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Effects of different storage protocols on cat testis tissue potential for xenografting and recovery of spermatogenesis

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Abstract

The loss of genetic diversity due to premature death of valuable individuals is a significant problem in animal conservation programs, including endangered felids. Testis tissue xenografting has emerged as a system to obtain spermatozoa from dead immature animals, however protocols to store this tissue before xenografting are still lacking. This study focused on testis tissue cryopreservation and storage from the domestic cat (*Felis catus*) classified as "pre-pubertal" and "pubertal" according to spermatogenesis development. Grafts from testis tissue cryopreserved with DMSO 1.4M, recovered after 10 weeks xenografting, presented seminiferous tubules with no germ cells. On the contrary, testis tissue from pre-pubertal animals preserved in ice-cold medium for 2 to 5 days presented no loss of viability or spermatogenic potential, while the number of grafts of pubertal cat testis tissue with germ cells after 10 weeks of xenografting decreased with increasing storage time. Nevertheless, even grafts from pre-pubertal cat testis tissue presented lower anti-DDX4 and anti-BOULE staining (proteins necessary for the meiosis completion), when compared with adult cat testis. Finally, a strong correlation found between testis weight and xenograft outcome may help choose good candidates for xenografting.

Keywords: Xenografting; Cat; Testis; Cryopreservation; Ice storage

1. Introduction

Every day young and pubertal endangered felids die in zoos and reserves. Since it is impossible to obtain sperm from the testes of these animals, their genetic pool is lost. Testis tissue xenografting provides a tool to obtain spermatozoa from immature animals, promoting spermatogenesis by keeping the microenvironment of the donor testis intact, as evaluated by comparing gene expression in development-matched grafted and donor tissue from pigs [1]. Xenografting also presents other major benefits such as the possibility of studying spermatogenesis and testicular maturation in species that are legally restricted [2,3] or in species that are difficult to manipulate due to their wildness, habitat or body constitution [4]. The production of viable and fertilizing sperm in testis xenografts from several mammals was first described by Honaramooz and colleagues [5]. Schlatt et al. extended the significance of the technique after obtaining live progeny from sperm extracted from

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grafts of immature mouse testes [6]. Snedaker and co-workers confirmed the applicability of xenografting in the domestic cat, after demonstrating the development of spermatogenesis and collection of sperm from grafts of immature testes [7]. Subsequently, cat testis tissue from immature (1 to 8 weeks), pre-pubertal (9 to 16 weeks), pubertal (5 to 7 mo), and adult animals was xenografted in the back of nude mice. After 50 weeks of xenografting, the authors observed an abrupt decrease in xenograft success when tissue was recovered from pubertal animals, while all grafts from adult animals had degenerated, thus establishing a clear age effect [8].

In both studies mentioned only fresh tissue was used [7,8]. One of the challenges of xenografting testis tissue is preserving the tissue to be used when and where necessary. There are many reports on cryopreservation of testis tissue, in humans [9,10], macaques [11], pigs [12], and rodents [13]. However, when critically assessing the literature it seems clear that there is not a universal cryoprotectant or cryopreservation protocol that can be used in all species. Furthermore, cryopreservation has variable effects in xenograft outcome, ranging from no obvious adverse effect in rodents [13] to a reduction of xenograft survival, increase in the number of seminiferous tubules with only Sertoli cells and reduced number of spermatogonia [11]. Another method to preserve testis tissue consists of cooling it to approximately 4 °C. Although it has some drawbacks when compared to cryopreservation, mainly reduced time to ship and xenograft the tissue, this methodology also has advantages, since it is a low cost low technology procedure and, as shown by Abrishami and authors using testis pig tissue, developmental competence of tissue cooled for 24, 48, or 72 is comparable or higher than that of fresh testis tissue [12].

We have therefore focused on tissue collection, cryopreservation and storage on ice-cold medium of testis tissue of domestic cat (*Felis catus*) in an attempt to optimize conditions for posterior application of this technique to endangered felids.

2. Materials and methods

2.1. Animals

Cat testes (aged 4 to 7 mo) were kindly provided by veterinarian clinics. The cats were castrated under general anesthesia and tissue was kept in ice-cold Leybovitz medium (L-15 Leibovitz; Mediatech Inc., Herndon, VA) supplemented with 5% fetal bovine serum (FBS), 1% nonessential aminoacids (NEAA) and 1% antibiotics (penicillin/streptomycin) until further manipulation.

Male immunodeficient nude mice (strain Nu/Nu *Foxn1nu*, aged 5–7 weeks) were obtained from Charles River Laboratories (Wilmington, MA) or from Janvier Europe (Le Genese St. Isle, France). Mice were maintained at the rodent housing facilities of the University of Pittsburgh School of Medicine and the Magee-Womens Research Institute under 12L:12D, with pellet food and water available ad libitum and at the central animal facilities of the School of Medicine of the University of Münster, Germany, under the same light conditions. Animal husbandry and all experimental procedures involving the animals were performed in compliance with the University of Pittsburgh and the Magee-Womens Research Institute Guidelines for the Care and Use of Laboratory Animals and in accord with the German Federal Law on the Care and Use of Laboratory Animals (license G24/2006).

2.2. Grafting experiments

Since use of age as a predictor of spermatogenesis development may result in a rather rough estimate (given that hierarchy, nutrition, housing and other factors may affect spermatogenesis development), domestic cat testis tissue used was classified as ranging from pre-pubertal to pubertal, according to spermatogenesis development (defined by histology) and by testis weight. Pre-pubertal cats were defined as having no meiotic germ cells and testes weight below 0.6g (n = 3) and pubertal animals presented higher or lower percentage of seminiferous tubules with meiotic germ cells and testes weight above 0.6g (n = 6).

Testes were removed from the L-15 medium used in transportation and dissected to remove the epididymides and a piece of testis tissue which was fixed in Bouin's solution. The remaining testis tissue was minced until $1mm^3$ pieces were obtained. This tissue was kept in ice-cold L-15 (≈ 4 °C) supplemented with 5% FBS, 1% NEAA and 1% antibiotics (penicillin/streptomycin) until the xenografting procedure (2 or 5 d) or cryopreserved in 1.4M DMSO. Briefly, the cryoprotective agent was added drop by drop, under constant manual agitation, to L-15 medium (supplemented with 10% FBS, 1% NEAA and 1% antibiotics) containing the tissue samples. The final concentration was reached after approximately 15 min. Tissue samples were then incubated on ice for an additional 15 min and shaken periodically to allow for complete cryoprotectant diffusion into the tissue samples. Approximately ten grafts were then transferred to each cryovial (Nunc)