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Transplantation of Goat Spermatogonial Stem Cells into the Mouse Rete Testis

Mohammad Sadra Shirazi[1], Banafsheh Heidari[1, *], Mohammad Mehdi Naderi[1], Bahareh Behzadi[1], Ali Sarvari[1], Sara Borjian-Boroujeni[1], Morteza Farab[1], Abolfazl Shirazi[1, 2]

[1]Reproductive Biotechnology Research Center, Avicenna Research Institute (ACECR), Tehran, Iran
[2]Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran

Abstract

Assisted reproductive techniques involving isolation, culture, and transplantation of spermatogonial stem cells offer unique approach to manipulate the male germline. The application of these techniques in farm animals has been the subject of an increasing number of studies, mostly because of its potential as an alternative strategy in producing transgenic livestock with higher efficiency and less time and capital requirement than the current methods. The aim of this study was to assess the colonization and differentiation potentials of enriched goat spermatogonia into the mouse testes. Moreover, because stem cells may need to be preserved for several years before re-introduction to the recipient testes, we developed the efficient cryopreservation technique for type A spermatogonoa. The enzymatically isolated SSCs obtained from one month old goats' testes were enriched by using discontinuous percoll density, and followed by cryopreservation protocol. After xenotransplantation of prepared goat testicular cells into the mouse rete testis, the proliferative activity and stemness potential of SSCs were evaluated and compared with in vitro culture condition. We demonstrated that the viability of testicular cells after cryopreservation was significantly lower than fresh cells, although these cells had normal structural and functional characteristics ($P < 0.001$). Donor goat spermatogonia were able to survive and colonize in depleted recipient's testis at 80 days after transplantation, but later stages of donor-derived spermatogenesis were not observed at this time. Although cross-species spermatogonial transplantation did not have the envisioned immediate practical application, it nonetheless provides a bioassay for stem cell potential of germ cells isolated from other species.

Keywords

Cryopreservation, Goat, Mouse, PGP9.5, Spermatogonial Stem Cells, Transplantation

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1. Introduction

Studies of spermatogenesis were long hampered because of a lack of powerful in vitro and in vivo assay systems until a method for the transplantation of germ cells (GCs) from one animal to another was established (Brinster and Zimmermann, 1994). Spermatogonial stem cell transplantation (SSCT), a procedure in which testis cells are harvested from a fertile male and microinjected into seminiferous tubules of an infertile recipient, offers unique approaches to explore basic biological aspects of male germ line stem cells, examine defects in spermatogenesis and treat male infertility (Dobrinski et al., 2006; Kim et al., 2008). This technique could enhance our understanding of developmental potential of Spermatogonial stem cell (SSCs) and increase the ability of male fertility preservation in both humane and animals. In cancer patients, GCs could be frozen prior to irradiation or chemotherapy treatment and subsequent re-introduction of

* Corresponding author
E-mail address: ban_heidari@yahoo.com (B. Heidari)

autologous GCs could restore fertility in the patient after recovery (Fujita et al., 2006). This technique also represents an efficient tool in valuable domestic and or endangered animals to maintain the genetic material from immature males that become sterile before they reach puberty (Kim et al., 2008). Another important application of SSCT is transgenesis through the male germ line using transplantation of transfected GCs. This approach has tremendous potential in species where embryonic stem cell technology is not available and or options to generate transgenic animals are inefficient. In addition, even if embryonic stem cell technology becomes available, the time required until transgenic sperm can be harvested will be significantly shorter using SSCT (Kim et al., 2008).

It was first reported in 1994, that transplantation of GCs from fertile donor mice to the testes of infertile recipient mice could result in donor derived spermatogenesis (Brinster and Zimmermann, 1994). Since the first report, isolated GCs from various donor including rat (Ogawa et al., 1997), hamster (Ogawa et al., 1999), rabbit (Dobrinski et al., 1999), dog (Kim et al., 2008), primate (Nagano et al., 2001), bull (Izadyar et al., 2002), cat (Kim et al., 2006), humane (Nagano et al., 2002), horse and pig (Dobrinski et al., 2000) have been transplanted into mouse testes. In rats and hamsters, donor derived SSCs were able to survive for long time, proliferate and differentiate into elongated spermatids and spermatozoa in the recipient mouse testes (Ogawa et al., 1997; 1999). Whereas, transplantation of GCs from non-rodent donors, as mentioned above, into the mice resulted in colonization or proliferation of SSCs into mouse testis but spermatogenesis became arrested at the stage of expansion (Dobrinski et al., 1999; 2000; Nagano et al., 2001; 2002; Izadyar et al., 2002; Kim et al., 2006). The success of SSCT requires the availability of stem cell niches in recipient testis and the enhancement of migration and proliferation potentials of donor cells (Dobrinski et al., 2006).The achievement of spermatogonial niches is depend on the relative size of the donor cells, host GCs population and the access of the transplanted cells to the basal lamina of the seminiferous tubules (Shinohara et al., 2002; Dobrinski et al., 2006). Several techniques have been developed to reduce the number of endogenous GCs in recipient testes, including irradiation (Kim et al., 2006), experimental cryptorchidism (shinohara et al., 2000), GnRH antagonist treatment (Schlatt et al., 1999) and sterilizing drug administration (Wang et al., 2010). Among the sterilizing drugs, busulfan, a DNA alkylating antineoplastic agent, was the most commonly used in rodents and administered by a single intraperitoneal (i.p.) injection. Busulfan can destroy primitive GCs and disrupt the junctions between Sertoli cells, thus promoting the immigration of transplanted spermatogonia into the basal lamina (Wang et al., 2010).

Transplantation of SSCs to clinical treatment of male infertility may require preservation of GCs before transplantation. Respecting to the lack of information on goat germ cell transplantation into rodent testes and the inadequate information regarding to the cryopreservation of goat SSCs, and also considering the application of transfected SSCs as an efficient tool in production of transgenic animals, the present study was aimed to study of the stemness, colonization and proliferation potentials of transplanted goat SSCs into the mouse testis and to investigate the developmental potential of cryopreserved SSCs to initiate spermatogenesis in vivo and in vitro.

2. Material and Methods

2.1. Ethics Statement

All experimental procedures were carried out with the recommendations in the guide for the care and use of animals by Avicenna Research Institute Animal Care and Use Committee.

2.2. Animals

Adult C57BL/6 mice (14-15 weeks of age), as recipient mice, were received a single i.p. injection of busulfan (30 mg kg^{-1}, Sigma, St. Louis, MO) to destroy endogenous germ cells and analyzed at 4, 6, 10, and 13 weeks after busulfan treatment by histological and immunohistochemical staining using an antibody against PGP9.5. Donor animal, one month old castrated goat was anaesthetized with Acepromazine (0.1 mg kg^{-1}, IM), followed 30 min later by Xylazine (0.22 mg kg^{-1}, IM) and then ketamine (10 mg kg^{-1}, IM).

2.3. Donor Cell Preparation

2.3.1. Cell Isolation

Following castration of a one month old goat, the testes were transported to the lab in transition media (PBS supplemented with 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin) at 37˚C. The tunica albuginea and visible connective tissues were aseptically removed. Single cell suspension was prepared using a protocol previously described (Heidari et al., 2014). Briefly, the testicular cells were isolated through two-step digestion method using collagenase type 1 (1 mg ml^{-1}) and trypsin $EDTA^{-1}$ (0.25% $1mM^{-1}$) for 60 and 25 min, respectively. The supernatant was processed by sequential filtration through 60 µm nylon mesh (Small part, F062N-08-C). After brief centrifugation, the pellet was resuspended in culture medium containing 10% FBS (Gibco) and 1% penicillin-streptomycin. In the final cell suspension, total cell number and viability were determined after Trypan Blue staining.

2.3.2. Enrichment of Donor Germ Cells

The goat SSCs were enriched from cell suspensions using a percoll gradient protocol as previously described (Heidari et al., 2014). Briefly, different percoll gradients of 20%, 28%, 30% and 32% were prepared and testicular suspension was slowly layered on the top of the above gradient, and then centrifuged at 800 x g for 30 min at 18ºC. After washing the collected cells, total cell number, viability rate, and different cell types were determined according to their morphological and immunocytochemical characteristics using PGP9.5 primary antibody.

2.3.3. Cryopreservation

Freshly collected cells from the gradients with the highest purity of undifferentiated type A spermatogonia were mixed with the freezing medium containing 10% dimethyl sulphoxide, 10% fetal calf serum and 0.07 mol l^{-1} sucrose. Cryovials containing 6×10^6 cells ml^{-1} were placed in an insulated (Nalgene Mr. Frosty freezing) container at -80 ºC for 1 day and then plunged into liquid nitrogen. The vials were thawed in 38ºC water for 60 s and centrifuged at 500 x g for 5 min. The viability and stemness potential of freeze-thaw cells was identified by Trypan Blue and immunocytochemical staining for detection of PGP9.5 positive cells.

2.3.4. Cell Culture and Proliferative Activity

Approximately 2-4 months after cryopreservation, the testicular cells were thawed and propagated at a concentration of 19×10^3 cells per cm^2 in 96-well cell chamber slides (Falcon, USA) at 38˚C, at humidified atmosphere with 5% CO_2 for two weeks and refreshed once a week. The basic culture system was consisted of high glucose DMEM (GibcoBRL) supplemented with FBS (10%), LIF (10 ng ml^{-1}), EGF (20 ng ml^{-1}), bFGF (10 ng ml^{-1}), GDNF (40 ng ml^{-1}) and penicillin-streptomycin (1%) (GibcoBRL). The different testicular cell types were identified by light and fluorescence microscopes for detection of PGP9.5 and vimentin positive cells using immunostaining techniques. The number and appearance of SSCs colonies were examined 7-14 days after culture initiation.

2.4. Transplantation Procedure

Immediately before transplantation, the testicular cells were thawed, resuspended in culture medium, and kept on ice until the transplantation. The prepared donor cells in a volume of approximately 50 µl of culture medium were transplanted into rete testis (450×10^3 cells $50\mu l^{-1}$ in each injection) using microinjection needle in one of the testes of each of the recipient mice; the other testis served as an internal control. Positive controls were mouse seminiferous tubules injected with donor goat testes cells one week before analysis. Negative controls were seminiferous tubules not receiving injection of donor testicular cells.

2.4.1. Assessment of Donor Spermatogonia Population in Recipient Testis

The testes of busulfan-injected mice were collected at 50 and 80 days after transplantation, fixed overnight at 4 °C in Bouin's solution, sectioned at 5 µm, and examined for histological and immunohistochemical staining. For histological evaluation, the sections were stained with HandE and examined under a light microscope for identification of cell types and their developmental stages.

2.4.2. Immunohistochemical and Immunocytochemical Staining

Undifferentiated spermatogonia were identified through immunohistochemical and immunocytochemical staining according to the protocol previously described (Heidari et al., 2014). In immunohistochemical staining, the prepared slides were incubated with unconjugated primary antibody including rabbit anti-PGP9.5 (Dako, Carpinteria, CA, USA) at 1:400 for 1 h at room temperature. After washing with TBS BSA^{-1}, the sections were exposed to secondary antibody (biotinylated sheep anti-rabbit IgG, Avicenna Research Institute, Iran) for 45 min. The sections were exposed to diluted HRP-conjugated streptavidin (Biosource, USA), 1:250 for 30 min and developed by the addition of 3, 3'-Diaminobenzidine (DAB; Roche, Germany) for 10 min. The slides were counterstained with hematoxylin for 30 s and mounted in Entellan (Merck, Germany).

For PGP9.5 immunocytochemical staining, approximately 4×10^4 cells were placed in each slide, cytospun at 400 rpm for 5 min, fixed in acetone at -70°C and then placed in 4°C for 90 min. After drying the slides, cells immunostained as described above except that the washing was done by Tween 20 (0.2% in PBS) and mounting with glycerol PBS^{-1}. The percentages of PGP9.5 positive and negative cells were evaluated by cell counting of prepared immunocytochemical slides. The Sertoli cells were identified through Vimentin immunocytochemical staining. For this purpose, two days after culture initiation, the monolayer was fixed in acetone and incubated with the primary antibody including anti-vimentin antibody (Abcam, Cambridge, UK) for 1 h and then the secondary antibody (Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG) for 45 min at room temperature. The nuclei were counterstained by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 µg ml^{-1}, Calbiochem, Nottingham, UK) for 20 min and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

2.5. Statistical Analysis

The statistical significance between the mean values was determined by one-way ANOVA analysis of variance (Tukey Test). A $P<0.05$ was defined as statistical significance.

3. Result

3.1. Effect of Busulfan on Morphological Characteristic of Testes

Injection of busulfan decreased the testis size and weight and caused enough space for cell injection. During the following 4 weeks (4-10 weeks), capsular thickness was significantly increased and the walls of the all seminiferous tubules became thinner because of the depletion of the spermatogenic cells (Table 1). The lowest diameter of seminiferous tubules were seen at 6 weeks after busulfan treatment (51.7±0.9 µm, $P<0.001$) (Table 1).

3.2. Effect of Busulfan on Male Germ Cell Development

In adult mouse testis, the seminiferous tubules showed a limited lumen with a thick wall consisting of several layers of epithelial cells. Histological and immunohistochemical examinations of the testes in busulfan-treated group showed that at 4 week after injection, more than 99% of the seminiferous tubules were spermatogenic with a little irregularity in the arrangement of germ cells (Fig. 1A, a). The PGP9.5 positive cells were found round with a spherical nucleus that arranged in singles or pairs at the base of the seminiferous epithelium (Fig. 1a). The optimal effect of busulfan on spermatogenic cells were seen at 6 week after treatment so that the seminiferous epithelium was composed of only a single, basal row of Sertoli cells (Fig. 1B, b). During the following 6 weeks (6-13 weeks after busulfan treatment) the seminiferous tubules were still devoid of spermatogonia and other germ cell lines (Fig. 1C-d).

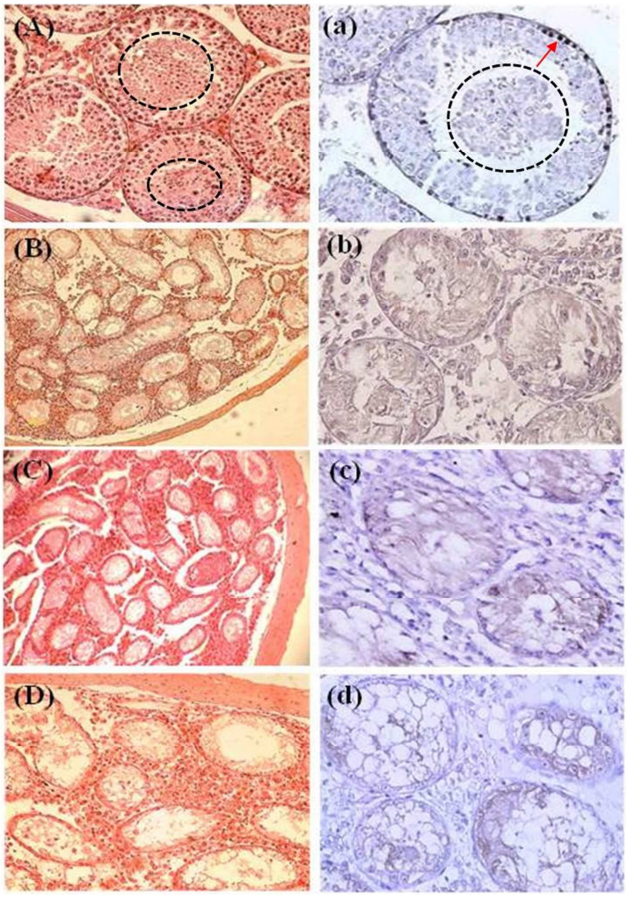

Fig. 1. Histological and immunohistochemical evaluations of mice testes after busulfan injection using an antibody against PGP9.5. The seminiferous epithelium had different layers of spermatogenic cells including type A spermatogonia (arrows, A, a) at 4 weeks after busulfan treatment. Note the shedding of cells in the lumen at this time (outlined, A, a). Busulfan treatment mice testes 6 (B, b), 10 (C, c), and 13 (D, d) weeks after injection. Note from 6 weeks all seminiferous tubules were devoid of spermatogenic cells and no indication of spermatogenesis was seen in recipient mouse. Scale bars = (B, C, D) 50µm, (A, b, c, d) 30µm, (a) 20µm.

Table 1. Changes in morphological characteristics of BALB/c mice with time after busulfan treatment.

Groups	Capsular Thickness (µm) mean ± SE	Seminiferous Tubules (µm) mean ± SE	Testicular Mass (mg) mean ± SE
Control	17.1±0.6a	130.7±1.5a	86.3±2.1a
4 weeks	18.6±0.44a	114.7±1.8b	78.3±0.9b
6 weeks	23.9±0.7b	51.7±0.9c	33.3±1.2c
10 weeks	47.25±1.2c	60±2.1d	36.3±1.4c
13 weeks	40.3±0.8d	65.7±1.7d	37.3±0.3c

[a-d] Numbers with different lower case superscript letters in the same column differ significantly ($P<0.001$).

3.3. Assessment of Undifferentiated Type a Spermatogonia Population

The highest and lowest number of collected cells, including somatic and stem cells, was achieved at 28% and 30% density, respectively (1147880 cells ml^{-1} vs. 121660 cells ml^{-1}). Concurrent with the increment of percoll gradient, the number of undifferentiated type A spermatogonia was increased ($P<0.001$, Fig. 2). Though, The maximum number (129×10^4 cells ml^{-1}) and percentage (94.6%±0.4) of undifferentiated type A SSCs was obtained at 32% percoll density ($P<0.001$, Fig. 2).

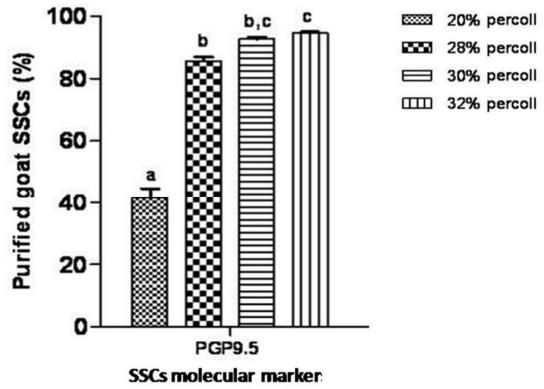

Fig. 2. Goat SSCs purification using different percoll gradients, confirmed by PGP9.5 molecular marker.

a-c columns with different lowercase letters differ significantly (P<0.001).

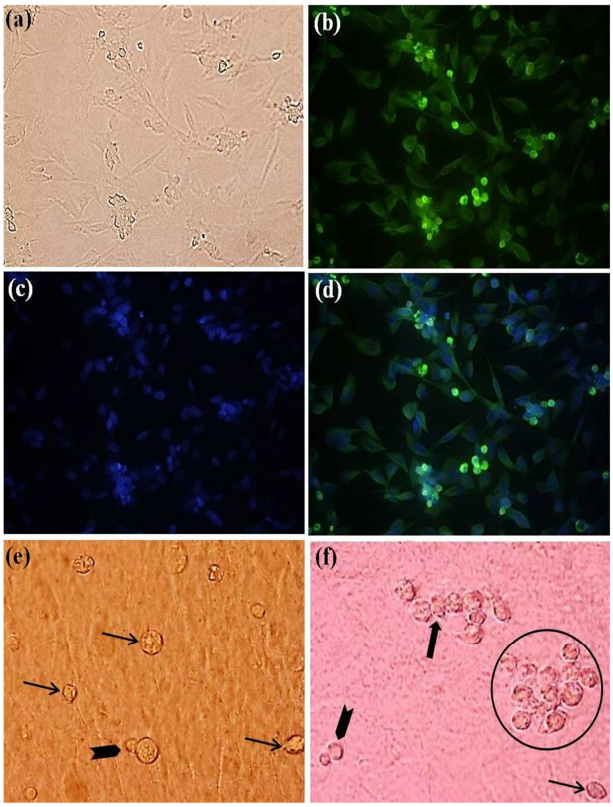

Fig. 3. Cytological and immunocytochemical evaluations of goat testicular cells after 2 days culture using an antibody against vimentin (a-d). The somatic cells including Sertoli cells constitute a feeder layer (a) that was vimentin positive (b-d). Typical development of SSCs colonies after 1 week (e) and 2 weeks (f) cultures. Many small colonies were formed mostly single (arrows, e) or paired (arrowhead, e) on top of the monolayer. After culturing for 2 weeks, some align chains with higher than 6-connected colonies (block arrow, f) and cluster form (encircle, f) were detected. Scale bars = (a-d) 30µm and (e, f) 45µm.

3.4. Qualitative and Quantitative Colonization and Stemness Capacity Following Cryopreservation

The viability rate of collected goat testicular cells after enzymatic digestion and purification was almost identical (>95%). After cryopreservation, viability of testicular suspension was significantly lower than fresh cells (58% ± 4.4% vs. 96.6% ± 2.4%, p <0.001). The proportion of PGP9.5 positive cells derived from fresh cells was not different from those obtained using cryopreserved cells at the first beginning and 2 weeks after culture initiation (P<0.001).

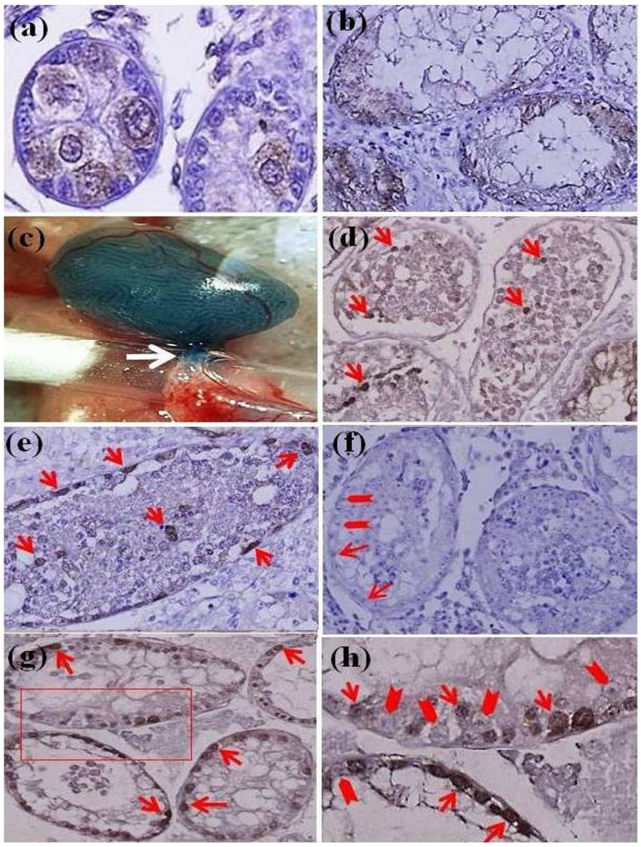

Fig. 4. Histological and immunohistochemical identification of goat spermatogonia in one month lamb (a) and mouse (b-h) seminiferous tubules using an antibody against PGP9.5. Injection of goat testicular suspension into the mouse rete testis immediately (c) and one week after injection (d). Busulfan-injected testis 50 (e, f) and 80 (g, h) days after transplantation. Note the accumulation of testicular cells including the undifferentiated type A spermatogonia in the lumen and at the base membrane after 50 days (arrows, e, f). Note the maintenance and colonization of goat undifferentiated spermatogonia (arrows, g, h) in proximity of Sertoli cells (arrowheads, g, h) at the base membrane of mouse seminiferous epithelium. Positive (d) and negative (b) controls were demonstrated. Scale bars = (a, h) 20µm, (b-g) 30µm.

3.5. Evaluation of Goat SSCs Colonization in Culture Condition

During the process cultivation, the somatic cells with the predominance of Sertoli cells were completely stuck to the culture plate within 12 h. After two days, these cells were constituted a monolayer with 80% confluency as a feeder layer (Fig. 3a). Immunocytochemical evaluation using an antibody against vimentin confirmed these cells (Fig. 3b-d). With increasing the duration of culture to one week, the cells (somatic and germ cells) and colonies number were increased

(Fig. 4e). Two weeks after cultivation, different forms of colonies (mostly single, sometimes paired, multi-cell chains and or small cluster) were determined (Fig. 3f).

3.6. Evaluation of Goat SSCs Colonization in Recipient Testes

We demonstrated that the mouse seminiferous tubules were completely devoid of spermatogonia and other germ cell lines 6 weeks after busulfan treatment (Fig. 1B, b and 4b). The accumulation of goat testicular cells including the undifferentiated type A spermatogonia were demonstrated 50 days after transplantation (Fig. 4e, f). Donor spermatogonia, PGP9.5 positive round cells with a spherical big nucleus, were able to survive and colonize in depleted recipient's testis after 80 days but later stages of donor-derived spermatogenesis were not observed at this time (Fig. 4g, h). Positive control mouse testes, those injected with goat testes cells one week after transplantation, showed an abundance of cells including the positively stained SSCs (Fig. 4d). Negative control mouse testes, receiving no injected cells, showed no significant cellular staining from the immunohistochemical procedure (Fig. 4b).

4. Discussion

The technique of SSCT was first reported by Brinster and Zimmermann in 1994. Although technically still at an experimental level, this technique provides a bioassay to study the genes and factors involved in regulation of spermatogenesis and to examine defects in male infertility (Fujita et al., 2006, Honaramooz et al., 2011).

In present study, the viability rate of collected goat testicular cells, >95%, was notably more than what reported in other species such as prepubertal boar SSCs; 45% (Dirami et al., 1999), prepubertal mouse germ cells; 30% (Sugiyama et al., 2001), pubescent mice spermatogonia; 68% (Creemers 2002), goat spermatogonial stem cells, 65%-70% (Kaul et al., 2012) and bovine SSCs; >80% (Izadyar et al., 2002).The higher percentage of viability rate in our study might be related to the type or concentration of used digestive enzymes, applied protocol, as well as the age and species of animals. We demonstrated a decrease in cell survival rate after thawing (58% ± 4.4%) which was consistent with observations in bovine (50–70%) and porcine (55–88%) SSCs (Izadyar et al., 2002; Abrishami et al., 2010). In our study, the proportion of undifferentiated type A spermatogonia (PGP9.5 positive cells) obtained after cryopreservation was not different from those obtained using fresh cells. It is reasonable to speculate that a large population of the undifferentiated type A spermatognia survived in the cryopreservation procedure and retained normal structural and functional attributes after cryogenic storage. Our finding was consistent with the previous results indicating that these cells are the last cell type to be destroyed after irradiation or chemical insult including cryoprotectants, and are able to regenerate via self-renewing division to complete spermatogenesis (Izadyar et al., 2002; Kanatsu-Shinohara et al., 2003).

It is well documented that the efficiency of SSCT is low and only 1% (Jiang et al., 2001) or 7–20% (Nagano et al., 1999) of the transplanted SSCs will actually colonize the recipient testis. Naturally, increasing the number of SSCs in donor cell population, depletion of endogenous germ cells by cytoablative methods and more access to the stem cell niche in the recipient testis can significantly improve the migration and colonization of donor SSCs in recipients (Shinohara et al., 2002). Therefore, we used the percoll density method for increasing the number of donor SSCs and achieved the maximum type A spermatogonia purification (94.6%±0.4) by using 32% gradient that was slightly higher than those reported in other species including: rat with 80 ± 6.1% SSCs purification using 30%-32% gradient (van Pelt et al., 1996), pig with 83.62±4.24% SSCs purification using 30-45% gradient (Marret et al., 2000), and humane with 86.7% SSCs using 27-35% gradient (Liu et al., 2011). I addition to increasing the transplantation efficiency, we depleted the endogenous germ cells by busulfan and showed that this sterilizing drug can create sufficient depleted niches which in turn could maintain the microenvironment for better colonization of donor spermatogonia for longer time periods. The histological and immunohistochemical examinations showed that at 6 weeks after injection, all seminiferous tubules became non-spermatogenic. This was consistent with the results of other researchers that demonstrated the endogenous germ cells were removed from the lumen of recipient seminiferous tubules 4 weeks after busulfan treatment and transplantation was usually performed 6 weeks after injection (Brinster and Zimmermann 1994; Nagano et al., 1999; Wang et al., 2010). Considering the exclusive properties of SSCs as the only testicular cells with colonizing and expanding capability following transplantation, we demonstrated the migration and colonization of undifferentiated type A spermatogonia at 80 days after transplantation through immunostaining for PGP9.5. Despite, the successful colonization of goats SSCs, but these cells did not produce more advanced germ cells in the recipient mouse testes. This is probably due to the goat SSCs most likely does not receive the proper signaling for this conversion within recipient mouse seminiferous tubules. These results were consistent with those obtained by other researchers as such xenotransplantation of germ cells from non-rodent donors ranging from rabbits, dogs, pigs, cattle, boar, horses, non-

human primates, and humans resulted in colonization and expansion of donor SSCs in mouse testis but not in their full differentiation presumably (Dobrinski et al., 1999; 2000; Nagano et al., 2001; 2002). This arrested at the first stage of spermatogonial development may be related to the phylogenetic distance between the donor and the recipient species and incompatibility of the recipient somatic cell compartment with donor germ cells (Kanatsu-Shinohara et al., 2003, Honaramooz et al., 2011; Tang et al., 2012). Although, SSCT from non-rodent species into the mouse testis (cross-species) did not result in complete spermatogenesis, it nonetheless provides the only available bioassay for detecting the migration and colonization potential of SSCs in a given population of donor testis cells from any species (Dobrinski et al., 1999; 2000; Izadyar et al., 2002; Honaramooz et al., 2011).

5. Conclusion

We developed an appropriate protocol for the cryopreservation of purified goat type A spermatogonia and demonstrated that goat SSCs are able to recover with full functionalcapability after freezing and thawing procedures. After transplantation, the undifferentiated goat spermatogonia were found to remain functional and able to colonize recipient mouse testes. Although, the mouse testis was capable of supporting the initial steps of goat spermatogonial colonization, but the complete spermatogenesis could no longer be achieved in the mouse.

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