Evaluation of in vivo conception after testicular stem cell transplantation in a mouse model shows altered post-implantation development

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BACKGROUND: Apart from research applications, testicular stem cell transplantation (TSCT) may one day also have valuable clinical applications. Therefore, it is important to investigate whether this technique is a safe method to have progeny. This controlled study aims at evaluating the fetuses and the live born offspring obtained after TSCT in male mice. METHODS: Male mice were mated with wild-type (WT) females after TSCT to produce offspring. First, fetuses were evaluated on the 17th gestational day. The length, weight and morphological age were compared to those of control mouse fetuses. The live born offspring were then investigated for their reproductive potential over three generations. RESULTS: The litter sizes after TSCT were decreased compared to controls. Fetuses showed developmental retardation of a quarter of a day, but no major external abnormalities were observed. The live born pups were able to produce normal litter sizes, at least until the third generation. CONCLUSIONS: Transplanted animals are able to reproduce naturally. Although litter sizes are lower and development is retarded, no major morphological or procreative abnormalities were observed.

Key words: fetus/offspring/spermatogenesis/testis/transplantation

Introduction

Brinster and colleagues introduced the technique of testicular stem cell transplantation (TSCT), which has now become an established research model to study and manipulate the testicular germ cell line (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Brinster, 2002). Using TSCT in mice, donor spermatogenesis can be established in the seminiferous tubules of an otherwise infertile recipient. Transplanted males are able to produce offspring after spontaneous mating, and this offspring has been proven to be fertile (Brinster and Avarbock, 1994; Ogawa et al., 2000; Kanatsu-Shinohara et al., 2003). Fertility has also been restored after TSCT using cryopreserved testicular stem cells (Avarbock et al., 1996). TSCT may therefore have important clinical applications in preserving the progenitive potential of young boys who need to undergo a sterilizing chemotherapy.

Although this technique seems very promising, one needs to be aware of the risks of TSCT. Jahnukainen et al. (2001) described that after transplantation, as few as 20 leukaemic cells could cause a cancer relapse in rats. However, it was recently reported that malignant contamination could be overcome by depleting the cell suspension from leukaemic cells by fluorescence activated cell sorting (FACS) before transplantation (Fujita et al., 2005).

Recently, some alternative methods for testicular stem cell preservation have been described, that is testicular tissue grafting of immature and adult murine and human tissue (Honaramooz et al., 2002; Geens et al., 2006; Schlatt et al., 2006) and long-term culture of testicular stem cells (Izadyar et al., 2003; Kanatsu-Shinohara et al., 2003; Nagano et al., 2003). However, there are still concerns about these alternatives. Apart from ethical issues, there is also the risk for zoonosis after xenografting or after using animal supplements in culture systems. Autologous grafting might be a way to avoid zoonosis after xenografting. It was recently found in primates that autologous grafting could start spermatogenesis. However, sperm maturation was arrested at early meiosis (Wistuba et al., 2006).

TSCT therefore remains the most promising method for preserving and using testicular stem cells for fertility restoration in pre-pubertal boys undergoing sterilizing cancer treatment.
Before accepting TSCT as a clinical technique, all safety concerns need to be carefully evaluated. Although TSCT has been reported to produce live offspring, we observed earlier that the litter size was significantly reduced compared to wild-type (WT) controls (Goossens et al., 2003). Recently, we reported a deficit blastocyst formation after IVF with spermatozoa obtained from transplanted animals. Blastocysts derived from TSCT-IVF had significantly lower inner-cell mass cell numbers and lower inner-cell mass/trophectoderm ratios compared to control blastocysts, suggesting an altered preimplantation development (Goossens et al., in press). Although TSCT has been reported to produce live offspring, this study examines the post-implantation development after in vivo conception in a controlled way.

Materials and methods

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

**Study design**
For this study, two independent experiments were performed. In the first part of the study, the litter sizes and fetal development were evaluated on day 17 of gestation. In the second part, the offspring was carried to term, and the live born young were used for the evaluation of their transgenerational reproductive potential.

**Transplantation**
Donor cells were obtained from 6-week-old B6CBAFl/Juco mice (Charles River, Belgium) made cryptorchid 2 months before transplantation. The testes were decapsulated, and the testicular tissue was digested as previously described (Brinster and Avarbock, 1994). Four- to 6-week-old W/W<sup>v</sup> mice (Jackson Labs, Bar Harbor, ME, USA) were used as recipients. Transplantation was performed through the efferent duct as previously described (Ogawa et al., 1997). Immediately after transplantation, animals were given an antibiotic at a dose of 100 μl s.c. [5% Baytril® (Bayer, Belgium) in saline].

**Evaluation of fetuses after in vivo conception**
Four months after transplantation, animals were mated with 6-week-old B6CBAFl/Juco hybrid females to evaluate in vivo conception. Control experiments were also performed using 6-month-old fertile B6CBAFl/Juco males. One male was housed together with two females for one night, and females were evaluated for the presence of a vaginal plug on the next morning. Males without a vaginal plug were switched to another male 1 week later. Females with a positive plug were selected and killed on day 17 of gestation. The conceptuses were removed from the uterus, and the number of implantation sites, resolving conceptuses and live fetuses were noted. The extra-embryonic membranes were removed, and the fetuses were examined for major external anomalies. Subsequently, the fetuses were examined by weight and length (crown to rump). The developmental age of the fetuses was determined using the following formulas (Wahlsten and Wainwright, 1977):

\[
\text{Age} = 12.36 + 9.89 \times \text{weight} - 5.09 \times \text{weight}^2
\]

\[
\text{Age} = 7.8 + 0.729 \times \text{length} - 0.0135 \times \text{length}^2
\]

Fetal development was staged according to the Wahlsten and Wainwright criteria, and scores for skin, limbs, eyes and ears were averaged to give an overall morphology score (Wahlsten and Wainwright, 1977) (Table I).

Finally, the average age of the three parameters (weight, length and morphology) was determined, which corresponded to the estimated age of the fetus.

**Histology**
To analyse the histological appearance of the tubules after transplantation, testes were fixed overnight in Bouin’s fixative at 4°C and embedded in paraffin. Four-micrometer thick sections were cut and stained with eosin and haematoxylin. The slides were analysed under an inverted microscope with a magnification of ×200.

**Evaluation of transgenerational reproduction**
Offspring (males as well as females) obtained from transplanted males and WT females were evaluated morphologically. They were also mated with WT mice to evaluate their reproductive capacity. The offspring of these mice were again evaluated on morphological characteristics and were further mated with WT mice so as to evaluate the safety of reproduction until the third generation after transplantation. Results were compared to the data from our routine B6CBA breeding.

**Statistical analysis**
Fisher’s exact test was used for comparing pregnancy rates. Mann–Whitney statistics were used for comparing litter sizes and developmental ages. \( P < 0.05 \) was considered significant.

**Results**

**In vivo conception**
Twenty-six females were mated with thirteen transplanted W/W<sup>v</sup> mice. Nineteen females revealed a copulating plug. Seven animals became pregnant (37%) and carried 24 pups, that is an average of 3.4 pups per female mouse. One female carried eight pups, another two carried six pups. The remaining four female mice carried one pup each. Unfortunately, one of them

| Table I. Criteria for judging morphological age on the basis of skin, limb, eye and ear characteristics |
|---|---|---|---|
| Age | Skin features | Limb features | Eye features | Ear features |
| 14 | Foecicles on the ventral abdomen | H digits webbed | Round, distinct cornea and dark pigment | Distinct pinna flap |
| 15 | Foecicles on head | Digits splayed, clear phalanges on F | Almond shaped | Almost over meatus |
| | Abdomen wrinkled | Digits separated on H | Beginning to close | Distinct pinna flap |
| 16 | Neck and limbs wrinkled | Digits parallel. Clear phalanges F and H | Closed. Eyelids light | Distinct pinna flap |
| 17–18 | Skin dark | Like 16, but limbs larger | Closed, eyelids dark, very fine wrinkles | Closed, well-fused and dark skin |
| | Temporal area of head wrinkled | | | |

F, forelimb; H, hindlimb (Wahlsten and Wainwright, 1977).
had a spontaneous abortion. In the control group, thirteen females were mated with thirteen control males, and nine females showed a copulating plug. Eight of nine mice became pregnant (89%) and carried 66 pups, that is an average of 8.3 pups per female mouse. Both pregnancy rates and litter sizes differed significantly ($P = 0.016$ and $P = 0.005$, respectively) (Table II).

**Histology**

Males ($n = 7$) that impregnated females showed spermatogenesis in at least one of the testes with a re-colonization between 3 and 55%. Males ($n = 6$) that failed to conceive also showed spermatogenesis in at least one of the testes with a re-colonization ranging from 2 to 38%. No correlation was found between degree of fertilization and male fertility.

**Fetuses**

The fetuses, obtained from transplanted males, were weighed, measured and examined morphologically on day 17 of gestation. The average fetal weight was 0.56 g, and the average fetal length was 17 mm, which corresponds to a developmental age of 16.2 for both parameters. Evaluation of skin, limb, ear and eye features revealed a morphological age of 15.7 days. The overall estimated age was 16.1 days.

The control fetuses were examined and showed an average weight of 0.68 g and an average length of 19 mm. This correlated with an estimated age of 16.6 and 16.7 days, respectively. Evaluation of skin, limb, eye and ear features revealed a morphological age of 16.0 days. The overall age was estimated to be 16.4 days.

Weight and length were significantly lower for TSCT fetuses ($P = 0.009$ and $P = 0.005$), as well as the overall estimated age. The latter parameter revealed a developmental retardation of a quarter a day in TSCT fetuses ($P = 0.006$) (Figure 1).

**Evaluation of breeding safety**

Live born offspring showed no major morphological anomalies. All animals (males and females) revealed normal fertility. Litter sizes ranged from five to 11 live born pups in the second generation and from six to eight in the third generation. All pups were morphologically normal, and no fertility problems were observed (Table III).

**Discussion**

Using TSCT in mice, donor spermatogenesis can be established in the seminiferous tubules of an infertile recipient.

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**Table II.** Natural fertilization capacity of transplanted male mice

<table>
<thead>
<tr>
<th>Origin of sperm</th>
<th>Number of females</th>
<th>Number of plugs</th>
<th>Pregnancy rate (%)</th>
<th>Number of fetuses</th>
<th>Number of aborted fetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplanted mice</td>
<td>26</td>
<td>19</td>
<td>7 (37)$^b$</td>
<td>23$^c$</td>
<td>1</td>
</tr>
<tr>
<td>Control mice</td>
<td>13</td>
<td>9</td>
<td>8 (89)$^b$</td>
<td>66$^c$</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$Pregnancy rate = (number of pregnant females)/(number of females with a copulating plug).

$^bP = 0.016$ by Fisher’s exact test.

$^cP = 0.005$ by Mann–Whitney.

Transplanted males were able to produce offspring after spontaneous mating, and the offspring were found to be fertile (Brinster and Avarbock, 1994; Ogawa *et al*., 2000; Kanatsu-Shinohara *et al*., 2003). In previous experiments, we observed that *in vivo* conception with transplanted mice resulted in smaller litter sizes. We also observed that mouse spermatozoa obtained after transplantation were able to fertilize oocytes by IVF but with reduced fertilization and development rates in the transplanted group (Goossens *et al*., 2003). Blastocysts, obtained after IVF with sperm from transplanted male mice, showed reduced numbers of inner-cell mass cells, implicating lower implantation potential (Goossens *et al*., in press). As a result of these observations, we set up this study aimed at evaluating the fetuses obtained after *in vivo* conception with transplanted male mice. Wahlsten and Wainwright (1977) introduced an elegant analytic scheme for assessing defects in mouse development and estimating the developmental age. This scheme allows for morphology and maturity to be...
analysed. In contrast with other studies evaluating offspring after TSCT, we included a fertile control group to compare data obtained from transplanted mice.

We again observed a lower pregnancy rate and a smaller litter size in females impregnated with TSCT males. Additionally, TSCT fetuses were significantly shorter and weighed less on day 17 of gestation, indicating developmental retardation. However, no major abnormalities were observed. Live born pups did not show anomalies, and first-generation pups, as well as pups of the second and third generations, were proven fertile with normal litter sizes and normal and fertile offspring.

The reduced litter size might be due to lower sperm concentration or poor motility. Even though concentration and motility were not assessed in this study, motility impairment was noticed in previous studies (Goossens et al., 2003, in press). Hence, a more detailed analysis of the motility kinematics is mandatory to clarify this hypothesis.

Developmental disorders might result from genomic modifications in the paternal DNA or from sperm with aneuploidy. It would be interesting to compare the proportion of spermatozoa with aneuploidy in the semen of fertile and transplanted males.

Considering the aforementioned remarks, we believe that TSCT in the human can be used for fertility preservation. However, how this technique can be implemented in the clinic is still unclear. Human testes have another tubular structure and a different hormonal environment compared to rodents. It is obvious that research on more human-related species is necessary before TSCT can be safely applied in the clinic.

It is possible that the outcome of this study relies mostly on the murine model used for the experiments. It is obvious that for any clinical application, an autologous transplantation will be performed instead of a heterologous one, minimizing possible immune responses that can influence concentration and motility. However, 4 months after transplantation, no signs of rejection or inflammation were noticed. Nevertheless, this limitation may be the cause for perturbed sperm maturity. Another difference with our mouse model is the use of cryptorchid instead of pre-pubertal tissue. Testicular stem cells derived from cryptorchid tissue may have undergone genetic or structural changes because of the abnormal environment, and this may have caused atypical sperm maturation. In addition, the somatic testicular environment of the W/W recipients might be inadequate, resulting in impaired spermatogenesis.

It is also plausible that the 4-month period of recovery after transplantation is too short to re-establish regular spermatogenesis. It was reported earlier that host spermatogenesis is required to restore fertility as donor cells alone are too few for fertility restoration (Brinster et al., 2003). Obviously, a much longer recovery period would exist in the human application when TSCT would be performed shortly after the patient has been cured.

In summary, from the results of this study, we may conclude that infertile mice, injected with a testicular cell suspension, can father offspring, but that their litter sizes are decreased and their fetal development is retarded compared to controls. No morphological, developmental or fertility problems, however, were observed in subsequent generations. These findings encourage studies on human-related species, such as primates, to investigate the future clinical use of the technique.

**Acknowledgements**

The authors thank Mr Michael Whitburn for proofreading the manuscript. This study was supported by grants from the Dutch-speaking Brussels Free University (OZR 318) and the Fund for Scientific Research Flanders (FWO).

**References**


