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Xenogeneic transplantation of equine testicular cells into seminiferous tubules of immunocompetent rats

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Abstract

The objectives were to develop a transplantation assay for equine testicular cells using busulfan-treated prepubertal immunocompetent rats as recipients, and to determine if putative equine spermatogonial stem cells (SSCs) could be enriched by flow cytometric cell sorting (based on light scattering properties), thereby improving engraftment efficiency. Four weeks after transplantation of frozen/thawed PKH26-labeled equine testicular cells, $0.029 \pm 0.045\%$ (mean $\pm$ SD) of viable donor cells transplanted had engrafted. Donor cells were present in seminiferous tubules of all recipient rats forming chains, pairs, mesh structures, or clusters (with 2 to >30 cells/structure). Cells were localized to the basal compartment by the basement membrane. Although equine cells proliferated within rat seminiferous tubules, no donor-derived spermatogenesis was evident. Furthermore, there was no histologic evidence of acute cellular rejection. No fluorescent cells were present in control testes. When equine testicular cells were sorted based on light scattering properties, the percentage of transplanted donor cells that engrafted was higher after injection of cells from the small, low complexity fraction (II; $0.169 \pm 0.099\%$) than from either the large, high complexity fraction (I; $0.046 \pm 0.051\%$) or unsorted cells ($0.009 \pm 0.007\%$; $P < 0.05$). Seminiferous tubules of busulfan-treated prepubertal immunocompetent rats provided a suitable niche for engraftment and proliferation, but not differentiation, of equine testicular cells. Sorting equine testicular cells based on light scattering properties resulted in a 19-fold improvement in colonization efficiency by cells with high forward scatter and low side scatter, which may represent putative equine SSCs.

Keywords

Spermatogonia; Germ cell; Equine; Xenogeneic; Transplantation

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1. Introduction

Stem cells have the capacity for self-renewal and tissue regeneration. Within the testis, spermatogonial stem cells (SSCs) are responsible for maintaining spermatogenesis throughout adult life, and for repopulating the testis after an insult. When SSCs from a donor male are transplanted into the testis of a recipient male, donor SSCs colonize the seminiferous tubules, and initiate donor-derived spermatogenesis [1]. Successful SSC transplantation has been reported in several species, but not in horses. The technique has been proposed for preservation or expansion of genetics of valuable males, preservation of endangered species, generation of transgenic animals, and treatment of azoospermia caused by Sertoli cell dysfunction [2]. In horses, SSC transplantation could enhance understanding of equine spermatogenesis and its regulation. Clinically, equine SSC cryopreservation and transplantation has potential in preservation of male genetics in cases of death or debilitating disease of a valuable stallion. Idiopathic testicular degeneration (ITD) is thought to result from a primary Sertoli cell dysfunction [3] and SSC transplantation could enhance understanding of the pathogenesis of ITD in stallions.

To implement SSC transplantation in the horse, it is necessary first to develop a transplantation assay to identify equine SSCs and test the effects of various cell treatments on their ability to engraft. Transplantation assays have been developed in mice and rats to identify SSCs, based on their ability to colonize the seminiferous tubules of recipient testes [1,4]. Xenogeneic transplantation of testicular cells derived from large domestic species into mouse testes is also a well-established transplantation assay that provides economical and logistical advantage relative to using large domestic animals as recipients. Testicular cells from hamsters [5], dogs, rabbits [6], baboons [7], bulls, pigs, and horses [8] have been shown to engraft and colonize mouse seminiferous tubules. However, colonization of mouse seminiferous tubules by equine testicular cells was reportedly inefficient [8].

Mouse and rat SSCs have been identified by flow cytometric cell sorting based on light scattering properties. Mouse and rat SSCs were reported to have high forward scatter and low side scatter [9,10]. Cell sorting based on these properties resulted in a 3.7-fold improved colonization efficiency by rat [10], but not mouse testicular cells [9]. No information is available on cell characteristics of equine SSCs and it is unknown if flow cytometric cell sorting based on light scattering properties would result in enrichment of equine testicular cells capable of engrafting. The objectives of this preliminary study were to develop a transplantation assay for equine testicular cells using busulfan-treated prepubertal immunocompetent rats as recipients, and to determine if putative equine SSCs could be enriched by flow cytometric cell sorting based on light scattering properties, thereby improving engraftment efficiency.

2. Materials and methods

2.1. Evaluation of the ability of rat seminiferous tubules to provide an environment suitable for engraftment of equine testicular cells (Experiment 1)

Frozen/thawed equine testicular cells were labeled with the fluorescent cell tracer PKH26 (Sigma-Aldrich Co., St. Louis, MO, USA; Product No. MINI26). This lipophilic red fluorescent dye stably integrates into the cell membrane. The left testis of recipient rats was transplanted with the PKH26-labeled cells. The right testis injected with transplantation medium served as a sham control (see recipient preparation and transplantation methods in Section 2.5). Testes were recovered 4 wk after transplantation and were evaluated using light, fluorescence, and confocal laser microscopy.

2.2. Evaluation of engraftment efficiency of equine testicular cells sorted by flow cytometry based on light scattering properties (Experiment 2)

Frozen/thawed testicular cells (20 to 59×10^6) from each stallion were divided into two aliquots. An aliquot containing 0.088 to 7×10^6 cells was labeled with PKH26 and transferred unsorted into the left testis of a recipient rat. Injection of transplantation medium into the right testis served as a sham control. The remaining cells were sorted with a FACSVantage SE flow cytometer (BDIS, San Jose, CA, USA), based on forward and 90° scatter of the 488 nm laser. The dot plots of the scatters showed three populations of cells. The larger, higher complexity cell population (Fraction I) and the smaller, lower complexity cells (Fraction II) were separated from the total population (Fig. 1). The separated fractions (I and II) were labeled with PKH26. The fluorescently labeled fractions were then transplanted into the right (Fraction I) and left (Fraction II) testis of a recipient rat. Rat testes were evaluated with fluorescence microscopy 4 wk after transplantation. Fraction III containing cells with low forward scatter was considered to represent red blood cells and cell debris [11] and therefore was discarded.

2.3. Isolation of equine testicular cells

Donor stallions were presented to the Kansas State University Veterinary Medical Teaching Hospital for routine orchiectomy in August, October, and January. Stallions were 6 to 33 mo old (Exp. 1; $n=5$) and 8 to 33 mo old (Exp. 2; $n=4$), of Warmblood and American Quarter Horse breeds. All stallions had bilateral scrotal testes, with no gross testicular abnormalities. Testes were collected aseptically and cell isolation was initiated within 30 min after orchiectomy. The tunica albuginea was removed and 10 g of testicular parenchyma was minced with a scalpel. Minced tissue was suspended in Dulbecco Modified Eagle medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) with 100 IU/mL penicillin, 100 μ g/mL streptomycin (Invitrogen Corp.), 1 mg/mL collagenase type I (Invitrogen Corp.), 1 mg/mL trypsin (Invitrogen Corp.) and 1 mg/mL hyaluronidase type II (Fisher Scientific Inc., Pittsburgh, PA, USA). The tissue was incubated at 37°C for 60 min. Seminiferous tubule fragments were centrifuged at $300 \times g$ for 1 min, and the supernatant was discarded. Tubule fragments were washed in DMEM three times by centrifugation at $1000 \times g$ for 3 min. After the third wash, seminiferous tubule fragments were further incubated with enzymes for 45 min as above. Undigested fragments were removed by filtration through a 60- μ m nylon filter. The filtrate was centrifuged at $1000 \times g$ for 3 min. The cell pellet was resuspended in DMEM with 1% bovine serum albumin (BSA) (w/v) (Sigma-Aldrich Co.) to a concentration of 6×10^6 cells/mL for cryopreservation.

2.4. Cryopreservation of equine testicular cells

Re-suspended cells were added an equal volume of 2x concentrated freezing medium to achieve a final concentration of 3×10^6 cells/mL. Freezing medium was DMEM with 1% BSA containing a final concentration of 10% fetal bovine serum (FBS, v:v; Fisher Scientific Inc.), 1.4 M dimethyl sulfoxide (Sigma-Aldrich Co.) and 0.07 M sucrose (Sigma-Aldrich Co.) [12]. The cell suspension was placed in 1.8-mL cryovials in 1-mL aliquots. The cryovials were placed at -80°C for 24 h, and then transferred to liquid nitrogen at -196°C until transplantation. Equine testicular cells were thawed the day of transplantation in a water bath at 38°C for 2 min. After adding 2 mL of DMEM with 10% FBS, cells were centrifuged at $1000 \times g$ for 5 min, the supernatant was removed and the pellet resuspended in DMEM for PKH26 labeling or DMEM with 1% BSA for flow cytometry. Testicular cells were counted with a hemacytometer and cell viability was evaluated by Trypan blue exclusion prior to freezing and after thawing.

2.5. Xenogeneic transplantation of equine testicular cells

Prepubertal immunocompetent Fisher 344 male rats were used as recipients in Exp. 1 (n=5) and Exp. 2 (n=8). Endogenous spermatogenesis was ablated at 10 d of age with Busulfan (Sigma-Aldrich Co.) 10 mg/kg ip [13]. Rats were transplanted with equine testicular cells when they were 18 to 22 d old. Equine testicular cells were labeled with the fluorescent cell tracer PKH26 immediately prior to transplantation following the instructions of the manufacturer (Sigma-Aldrich Co.). Briefly, cells were washed by centrifugation at 400 X g for 5 min in DMEM. The pellet was resuspended in Diluent C supplied with the kit. A 30×10^{-6} molar working solution of PKH26 was prepared in Diluent C, and 10 μ L was added per every 1×10^6 cells. Cells were incubated at room temperature for 3 min. The labeling reaction was stopped by adding FBS. Labeled cells were washed three times by centrifugation to remove unbound dye.

After labeling, cells were re-suspended to 0.2 to 140×10^6 cells/mL in transplantation medium. Transplantation medium consisted on DMEM with 10% FBS and 0.05% trypan blue (w/v). To determine if any residual amount of dye left with the medium after staining and washing the cells would result in staining of endogenous testicular cells, 0.04% PKH26 (v:v) was added to the transplantation medium of control testes. This concentration was estimated to be the approximate residual concentration of dye remaining with the cell pellet after washing by centrifugation. General anesthesia was induced and maintained with 3 to 5% and 1.5 to 3% isoflurane (in 100% oxygen), respectively. A 5- μ m fire-polished glass needle was used to inject the labeled cell suspension into the rete testis until most seminiferous tubules were filled. The volume injected ranged from 50 to 80 μ L per testis. The Institutional Animal Care and Use Committee of Kansas State University approved all animal experiments.

2.6. Analysis of recipient testes

Rats were killed 4 wk after transplantation to collect testes. A preliminary trial showed that, while equine testicular cells had already engrafted by the basement membrane of rat seminiferous tubules 2 wk post-transplantation, cells were found in singles with no evidence of proliferation. The SSCs are defined by their ability to engraft and colonize the recipient's testes. Therefore, 4 wk were allowed before evaluation, to increase the likelihood of detecting proliferation. Testes were fixed in Bouin's solution. Following equilibration in a cryopreservative solution of buffered 20% sucrose, frozen sections from the testes were obtained after removing the tunica albuginea. Half of each testis was cut into 10- μ m thick longitudinal sections. Sections were rinsed with Dulbecco's phosphate buffered saline (DPBS) and stored at 4 °C in the dark until analysis. Five hundred randomly selected non-adjacent cross sections of the seminiferous tubules from five to nine tissue sections were evaluated per testis. The number of red fluorescent PKH26-labeled cells in 500 seminiferous tubules was determined using an epifluorescence microscope at high power (X 400; Olympus B-Max 60, Olympus America Inc., Melville, NY, USA). The number of PKH26-labeled cells was normalized to 100 cells transplanted. During Experiment 1, one 5- μ m thick longitudinal section from each testis was also submitted to the Kansas State University Veterinary Diagnostic Laboratory for routine staining with hematoxylin and eosin; these sections were evaluated under light microscopy for histologic evidence of acute cellular rejection defined by presence of perivascular, vascular, interstitial and/or intratubular mononuclear inflammatory infiltration [14]. The number of mononuclear cells was counted in 10 high power fields (X 400). The remaining half of each recipient testis was evaluated under confocal laser microscopy for morphologic assessment of colonies. The tissue was washed in DPBS and seminiferous tubules were dispersed on a slide and mounted under a coverslip with a small amount of DPBS. Tissue was evaluated at low power (X 100) and

optical sections were collected (510 META; Carl Zeiss Inc., Thornwood, NY, USA). All evaluations were performed by one operator, who was blind to the identity of the samples.

2.7. Statistical analysis

The percentage of seminiferous tubule sections with PKH26-labeled cells, number of PKH26-labeled cells/seminiferous tubule section, number of PKH26-labeled cells/100 viable cells transplanted and number of viable cells transplanted were reported as mean $\pm$ SD. The number of mononuclear cells/high power field was compared between control and transplanted testes using a Student's *t*-test. The number of PKH26-labeled cells/500 seminiferous tubules and PKH26-labeled cells/100 cells transplanted were compared between groups in Experiment 2 using ANOVA. Where significant differences were present, paired comparisons were done using Fisher's test. The level of significance was set at 5%. Power of the analysis was 0.949. Data were expressed as mean $\pm$ SD.

3. Results

3.1. Equine testicular cells engraft in rat seminiferous tubules

Based upon visualization of fluorescently labeled cells, xenogeneic transplantation of equine testicular cells into the seminiferous tubules of busulfan-treated immunocompetent prepubertal rats resulted in engraftment in all recipient animals. Fluorescent cells were localized in histologic sections in the basal compartment of the seminiferous tubules by the basement membrane (Fig. 2A-C). Fluorescently labeled cells were present in $15.64 \pm 11.84\%$ of rat seminiferous tubule sections, and each seminiferous tubule section contained 1.52 ± 0.52 fluorescent cells. Viability of equine testicular cells was 95.9 ± 4.3 and $91.4 \pm 10.5\%$ before and after freezing/thawing, respectively. Within rat seminiferous tubules, 0.029 ± 0.045 equine testicular cells were identified per 100 viable cells transplanted. Equine testicular cells were frequently found in pairs and chains of cells. Donor cells also formed mesh structures and, more rarely, clusters (Fig. 2D-F). The number of cells in each structure varied between two and > 30 . Patches of fluorescent cells were also observed. No vertical expansion or spermatogenesis was evident and all structures had only one layer of cells. No fluorescent cells were present in the seminiferous tubules of testes injected with transplantation medium.

No evidence of acute cellular rejection was seen on histologic sections. There was no perivascular, vascular, interstitial or intratubular mononuclear inflammatory infiltration in any of the recipient testes. There was no significant difference in the number of mononuclear cells present in the interstitial compartment of recipient testes injected with transplantation medium (1.2 ± 0.5 cells/high power field) or equine testicular cells (1.4 ± 0.5 cells/high power field). All mononuclear cells were lymphocytes.

3.2. Engraftment is higher by equine testicular cells with high forward scatter and low side scatter

Unsorted cells and both sorted fractions contained donor cells with the ability to engraft, as evidenced by presence of fluorescent cells in the seminiferous tubules of all recipient rats. Conversely, no fluorescent cells were seen in testes of recipient rats injected with transplantation medium. Cells in Fractions I and II represented $0.34 \pm 0.21\%$ and $0.13 \pm 0.06\%$ of the initial cell population, respectively. The number of viable unsorted equine testicular cells, and viable cells from Fractions I and II transplanted per testis was 2 ± 2.69 , 0.09 ± 0.13 and $0.04 \pm 0.05 \times 10^6/\text{mL}$, respectively. More PKH26-labeled cells were present in 500 seminiferous tubule sections of recipient rats transplanted with unsorted cells (123.5 ± 121.9) than with cells from Fraction I (16.5 ± 17.14) but not Fraction II (39.2 ± 17.04) ($P < 0.05$). This may reflect the larger number of unsorted cells transplanted. However, when

the number of PKH26-labeled cells in rat testis was normalized to 100 viable equine testicular cells transplanted, cells from Fraction II ($0.169 \pm 0.099\%$) had higher engraftment efficiency than cells from Fraction I ($0.046 \pm 0.051\%$) or unsorted cells ($0.009 \pm 0.007\%$; $P < 0.05$). Testes transplanted with cells with high forward scatter and low side scatter (Fraction II), corresponding to the small, low complexity cell population, contained 19-fold more donor cells.

4. Discussion

In the present study, a transplantation assay for equine testicular cells was developed that used busulfan-treated immunocompetent prepubertal rats as recipients, and quantitative data on efficiency of colonization was presented. Equine PKH26-labeled cells were readily identified within rat seminiferous tubules 4 wk after transplantation in both histologic sections and whole mounts. Equine testicular cells showed a pattern of colonization in rat testes similar to rabbit, dog, pig, bull and horse testicular cells after transplantation into mouse testes [6,8]. Equine testicular cells were present in moderate to large numbers in pairs, chains, mesh structures and patches 4 wk after transplantation. Although rat seminiferous tubules provided an environment suitable for equine testicular cells to apparently undergo longitudinal expansion, spermatogenesis was not supported. It has been previously suggested that the extent of xenogeneic spermatogenesis decreased with increasing phylogenetic distance between donor and recipient [6,8] and rat seminiferous tubules did not provide an environmental advantage over mouse tubules for equine testicular cells to differentiate. Less likely, 4 wk may have been insufficient to detect donor-derived spermatogenesis. Colonization of mouse seminiferous tubules occurred in three phases: migration towards the basement membrane during the first week, proliferation of the SSCs along the basement membrane during the second week, and spermatogenesis beginning 1 mo post-transplantation [15]. The pattern of SSC colonization after transplantation into rat testes is not known. In a preliminary trial, equine testicular cells were identified as singles by the basement membrane of rat seminiferous tubules 2 wk post-transplantation, with no evidence of proliferation (data not shown). Because duration of spermatogenesis is 50% longer in rats (52 d) and horses (57 d) than in mice (35 d) [16], we inferred that the phases of colonization of rat seminiferous tubules may be delayed.

The previously described horse-in-mouse assay resulted in low efficiency of colonization [8]. Efficiency of colonization is influenced by the number of open niches available for engraftment in the recipient testis. Rat testes contain 120-fold more SSCs than mouse testes [4]. Because of the higher number of potential niches present in rat seminiferous tubules, it was speculated that rats may be better suited for xenogeneic transplantation of equine testicular cells than mice. This could not be proven, since the two species were not directly compared in the present study. Nevertheless, rat testes provided a suitable niche for equine testicular cells and a moderate to large number of donor cells were present within the recipient testes.

Efficiency of colonization is also influenced by the number of SSCs contained within the suspension of cells transplanted. Forward scatter and side scatter of incident light are indicators of cell size and shape or complexity, respectively. Therefore, light scattering properties allow evaluation and identification of distinct cell populations. Sorting equine testicular cells based on light scattering properties resulted in a 19-fold improvement in engraftment efficiency by cells with high forward scatter and low side scatter compared with unsorted testicular cells. Perhaps putative equine SSCs were represented primarily by this cell population. Rat and mouse SSCs had high forward scatter and low side scatter [9,10]. Although cell sorting based on light scattering properties did not improve colonization efficiency by mouse testicular cells [9], it resulted in a 3.7-fold enrichment of rat SSCs [10].

In xenogeneic transplantation, immunosuppression or use of immunodeficient recipients is required to avoid rejection of transplanted cells by the host's immune system [17]. The immunodeficient status of nude rats may not be as complete as in nude mice, since nude rats are not totally deprived of T cells [18]. Therefore, immunosuppression, with its undesirable side effects [6,8,17], is still recommended [13]. Use of immunocompetent recipients eliminates potential threats to animal welfare imposed by side effects of immunosuppression and the fragile nature of immunodeficient animals. Failure of experiments secondary to acquired infections is also less likely when using immunocompetent animals. Acute graft rejection manifests after the first week post-transplantation. The risk for acute graft rejection is highest during the first month. Both cellular and humoral rejection can occur. However, humoral rejection occurs in pre-sensitized patients and it is unlikely to have occurred in recipient rats here. Biopsy remains the gold standard for diagnosis of acute cellular rejection [14]. Although guidelines for interpretation and classification of acute cellular rejection are available in human patients undergoing organ transplantation, no guidelines are available to interpret rejection to transplanted testicular cells. Here, acute cellular rejection was defined based on presence and quantity of mononuclear inflammatory infiltration of the vascular, perivascular, interstitial and/or intratubular compartments. Equine testicular cells colonized the seminiferous tubules of immunocompetent rats without evidence of acute cellular rejection. In humans, the lowest incidence of acute rejection is seen in infants [19]. Rats used previously were 30 to 85 d old at the time of transplantation [17], and 18 to 22 d old here. Perhaps using younger animals decreased the likelihood of acute cellular rejection after xenogeneic transplantation. Although acute cellular rejection did not occur in recipient rats here, chronic cellular rejection cannot be ruled out, since it manifests after the first 1 to 6 mo post-transplantation.

Further work is needed to confirm the identity of engrafted donor cells based on expression of molecular and/or genetic markers, and the occurrence of chronic cellular rejection after xenogeneic transplantation using immunocompetent recipient rats. Nevertheless, we concluded that seminiferous tubules of busulfan-treated prepubertal immunocompetent rats provided a suitable niche for engraftment and proliferation of equine testicular cells. This was apparently the first report of xenogeneic transplantation of testicular cells derived from any domestic animal species into the seminiferous tubules of rats. The large number of donor cells engrafted in rat seminiferous tubules and labeling of donor cells with the fluorescent cell tracer PKH26 allowed easy identification of engrafted donor cells 4 wk after transplantation. Use of immunocompetent recipients eliminated the need for immunosuppression and its potentially undesirable side effects. Sorting equine testicular cells based on light scattering properties resulted in a 19-fold improvement in colonization efficiency by cells with high forward scatter and low side scatter, which may represent putative equine SSCs.

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Fig. 1.

Light scattering properties of equine testicular cells. Each dot represents the forward scatter and side scatter value of a single cell. Cells in Fractions I and II were separated from the total population and transplanted into seminiferous tubules of recipient rats. Fraction III was discarded.

Fig. 2.

PKH26-labeled equine testicular cell by the basement membrane (arrows) in a histologic section of a rat seminiferous tubule 4 wk after transplantation (A to C). Whole mount of rat seminiferous tubules with fluorescent equine testicular cells forming a mesh structure and chain of cells (arrow) (D to F). The images correspond to fluorescence (A, D), bright field (B, E) and composite of fluorescence and bright field microscopy (C, F). Please refer to the online version of the manuscript for color images.