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ScienceDirect
Theriogenology 66 (2006) 2091–2103
Theriogenology
www.journals.elsevierhealth.com/periodicals/the

Isolation and transplantation of spermatogonia in sheep

Jose Rafael Rodriguez-Sosa [a], Howard Dobson [b], Ann Hahnel [a,*]

[a] Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1
[b] Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received 7 March 2006; accepted 25 March 2006

Abstract

Studies in rodents show that spermatogonial transplantation is an excellent new tool for studying spermatogenesis and for preservation and dissemination of genetics. The aim of this study was to adapt the technique to rams. Two issues were addressed: purification of stem cell spermatogonia, and efficient injection of donor spermatogonia into the seminiferous tubules of rams. We compared differential plating and Percoll gradient methods for purifying donor spermatogonia from ram lamb testes. Spermatogonia were identified with an antibody against PGP 9.5, a ubiquitin C-terminal hydrolase. Both purity and total number of spermatogonia recovered were higher after purification by Percoll gradient than by differential plating. Four approaches for injecting cells into the seminiferous tubules of ram testes were compared ex vivo: insertion of a needle into the extra-testicular rete testis after reflection of the head of the epididymis ('surgical' approach), and ultrasound-guided insertion of a needle into the extra-testicular rete, and the proximal and distal parts of the intra-testicular rete testis. 'Surgical' and ultrasound-guided approaches into the extra-testicular rete resulted in highest success rates and best filling of the seminiferous tubules. Finally, the ultrasound guided approach into the extra-testicular rete testis was validated in vivo by transplanting purified spermatogonia previously labeled with a fluorescent molecule (CFDA-SE). In seven of eight testes injected, donor cells were identified within the seminiferous epithelium for up to 2 wk after transplantation, indicating the integration of donor cells.
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Keywords: Spermatogonial transplantation; Spermatogonia; Spermatogenesis; Sheep

1. Introduction

Experiments using spermatogonial transplantation in rodents have shown the technique to be an important new tool for studying spermatogenesis in mammals and have opened the possibility of using spermatogonial stem cells in domestic animals for preservation of fertility and dissemination of genetics in domestic animal production [1,2]. Research to translate this technique to other mammals is in its initial stages. The purpose of this study is to adapt spermatogonial transplantation to the anatomy of sheep. The sheep industry would particularly benefit from an additional means to transport and disseminate genetics because of the difficulties of freezing sperm and in performing insemination and embryo transfer [3]. Also, there is a ready supply of ram lamb testes from the abattoir for ex vivo studies. The challenges include developing methods to purify donor stem cell spermatogonia, establishing efficient routes for injecting donor spermatogonia into the seminiferous tubules of recipient testes, developing strategies to reduce competition by endogenous spermatogonia, and finding ways to detect donor spermatogonia after transplantation.

The amount of spermatogenesis from donor cells depends on the number of stem cell spermatogonia

* Corresponding author. Tel.: +1 519 824 4120x58399; fax: +1 519 767 1450.
E-mail address: ahahnel@ovc.uoguelph.ca (A. Hahnel).

0093-691X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2006.03.039

among the transplanted cells [4], and is functionally defined in rodents by number of donor colonies. Up to 166-fold purification of rodent stem cell spermatogonia has been reported by antibody mediated cell sorting [5–8]. Molecular markers of 'undifferentiated' spermatogonia in rodents include α6β1 integrin, Thy-1, CD9, Ep-CAM, Notch-1, and GDNFR [4–10]. However, there is no information in the literature that any of these antigens can be used for isolation of spermatogonia in other species. Purification of spermatogonia prior to transplantation in farm animals has depended on techniques such as Percoll or BSA gradients or differential adhesion, and assessment of purity of spermatogonial fractions has been based on morphological criteria in pig [11] and binding of Dolichos biflorus agglutinin (DBA) in cattle [12].

In mice, donor spermatogonia have been transplanted by multiple injections into the seminiferous tubules, or by insertion into the rete testis, either directly or via an efferent duct [13]. Anatomical differences hamper the use of these approaches in other species. Mice have a thin tunica albuginea and unusually large seminiferous tubules, the rete testis is laterally placed, and the efferent ducts run from the testis through a fat pad to the caput epididymis. In sheep, as in most farm species, the head of the epididymis is tightly adherent to the tunica albuginea; the efferent ducts are within the head of the epididymis and connected to a central intra-testicular rete testis by the short extra-testicular rete testis [14]. In previous ex vivo and in vivo studies in cattle, pig, goat, monkey and human, both multiple injections into the seminiferous tubules and the efferent duct approach were found to be impractical [15–19]. However, guiding a needle into the intra-testicular rete using ultrasound was successful in 75–100% of testes, although only a maximum of 35–50% of the seminiferous tubules were filled [17]. Interestingly, Ogawa et al. [13] described the best site for direct injection into the rete testis of mouse to be into a small area of the rete cranial to the vascular pedicle from which the efferent ducts arise. This site is comparable to the extra-testicular rete testis described in larger animals [14]. The extra-testicular rete testis has been cannulated to obtain fluid from the rete in live rams [20,21], and a detailed description of the procedure was published by Brown et al. [22] in the goat.

In rodents, competition by endogenous spermatogonia has been decreased by depleting recipients of spermatogonia with busulfan or with irradiation prior to transplantation, or by transplanting cells into juveniles [13,23,24]. While all of these techniques have been tried in farm animals, irradiation or transfer into juveniles seem to be better techniques for the larger domestic species.

A final challenge for translation of the rodent technique to sheep is to be able to follow colonization of the donor cells in the absence of genetic markers. In pigs [17] and goats [18], donor cells were labeled with the fluorescent lipophilic dye PKH26. The donor cells from the goats were from a transgenic animal, and successful colonization was also evidenced by the presence of donor sperm and generation of donor offspring [25]. In the one bovine study, recipients were depleted of endogenous spermatogonia by irradiation, and an increase over controls in number of tubules with spermatogenesis was used to estimate the amount of colonization by donor spermatogonia [19].

The first objective of this study was to identify a molecular marker of ram spermatogonia and to compare the techniques of differential plating and discontinuous Percoll gradient for purifying ram spermatogonia. The second objective was to compare ex vivo four routes for injecting spermatogonia into the seminiferous tubules of ram lambs at various stages of development. Finally, we tested in vivo the ultrasound-guided approach that we had developed for injection into the extra-testicular rete for transplanting spermatogonia into ram testes.

2. Materials and methods

2.1. Sheep testes and rams

Sheep testes were obtained over a period of 1.5 yr from a local abattoir and transported to the laboratory on ice. Testicular volume (TV) was determined according to the formula described by Steger and Wrobel [26]: TV = $1/6\pi a^2 bk$ (cm^3), where: a = width, b = length and $k = 0.945$. This technique was chosen since it is applicable to live sheep. However, volume determined as above and by water displacement were compared for 20 testes; the average difference between the two volume determinations for each testis was less than 2% and this difference was not significant ($P > 0.87$). Six testes (47.5–51.3 cm^3) were used for purification of spermatogonia and for immunohistochemistry, another six testes were used to correlate the location of cells with the appearance of dye in successful and unsuccessful injections, and a total of 126 testes were used to compare ways to inject cells into testes of various sizes. Five 20 wk-old rams (Rideau-Arcott/Dorset mix from the Ontario Ministry of Agriculture and Food research farm) with testicular volume >150 cm^3 were the recipients in the in vivo transplantation study.

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