic in mice. Differences between control and transplanted animals may be attributed to deficiencies in the animal model used. Whenever applied in a human situation, most of these deficiencies would probably not exist. Our results confirm again the potential of the testicular stem cell transplantation technique for circumventing sterility in male childhood cancer survivors.

Although based on a limited series, inherent with the complexity of the transplantation IVF and ICSI model, we also showed that sperm obtained after testicular stem cell transplantation are able to fertilize oocytes *in vitro* by means of techniques of assisted reproduction. Albeit fertilization and blastocyst developmental rates after IVF were lower when post-transplantation epididymal sperm were used, again limitations of the experimental model used may account for this finding.

Although additional study, including post-implantation development, is needed, our data strengthen the hope for future application of this approach in the human.

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