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Germ cell transfer into rat, bovine, monkey and human testes

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Germ cell transplantation is a potentially valuable technique offering oncological patients gonadal protection by reinitiating spermatogenesis from stem cells which were reinfused into the seminiferous tubules. In order to achieve an intratubular germ cell transfer, intratubular microinjection, efferent duct injections and rete testis injections were applied on dissected testes of four different species: rat, bull, monkey and man. Ultrasound-guided intratesticular rete testis injection was the best and least invasive injection technique with maximal infusion efficiency for larger testes. Deep infiltration of seminiferous tubules was only achieved in immature or partially regressed testes. This technique was applied *in vivo* on two cynomolgus monkeys. In the first monkey a deep infusion of injected cells and dye into the lumen of the seminiferous tubules was achieved. In the second, transplanted germ cells were present in the seminiferous epithelium 4 weeks after the transfer. These cells were morphologically identified as B-spermatogonia and located at the base of the seminiferous epithelium. In summary, this paper describes a promising approach for germ cell infusion into large testes. The application of this technique is the first successful attempt of a germ cell transfer in a primate.

Key words: germ cell transplantation/fertility/gonadal protection/spermatogonia/testis

Introduction

In patients suffering from malignancies, chemotherapy or X-ray treatment may lead to permanent azoospermia or long azoospermic periods before recovery of spermatogenesis occurs (Rowley *et al*., 1974; Hahn *et al*., 1982; Clifton and Bremner, 1983; da Cunha *et al*., 1984; Marmor *et al*., 1992; Meistrich *et al*., 1992). In adult oncology patients, fertility can be maintained by cryopreservation of a semen sample. The introduction of intracytoplasmic sperm injection of single spermatozoa (ICSI) allows even patients with severely impaired sperm characteristics to be fertile (Palermo *et al*., 1992). ICSI can be applied to cryopreserved semen samples which will lead to higher pregnancy rates using cryopreserved sperm from oncological patients. However, hormonal pretreatment to protect testicular stem cells would offer an alternative way of maintaining the patients fertility. The extensive search for such pretreatments has resulted in hormonal deprivation schemes which down-regulate the proliferative activity of stem cells and thereby decrease their sensitivity to X-ray damage and chemotherapy (Velez de la Call and Jegou, 1990; Ward *et al*., 1990; Jegou *et al*., 1991; Kangasniemi, 1995a,b; Meistrich *et al*., 1996). However, these protective treatment regimens are not always effective and can hardly be applied when immediate oncological therapy is necessary.

Autologous transplantation of testicular stem cells is potentially a clinically relevant method for gonadal protection of tumour patients. Removal of testicular stem cells before treatment and subsequent retransplantation after recovery might implement fast and efficient restitution of spermatogenesis. Techniques for homologous and xenogeneic transplantation of testicular stem cells have been described for mice. Retransplantation was performed by microinjection into the seminiferous tubules. This technique succeeded in filling up to 80% of the surface area of the mouse testis (Brinster and Avarbock, 1994). After transfer of cells isolated from immunocompetent healthy donors, genetically and experimentally infertile recipient mice showed a restimulation of spermatogenesis and some of them produced offspring with the genetic background of the donor animals (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Moreover xenogeneic transplantation of rat stem cells into immunodeficient mice was also successful (Clouthier *et al*., 1996).

In germ cell transplantation experiments the donor cell preparations consisted of single cells and small fragments of all testicular germ and somatic cell types (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Clouthier *et al*., 1996). The initial method for transplantation was multiple microinjection into superficial seminiferous tubules. Subsequently other routes of injection, either into the efferent ducts or the rete testis, were also described and shown to be as efficient as intratubular microinjection (Ogawa *et al*., 1997; Russel *et al*., 1998). Donor germ cells were either freshly prepared before transplantation or stored in long-term culture systems (Nagano and Brinster, 1998) or by cryopreservation (Avarbock *et al*., 1996).

This study attempts to develop a strategy for the intratubular transfer of cell suspensions into the testes from ruminants and primates in which the testicular volume-to-surface ratio is much larger than in mice. A technique was developed in these animal models which could be used for germ cell transplantation in oncological patients.

Table I. Feasibility[a] of injection techniques for intratubular infusion

Injection technique	Rat	Bull	Monkey	Human
Microinjection of seminiferous tubules	4/4	1/4	1/3	1/3
Injection into efferent ducts	1/3	2/6	1/3	1/3
Injection into the intratesticular rete testis	NP	10/15	1/3	1/3

[a]Feasibility is expressed as the number of succesful attempts in relation to the number of total attempts performed. An injection was classified as successful when any injected dye was observed either in the intratesticular rete testis or the seminiferous tubules after dissection of the testis.
NP = not performed.

Materials and methods

Source of testes

In a first series of experiments, freshly dissected testes were used for the injection and localization of dye and cell suspension. Fifty bull and 20 calf testes were obtained from the local slaughterhouse. Four cynomolgus monkey and four rat testes were obtained from animals of our institutional colonies. Six human testes were donated by men undergoing orchidectomy for treatment of prostate carcinoma. All testes were kept on ice before the actual injection procedure started.

Preparation of donor cell injections

Cell suspensions of testicular cells were prepared by sequential digestion as previously described (Schlatt *et al*., 1996). Briefly, the testes were decapsulated and the tissue was minced using fine scissors. A first digestion was performed using collagenase type I (1 mg/ml; Sigma, Steinheim, Germany) and DNase (5 μg/ml) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) suppplemented with MEM (Gibco) and antibiotics (Gibco). This digestion was performed for 5 min at 37°C in a shaking water bath (90 cycles/min). The tissue fragments were aspirated six times through a 10 ml pipette using an automatic pipettor. The interstitial cells were discarded with the supernatant after sedimentation at unit gravity for 2 min. The separation step was repeated after addition of fresh DMEM. A second digestion using collagenase type IA (Sigma), DNase I (Sigma) and hyaluronidase type II (0.5 mg/ml Sigma) was performed for 20 min. Every 5 min cells were aspirated through a 10 ml pipette using an automatic pipettor. The cells were pelleted by centrifugation (5 min at 500 *g*), resuspended in phosphate-buffered saline (PBS, 50 mM, 150 mM NaCl, pH 7.2) and killed by heating to 70°C for 2 min. Before being used for microinjection they were mixed with trypan blue solution (final concentration: 0.04%).

Development of the injection procedure on dissected testes

For comparison of the injection techniques the testes were dissected after infusion under a dissection microscope, macroscopically analysed and documented by photography for the presence and distribution of the trypan blue solution. Representative pieces of tissue were fixed in Bouin's solution.

Microinjections into seminiferous tubules and efferent ducts were performed in all species using glass needles with a luminal diameter of 30–40 μm. Movements of the needle were controlled with a mechanical micromanipulator. In bull, monkey and man, but not in the rat, intratubular microinjections using glass needles were hampered by the very resistent lamina propria. In addition, the stronger convolution of the seminiferous tubules made it difficult to perform the injections (Table I). In all species, when the injection was succesful only small amounts of fluid (<50 μl) could be injected at each attempt into individual seminiferous tubules. The larger the testes the smaller was the area in which the seminiferous tubules were filled by tubular microinjection (bull < man, monkey < rat). Surgical preparation, localization and cannulation of the efferent ducts was difficult in all species. The head of the epididymis had to be partially dissected from the apical pole of the testis to exteriorize the efferent ducts. The fragile ducts were hard to recognize in the fat and connective tissue present in this area. After successful attempts, small amounts of coloured solution were detected in the intratesticular rete close to the apical pole of the testis (Table I). The amount of fluid which could be injected into the ducts was small (<100 μl). Some of the fluid entered the head of the epididymis where stained tubules could be observed. No coloured fluid or germ cells were observed in the seminiferous tubules by macroscopic observation after dissection. Rete testis injections were perfomed by hand with an injection needle (28 gauge). The injection system was connected to a tube and an infusion bag functioning as a reservoir for the germ cell preparation. The hydrostatic pressure during the injections was low. Rete testis injection was applied for intratubular germ cell transfer in testes from bull, monkey and human (Table I). The rate of successful attempts was low due to the difficulty of positioning the injection needle into the rete testis. In many attempts the dye floated the interstitial space of the testis but no dye was observed in the rete testis or the seminiferous tubules. However, in all cases when the correct injection site was used, the rete testis was filled at its full length throughout the testis and seminiferous tubules close to the rete testis also contained injected dye.

In order to optimize the infusion technique via the rete testis, further studies were performed. An attempt was made to use ultrasonography to localize the rete testis and guide the injection needle during rete testis injections. It was furthermore analysed whether the infusion would be effected using immature calf testes or regressed monkey testes.

Application of the injection technique in intact monkeys

Two cynomolgus monkeys were used to test the injection procedure in the intact animal. The monkeys were pretreated for 6 weeks with a gonadotrophin releasing hormone (GnRH) antagonist (Antide, 450 μg/kg/day; Weinbauer *et al*., 1989) resulting in a 40% reduction of testis volume. Both monkeys were then hemiorchidectomized. Animal maintenance and handling were performed in accordance with the German Federal Law for Care and Use of Laboratory Animals.

The testicular tissue was decapsulated and digested in a similar way as described above. The first digestion step was a mixture of collagenase Type I (Sigma) and DNase (Sigma). Thereafter the seminiferous tubules were separated, washed and incubated overnight in DMEM supplemented with antibiotics, glutamine, MEM (Gibco), bromodeoxyuridine (BrdU, 30 μg/ml; Sigma) and 500 mIU/ml urogonadotrophin (Pergonal; Serono, Freiburg, Germany). BrdU was added to the culture medium as a marker for the donor cells. The incorporation of the substance into cells in S-phase of the cell cycle during the culture period allowed these cells to be localized immunohistochemically in tissue sections of the injected testis. On the next day, a single-cell suspension was obtained by digestion in a

mixture of collagenase Type I, DNase and hyaluronidase (Sigma). After washing the cells in fresh culture medium they were transferred into PBS immediately before the injection procedure. Ultrasonography was used to localize the rete testis and to guide and position an injection needle through the scrotum into the intratesticular rete testis.

Ultrasonography

All ultrasonographical measurements were performed by a 7.5 MHz sector scanner allowing high resolution (Siemens Sonoline Versa Pro, Erlangen, Germany). The anaesthetized monkey was placed on a table lying on his back. The ultrasound transmission gel was applied to the scrotum. Systematic longitudinal and transversal scans identified the rete testis due to its higher echogeneity. Injections were performed from the lower testicular pole under continuous sonographic monitoring. The injections required ~30 min. All sonographies and injections were performed by the same scientist.

Histological techniques

Paraplast (Monoject Scientific Inc., Kildare, Ireland) was used for embedding the tissue. After overnight fixation in Bouin's solution the tissue was stored in 70% ethanol. After complete dehydration in graded series of ethanol, *N*-butylacetate was used as an intermedium before the tissue was infiltrated with paraplast, sectioned at 5 μm thickness onto sialinated slides. For histological analysis, the periodic acid–Schiff reaction was performed, resulting in an intense red staining of the acrosome of spermatids and the lamina propria of the seminiferous tubules and rete testis cords. The omission of counterstaining with haematoxylin allowed recognition of cells which had been labelled with trypan blue. Micrographs were produced with 64 tungsten slide film on a Zeiss photomicroscope.

Sections of the testis which had been injected with trypan blue-labelled cells were analysed for absolute changes in seminiferous tubule diameter and luminal diameter. For each parameter a final number of 40 tubules was evaluated in eight areas containing labelled cells in the centre of the seminiferous tubules. As control the same number of seminiferous tubules was scored in those areas in which no labelled cells were observed. A *t*-test was performed to determine statistically significant differences between the two groups analysed for each parameter.

The immunohistochemical localization of bromodeoxyuridine (BrdU) was performed as described previously (Rosiepen *et al*., 1994). After hydrolysis with 1 N HCl at 70°C in the microwave oven and trypsin digestion, the primary antibody (DAKO code No. M 0744, diluted 1:50) was incubated for 60 min after blocking of unspecific backgound staining with 5% normal goat serum. The next incubation step was a biotinylated anti-mouse antibody followed by a streptavidin–peroxidase conjugate. Finally, BrdU immunoreactivity was visualized with 3′3′-diaminobenzidine as a brown precipitate in the nuclei of cells.

Results

Injections into the intratesticular rete of dissected testes

Ultrasonography allowed the localization of the rete testis throughout the length of the testis and guidance of the injection needle into the rete testis. Under ultrasound guidance, two to three scrotal injection sites were selected. The injected volume for each injection was 2–5 ml. The injection into the bull rete testis filled the rete at its full length throughout the testis (Figure 1). Clusters of injected cells were recognized in histological rete testis cross-sections (Figure 2). Injected fluid and cells were found in the interstitium close to the injection sites (Figure 3). Occasionally and only very close to the rete tubuli, labelled cells or coloured fluid were observed in the lumen of seminiferous tubules of adult bull testes (Figure 4). The introduction of ultrasonography prevented unsuccessful attempts in which all of the injected cells and dye was injected into the interstitium.

In the calf, intratesticular rete injections of 2–3 ml cell suspension plus trypan blue resulted in the appearance of most of the coloured fluid and labelled germ cells in the lumen of the seminiferous tubules. Close to the injection site, cells were also seen in the interstitium (Figure 5). In contrast to the adult testis, large areas of the testis were reached by the injected cells and dye but always depended on the injected volume and the accuracy of placing the injection site correctly into the rete testis area. Many seminiferous tubules in large areas of the testis were infiltrated with the injected fluid even in areas distant from the injection site (Figure 6).

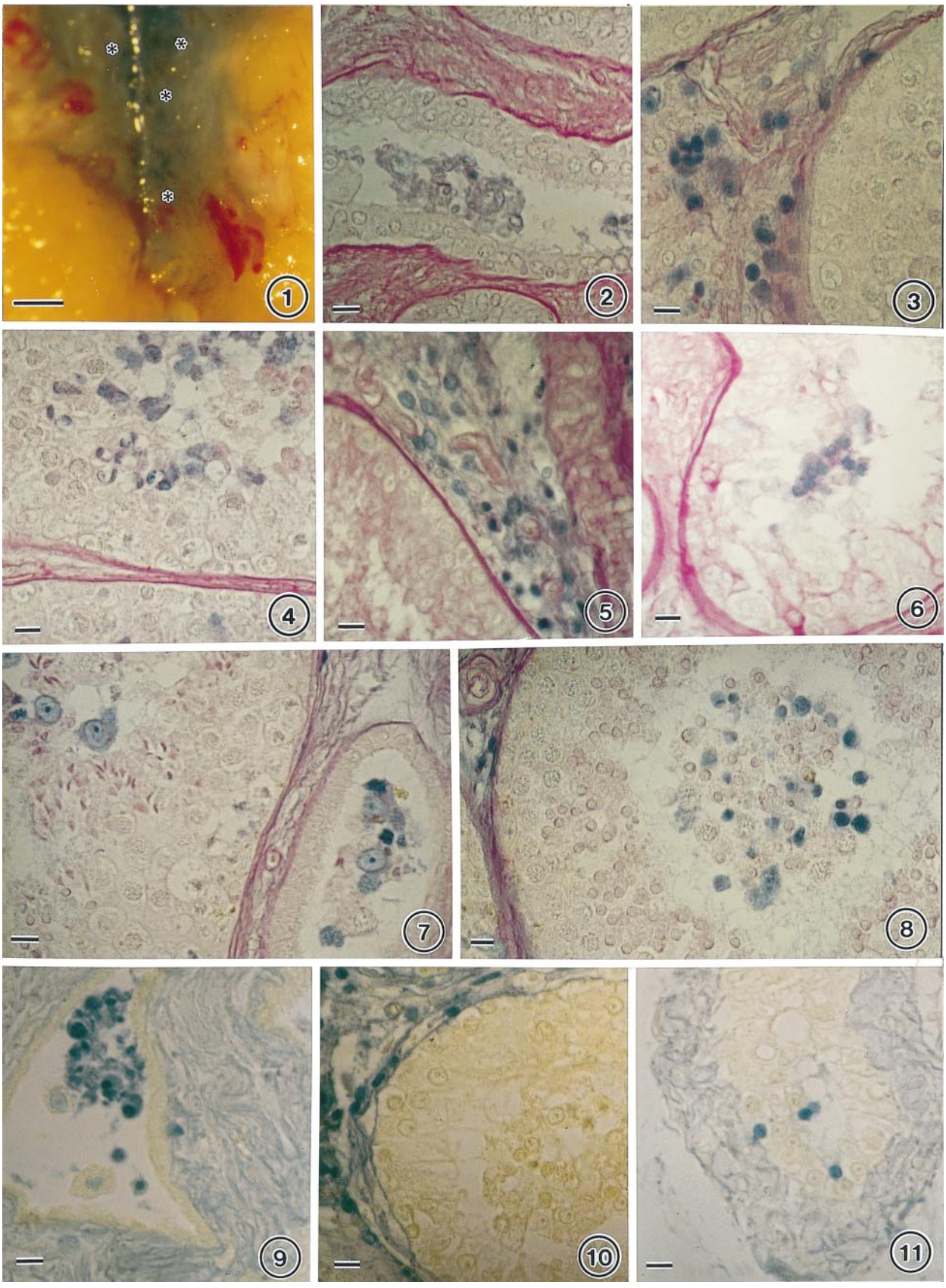
One cynomolgus monkey and four human testes were injected using the ultrasound-guided rete testis injection. These infusions resulted in a complete filling of the rete testis throughout the organ (Figure 7). In histological preparations, injected germ cells were localized in the lumen of a few seminiferous tubules of the monkey (Figure 7 and 8). In men rete testis injection led to a complete filling of the rete testis area (Figure 9). The extent of tubular infusion was variable. Deeper infusion of germ cells into the seminiferous tubules was observed in small testes revealing poor spermatogenesis. As in the other species, clusters of injected cells could be observed in the interstitium close to the injection site (Figure 10) and in the seminiferous tubules (Figure 11).

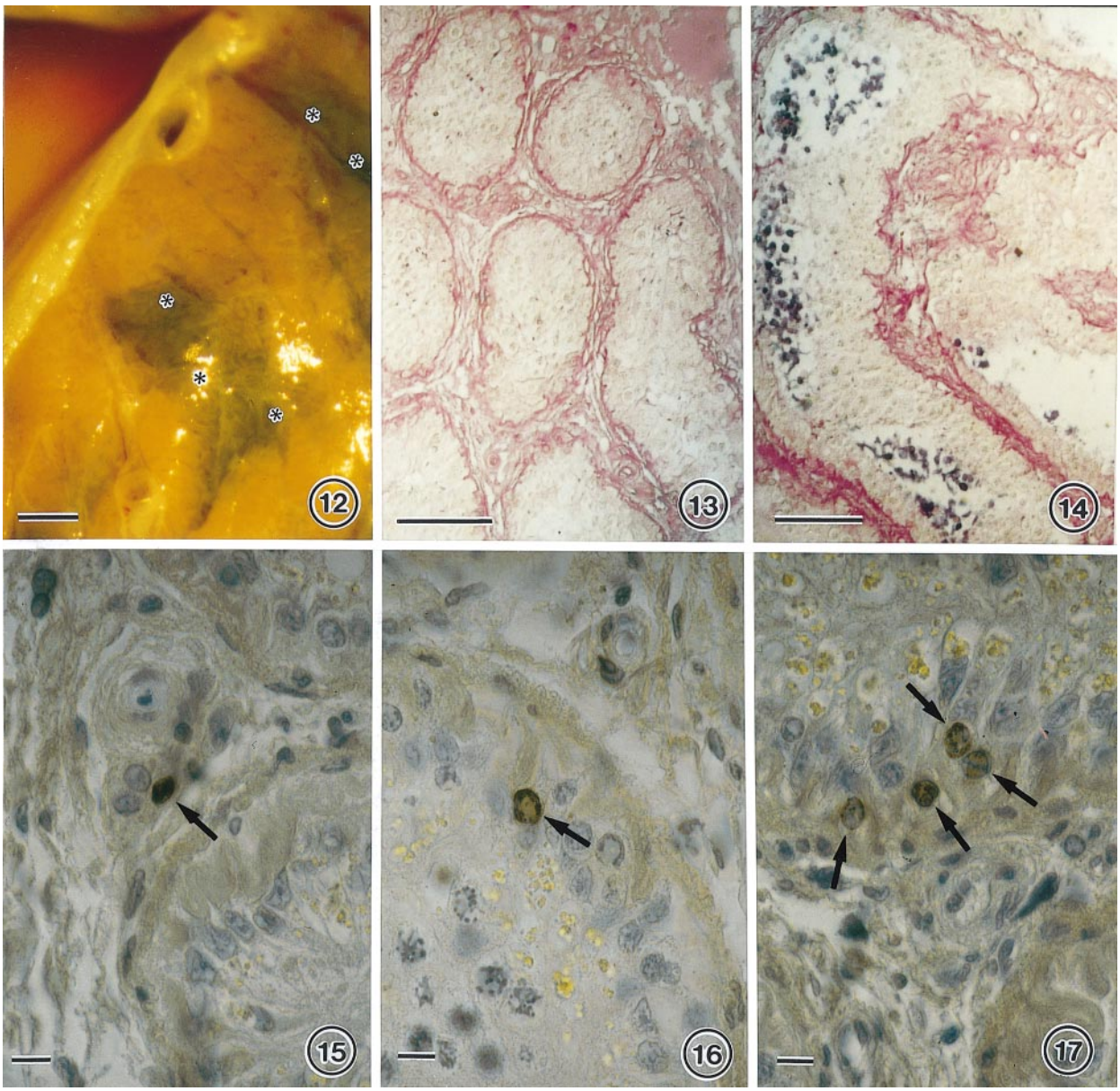
Germ cell transfer into monkey testes in vivo

After considering all results of the injection experiments on dissected testes, the experiments on autologous germ cell

Figures 1–11. Micrographs documenting the transfer of dye (trypan blue) and cells into the testis of bull (Figures 1–4), calf (Figures 5, 6), monkey (Figures 7, 8) and man (Figures 9–11) after ultrasound-guided injection into the rete testis. **Figure 1.** Bull testis. The testis was freshly dissected directly after trypan blue injection via the intratesticular rete testis. The blue colour is observed in the rete testis area (stars) but does not proceed deep into the tubule area. **Figure 2.** Bull rete testis. Labelled cells can be seen in the lumen of the rete testis [periodic acid–Schiff (PAS) staining]. **Figure 3.** Bull testis. Labelled cells are observed in the interstitium close to the injection site (PAS staining). **Figure 4.** Bull testis. Occasionally, seminiferous tubules with labelled cells can be seen (PAS staining). **Figure 5.** Calf testis. Labelled cells can be seen in the interstitium around the injection site (PAS staining). **Figure 6.** Calf testis. A seminiferous tubule containing labelled cells in the lumen (PAS staining). **Figure 7.** Monkey testis. A rete testis duct and a seminiferous tubule contain injected cells (PAS staining). **Figure 8.** Monkey testis. Normal seminiferous tubules with labelled cells (PAS staining). **Figure 9.** Human rete testis. Clusters of labelled cells are observed in the lumen of the rete testis (no counterstain). **Figure 10.** Human testis. Intense blue staining and labelled cells are seen in the interstitium close to the injection site (no counterstain). **Figure 11.** Human testis. Blue cells can be observed in seminiferous tubules of the human testis (no counterstain). Scale bars: Figure 1: 1 mm; Figures 2–11: 10 μm.

transfer in the cynomolgus monkey were designed. In order to reduce the secretory activity of the Sertoli cells, two cynomolgus monkeys were treated with GnRH antagonist before the injection technique was applied. The volume of 2–3 ml of the cell suspension which was injected into the remaining testis of both monkeys induced striking increases in the volume and the resistance of both testes. The first monkey received a suspension of dead cells dissolved in PBS





Figures 12–17. Micrographs showing the results of the germ cell transfer into the testes of cynomolgus monkeys treated for 6 weeks with gonadotrophin releasing hormone (GnRH) antagonist. **Figure 12.** Focal appearance of blue labelled areas (stars) containing the infused dye in the freshly dissected testis 2 h after the injection of dye and cells. **Figures 13 and 14.** Testis tissue fixed 2 h after infusion of dye and dead cells (periodic acid–Schiff staining, no counterstain). The seminiferous tubules are regressed due to the GnRH antagonist treatment. Either no or only very small lumina are observed in the seminiferous tubules which are devoid of later germ cell types (Figure 13). The lumen reappeared and was filled with clusters of labelled cells in tubules located in areas where the infiltration took place (Figure 14). **Figures 15–17.** Testis tissue fixed 4 weeks after infusion of germ cells (haematoxylin counterstain). Bromodeoxyuridine (BrdU)-positive germ cells are recognized by a dark brown nuclear staining (arrows). They are located in the interstitium close to the injection site (Figure 15) and in some seminiferous tubules (Figures 16, 17). BrdU-positive cells in the tubules appear as single cells (Figure 16) or as small clusters (Figure 17) and are morphologically identified as B-spermatogonia. Scale bars: Figure 12: 1 mm; Figures 13, 14: 100 μm; Figures 15–17: 10 μm.

supplemented with 0.4% trypan blue. Close to the injection site, the interstitium was filled with the coloured solution. Throughout the organ, focal areas containing blue-labelled seminiferous tubules were observed (Figure 12). Histological preparation showed that the seminiferous epithelium was involuted due to the GnRH antagonist treatment. Neither spermatocytes nor round spermatids were present in the seminiferous epithelium. The size of the tubular lumen was reduced and in many areas the lumen had almost disappeared (Figure 13). In areas where cells had been flushed in after the injection, the tubular lumen reappeared. Although this effect was highly significant, no significant increase in seminiferous tubule diameter was measured in the same subset of tubules (Table II; Figure 14). Throughout the organ, the lumen of many seminiferous tubules was filled with the injected solution in areas both close to and more distant from the injection site.

The second monkey had received an injection of living cells without trypan blue. No further treatment was performed

Table II. Changes in luminal diameter and seminiferous tubule diameter 2 h after germ cell transfer into a cynomolgus monkey testis

	Luminal diameter (μm)	Seminiferous tubule diameter (μm)
Seminiferous tubules containing injected cells	79.2 ± 14.8	131.7 ± 17.6
Seminiferous tubules without injected cells	48.4 ± 33.6	126.6 ± 15.5
P-value of difference between groups	< 0.001	NS

NS = not significant.

during the next 4 weeks when the testis was dissected, fixed and prepared for histological analysis. Immunostaining of BrdU was performed in order to recognize those cells which had incorporated this thymidine analogue into their nuclei before injection. Staining revealed the presence of some labelled cells in the interstitium in those areas close to the injection site (Figure 15). BrdU-positive cells were also localized in some seminiferous tubules (Figures. 16, 17). The positive cells were located at the base of the epithelium and identified as B-spermatogonia based on morphological criteria. They appeared both as single cells (Figure 16) as well as small clusters of cells (Figure 17).

Discussion

Three infusion routes for the transfer of cell preparations into the seminiferous tubules of larger testes are described. The transfer of germ cells by microinjection of seminiferous tubules or cannulation of efferent ducts was difficult and inefficient in the bull, in the monkey and in the human. In contrast, all three strategies have been reported to be useful and equally effective in the mouse testis (Ogawa *et al*., 1997; Russell *et al*., 1998). Microinjection into seminiferous tubules as well as into the efferent ducts is therefore not the most favourable approach for germ cell transfer into large testes. In contrast, injection into the rete testis allowed the infusion of a large injection volume into the testis. This method was optimized by the application of ultrasonography to localize the rete testis area and to guide the injection needle. This methodology avoided any open surgery and the injection could be performed by intratesticular injection at three or more locations through the scrotum. Intratesticular rete injection might be the most promising approach to be tested for a clinical application.

Only very few seminiferous tubules near the rete testes area were infiltrated in normal adult bull and monkey testes or in human testis derived from prostate carcinoma patients. However, in immature calf testes and regressed monkey testes, entry of the fluid into the seminiferous tubules was observed in large areas of the testis even quite distant from the injection site. Obviously, the intratubular fluid pressure is too high to allow more fluid to enter the seminiferous tubules in the retrograde direction. In contrast, in immature or regressed testes the fluid production of Sertoli cells is low and the lumen of the seminiferous tubules is very small or absent. In these testes the seminiferous tubules can be infiltrated by the retrograde flow of the injected solution. The GnRH antagonist treatment induced a strong involution of the tubule diameter from 180–210 μm in normal monkeys (Weinbauer *et al*., 1987) to ~130 μm after 6 weeks of treatment. Interestingly, although the lumen was restored, the seminiferous tubules did not increase much in size, indicating that either the intratubular pressure does not increase much or that the lamina propria, with its peritubular smooth muscle cells, is resistent to the increased intratubular pressure. The advantage of this effect is that a deep infusion of the cell suspension is achieved along the length of the seminiferous tubules. In fact, the focal appearance of up to 70% stained areas in the monkey testis indicates that in these lobuli most of the tubules are substantially filled. The same observation was made in the excised human testis where up to 70% of the lobuli could be reached by the dye. The time needed for the rete testis injections was ~30 min, which is similar to the rodent model in which a 70–100% filling of the surface seminiferous tubules was achieved in 5–30 min (Russell *et al*., 1998). As the testes of oncological patients are regressed due to the effects of chemo- or radiotherapy, the infiltration of seminiferous tubules in large areas of the testis should be possible using the technique described here.

The application of our infusion technique to the intact cynomolgus monkey marked the first successful transfer of germ cells into the primate testis. So far, only mice have been used as recipients in germ cell transplantation studies (Russell *et al*., 1998). The use of an immunohistochemically detectable marker for transplanted cells allowed the detection of some of the transplanted cells at the base of the seminiferous epithelium 4 weeks after the transfer. In contrast to the rodent model, in which transgenic donor cells expressing a genetic marker can be used (Brinster and Zimmermann, 1994), our cells were labelled by incorporation of BrdU. The disadvantage of this labelling approach is that only a small number of spermatogonial stem cells will have been in S-phase of the cell cycle during the culture period and will have incorporated BrdU. Therefore, only a small percentage of cells which had actually been transplanted will be BrdU-positive. However, the presence of a few BrdU-positive cells in the interstitium close to the injection site as well as a small number of cells at the base of the seminiferous tubules shows that the germ cell transfer technique worked in principle. These cells in the seminiferous tubules appeared to be B-spermatogonia, indicating that spermatogenesis had been reinitiated from transplanted stem cells. A similar 4 week period for the first localization of transplanted spermatogenic cells is reported in the mouse (Russell *et al*., 1998). The mechanism by which these germ cells migrated from the luminal compartment through the blood–testis barrier to the base of the seminiferous epithelium remains unexplained. Further studies are needed to establish

whether these early germ cells will lead to an efficient repopulation of the testis with germ cells and whether they will develop into mature sperm. In addition, the efficiency of the transplantation procedure has to be analysed by the use of better donor cell markers.

Today, male oncology patients who undergo potentially sterilizing cytotoxic treatments rely on pretreatment cryo-preservation of semen for fertility preservation. The semen sample can be used in assisted reproductive technologies such as insemination, in-vitro fertilization or ICSI in cases of poor sperm characteristics (Palermo *et al*., 1992). In the future, germ cell transplantation might become an alternative approach for the preservation of fertility. Similar to gonadal protection by pretreatment regimens before cancer therapy (Velez de la Call and Jegou, 1990; Ward *et al*., 1990; Jegou *et al*., 1991; Kangasniemi 1995a,b; Meistrich *et al*., 1996), the advantage of this approach would be that the treatment initiates a restoration of testicular function and therefore leads to a permanent cure of the patient. For some prepubertal patients or other patients who are not able to donate a semen sample this technique might be the only opportunity to maintain their fertilty.

In summary our data suggest that the most promising infusion technique for germ cells into larger testes is ultrasound-guided injection into the rete testis. The intensity of infiltration of seminiferous tubules depends on the intratubular pressure which is lower in immature and regressed testes. Using this technique, an efficient germ cell transfer into the seminiferous tubules of a regressed monkey testes was achieved. The detection of transplanted germ cells in seminiferous tubules 4 weeks after autologous transfer highlights the potential of this technique.

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