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Germ cell survival and differentiation after xenotransplantation of testis tissue from three endangered species: Iberian lynx (*Lynx pardinus*), Cuvier's gazelle (*Gazella cuvieri*) and Mohor gazelle (*G. dama mhorr*)

Lucía Arregui[A,C], **Ina Dobrinski**[B], and **Eduardo R. S. Roldan**[A,D]

[A]Reproductive Ecology and Biology Group, Museo Nacional de Ciencias Naturales (CSIC), C/ José Gutierrez Abascal 2, 28006 Madrid, Spain

[B]Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary AB T2N 4N1, Canada

Abstract

The use of assisted reproductive techniques for endangered species is a major goal for conservation. One of these techniques, testis tissue xenografting, allows for the development of spermatozoa from animals that die before reaching sexual maturity. To assess the potential use of this technique with endangered species, testis tissue from six Iberian lynxes (one fetus, two perinatal cubs, two 6-month-old and one 2-year-old lynx), two Cuvier's gazelle fetuses and one 8-month-old Mohor gazelle were transplanted ectopically into nude mice. Tissue from the lynx fetus, perinatal cubs and 2-year-old donors degenerated, whereas spermatogonia were present in 15% of seminiferous tubules more than 70 weeks after grafting in transplanted testis tissue from 6-month-old donors. Seminal vesicle weights (indicative of testosterone production) increased over time in mice transplanted with tissue from 6-month-old lynxes. Progression of spermatogenesis was observed in xenografts from gazelles and was donor age dependent. Tissue from Cuvier's gazelle fetuses contained spermatocytes 40 weeks after grafting. Finally, round spermatids were found 28 weeks after transplantation in grafts from the 8-month-old Mohor gazelle. This is the first time that xenotransplantation of testicular tissue has been performed with an endangered felid and the first successful xenotransplantation in an endangered species. Our results open important options for the preservation of biological diversity.

Additional keywords

conservation; testicular tissue; threatened species; xenografting

Introduction

The development of assisted reproductive techniques plays an important role in the conservation and management of threatened species because they could benefit free and

[D]Corresponding author. roldane@mncn.csic.es.

[C]Present address: Department of Biology, Universidad Autónoma de Madrid, C/Darwin 2, 28049 Madrid, Spain.

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captive populations of highly endangered taxa. Assisted reproductive techniques aid in the rescue of reproductive cells and, thus, allow for the conservation of genetic resources. The most commonly used assisted reproductive technique in males is the collection and cryopreservation of spermatozoa. Spermatozoa can be recovered from live or recently deceased adult males (Garde *et al.* 1998, 2003; Martinez-Pastor *et al.* 2005; Gañán *et al.* 2009*a*, 2010), and offspring of threatened felids and ungulates have been born after intrauterine insemination of females with frozen–thawed spermatozoa (Densmore *et al.* 1987; Holt *et al.* 1988; Garland 1989; Junior *et al.* 1990; Swanson *et al.* 1996; Johnston *et al.* 2002; Garde *et al.* 2006).

In contrast, spermatozoa cannot be collected from immature males and the death of these males represents the loss of their genetic resource forever. Relevant progress has been achieved in *in vitro* spermatogenesis, with the entire spermatogenic cycle from spermatogonia to spermatozoa obtained in a three-dimensional culture system (Stukenborg *et al.* 2009) and offspring obtained after culturing of immature mouse testis (Sato *et al.* 2011). Alternatively, somatic cells could be used for somatic cell nuclear transfer to clone a dead individual when host oocytes from related species are available (Lanza *et al.* 2000; Gómez *et al.* 2004). However, abnormal gene expression and epigenetic deregulation arise during cloning (Loi *et al.* 2007; Gómez *et al.* 2009), further conspiring against the success of the procedure.

Testicular tissue xenografting could provide an opportunity to rescue the genetic information of a juvenile male from an endangered species (Paris and Schlatt 2007). Testis tissue xenografting involves the transplantation of small pieces of immature testicular tissue subcutaneously to immunocompromised mice, as an *in vivo* culture system, to subsequently (after weeks or months) isolate spermatozoa from these tissue fragments, fertilise oocytes by intracytoplasmic sperm injection and transfer embryos into a female recipient (Honaramooz *et al.* 2002; Nakai *et al.* 2010). Xenografting of young testicular tissue has been used successfully to sustain complete spermatogenesis in several domestic animals, namely goat (Honaramooz *et al.* 2002), pig (Honaramooz *et al.* 2002), rabbit (Shinohara *et al.* 2002), bull (Oatley *et al.* 2004; Rathi *et al.* 2005), cat (Snedaker *et al.* 2004; Kim *et al.* 2007; Mota *et al.* 2012), horse (Rathi *et al.* 2006), sheep (Zeng *et al.* 2006; Arregui *et al.* 2008*a*), dog (Abrishami *et al.* 2010*a*) and bison (Abbasi and Honaramooz 2011), as well as in other non-domestic species, such as hamster (Schlatt *et al.* 2002), rhesus monkey (Honaramooz *et al.* 2004), ferret (Gourdon and Travis 2011), white-tailed deer (Abbasi and Honaramooz 2012) and humans (Wyns *et al.* 2008). Several of these species, namely cats, dogs, sheep, deer, bison and ferrets, have been proposed as model animals for endangered felids, canids, ungulates and small carnivores (Snedaker *et al.* 2004; Arregui *et al.* 2008*a*; Abrishami *et al.* 2010*a*; Abbasi and Honaramooz 2011, 2012; Gourdon and Travis 2011). However, thus far there is only one short report on xenografting of testis tissue from an endangered species: testis from the Javan banteng (*Bos javanicus*) were xenotransplanted, but complete spermatogenesis was not achieved (Honaramooz *et al.* 2005).

The world populations of Iberian lynx (*Lynx pardinus*), Cuvier's gazelle (*Gazella cuvieri*) and Mohor gazelle (*Gazella dama mhorr*) have been drastically reduced in recent decades and are still decreasing. The Iberian lynx is the most endangered felid in the world,

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categorised as 'critically endangered' by the International Union for Conservation of Nature (IUCN) since 2002 (IUCN 2012). It is an endemic species of the Iberian peninsula and the current total population has been estimated to be approximately 200 individuals scattered in several isolated subpopulations in the south of Spain (Guzmán *et al.* 2004; Alda *et al.* 2008; Sarmento *et al.* 2009). Only two populations reproduce regularly (Guzmán *et al.* 2004; Von Arx and Breitenmoser-Wursten 2008). Cuvier's gazelle has been regarded as 'endangered' since 1986 (IUCN 2012). It is an endemic species of the Atlas Mountains and has 1700–3000 individuals in fragmented populations in Morocco, Algeria and Tunisia, but none of them has more than 250 mature individuals (Mallon and Cuzin 2008). The current population trend is unknown. Dama gazelle (*Gazella* (=*Nanger*) *dama*) has been considered to be 'critically endangered' since 2006 (IUCN 2012), with very small and fragmented subpopulations and less than 500 individuals in the current wild population (Newby *et al.* 2008). The Mohor gazelle (*G. dama mhorr*) is considered to be extinct in the wild (Beudels *et al.* 2005).

Captive breeding programs have been established in Spain for these three species starting in 2004 for the Iberian lynx and between 1971 and 1975 for the gazelles. Thanks to the existence of these captive breeding programs, studies have been performed for the characterisation of sperm traits (Cassinello *et al.* 1998; Gañán *et al.* 2010), electrostimulation for sperm recovery (Cassinello *et al.* 1998; Garde *et al.* 2003; Gañán *et al.* 2009*b*) and sperm cryopreservation (Garde *et al.* 2003, 2008; Gañán *et al.* 2009*b*).

The premature death of young individuals is a significant problem in the conservation of these species because survival of lynx cubs and gazelle calves during the first months after birth is low. Average litter size in wild Iberian lynx is three cubs; after 3 months, 75% of cubs survive and less than 60% are alive 2 years after birth (Palomares *et al.* 2005). Cuvier's and Mohor gazelle calf mortality in captive populations is close to 50% in the former and 30% in the latter during the first months of life (Abaigar and Cano 2005; Barbosa and Espeso 2005). The development of a technique to recover the germ cells of these individuals will be an important tool to maintain their alleles in the population genetic pool.

Therefore, the aim of the present study was to test whether testis tissue xenografting could be an option to develop spermatozoa from juvenile Iberian lynx, Cuvier's and Mohor gazelles. The effect of donor age and freezing on testicular survival after grafting was also assessed.

Materials and methods

Lynx testes

Iberian lynx testes were obtained from necropsies at the Centro de Análisis y Diagnóstico de la Fauna Silvestre of the Junta de Andalucía (Seville, Spain) and sent to the laboratory at 5–10°C (Table 1). Donor tissue for xenografting was used from animals of different ages: one 6-week-old fetus, two perinatal cubs (1.5 and 3 days old), two 6-month-old cubs, and one 2-year-old sub-adult male. Testicular tissue from all specimens was grafted after cryopreservation, except for tissue from the 2-year-old animal, which was transplanted fresh.

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Gazelle testes

Testicular tissue was obtained from necropsies at Estación Experimental de Zonas Áridas (Consejo Superior de Investigaciones Cientificas (CSIC), Almeria, Spain) or at ZooAquarium Madrid (Madrid, Spain) and sent to the laboratory at 5–10°C (Table 1). Testes from two species of gazelles were used as donor tissue for the present study: two fetuses of Cuvier's gazelle (a mid-term and a full-term abortion) and one 8-month-old Mohor gazelle. Testicular tissue from all specimens was grafted after cryopreservation, but tissue from one Cuvier's gazelle was also transplanted fresh.

Testis tissue processing, cryopreservation, xenografting and recovery

After removal of the tunica albuginea, testes were cut into small fragments (~1 mm^3). As a reference for testis development, a piece of testicular tissue from each donor was fixed in Bouin's solution overnight followed by three changes of 70% ethanol before being processed for histology. Tissue was cryopreserved as described previously (Honaramooz *et al.* 2002). Freezing medium was prepared with fetal bovine serum (FBS; Gibco BRL, Madrid, Spain), Dulbecco's modified Eagle's medium (DMEM; Gibco) and dimethylsulfoxide (DMSO; Sigma, Madrid, Spain) at a ratio of 1 : 3 : 1 (v/v/v). One to 10 pieces of testicular tissue fragments were added to 0.5 mL freezing medium in 2-mL cryovials at room temperature. The vials were placed in a container with isopropyl alcohol at room temperature; the container ('Mr Frosty'; Nalgene, ThermoFisher, Madrid, Spain) is designed to provide a controlled cooling rate of −1°C min^{-1} when placed in a −80°C freezer. The tissue fragments were left at −80°C overnight and were subsequently transferred to liquid nitrogen.

Cryopreserved testes were stored for at least 1 month before use in xenografting. For thawing, vials were held at room temperature for 1 min to evaporate any remaining liquid nitrogen and placed in a water bath at 25°C for 1 min. Afterwards, 1.5 mL DMEM at 25°C was added to each vial and the contents were transferred to a centrifuge tube. Then, the tissue fragments were washed twice with DMEM to remove cryoprotectant by centrifugation (300*g*, 2 min), resuspended in DMEM and kept in this medium until grafting.

Male immunodeficient mice (NCR-nude; 7–12 weeks old) were anaesthetised by inhalation of isofluorane, castrated and, during the same surgery, two to eight fragments of donor testis tissue were implanted under their back skin. Recipient mice were killed by CO_2 inhalation and recovered grafts were fixed in Bouin's solution and analysed by histology. Four testicular tissue fragments from a lynx fetus were transplanted to two immunodeficient mice each, two pieces from perinatal cubs testes were transplanted to 10 mice, six to eight fragments from 6-month-old males were transplanted to 17 mice and eight tissue pieces from a 2-year-old male were transplanted to six mice. Testis tissue from Cuvier's gazelles was grafted in 15 mice, whereas testis tissue from the Mohor gazelle was transplanted to seven mice. Six to eight tissue pieces from gazelles were subcutaneously transplanted per host mouse. The number of grafted mice per donor and recovered grafts are given in Table 2. Seminal vesicle weights of recipient mice were recorded as an indicator of the presence of bioactive testosterone originating from the grafts (Schlatt *et al.* 2002, 2003).

Animal husbandry and procedures followed European Union Regulation 2003/65 and Spanish Animal Protection Regulation RD1201/2005.

Analysis of testicular tissue

Donor and graft tissue were examined using tissue sections stained with haematoxylin and eosin. A graft was considered to be successful when seminiferous tubules could be identified. All seminiferous tubules present in one section per sample were examined under ×200 magnification and the most advanced germ cell present was recorded. When gonocytes or differentiated germ cells were not observed, the presence of spermatogonia was verified after immunostaining for protein gene product (PGP) 9.5, which is specifically expressed in germ cells of several mammalian species (Wrobel *et al.* 1996; Luo *et al.* 2006). An antibody against PGP 9.5 was used as described by Arregui *et al.* (2008*a*). Briefly, citrate antigen retrieval was used after deparaffinisation by boiling in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA) for 10 min. Then, slides were treated with 3% H_2O_2 (Sigma) in distilled water for 10 min and blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, Newmarket, Suffolk, UK) in phosphate-buffered saline (PBS) for 40 min at room temperature, avidin block for 10 min and biotin block for 10 min (Zymed, Invitrogen, Madrid, Spain). Subsequently, sections were incubated overnight at 4°C in a humidified chamber with the primary antibody (rabbit anti-PGP 9.5; AbD Serotec, Kidlington, Oxford, UK) diluted 1 : 500 in PBS. The following day, samples were treated for 30 min with the secondary antibody (biotinylated goat anti-rabbit IgG; 1.5 mg mL^{-1}; Vector) diluted to 6 μg mL^{-1} in PBS and exposed for 30 min to streptavidin horseradish peroxidise (1 mg mL^{-1}; Vector) at a concentration of 3 μg mL^{-1} in PBS. Finally, peroxidase activity was detected with a VIP substrate kit for peroxidase (Vector) for 2 min and samples were mounted.

Graft tissues from Iberian lynx and Cuvier's gazelle were analysed at two time points: (1) within 40 weeks of grafting (grafts recovered between 25 and 38 weeks after grafting); and (2) more than 40 weeks after transplantation (range 42–71 weeks). Data between these two groups were compared using a *t*-test implemented in SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Mohor gazelle tissue was recovered and analysed from one mouse at 12 weeks after grafting and in two mice each at 16, 20 and 28 weeks after grafting.

Results

Testes size, cause of death and the origin of the animals in the present study are summarised in Table 1.

Lynx testes

Histological analysis of testes from the 6-week-old fetus showed cubic epithelia that did not correspond to seminiferous cords (Fig. 1*a*). This epithelium was probably excurrent duct, such as epididymis, that in very young testes occupies a high volume. Testicular tissue from 1–3-day-old lynxes showed the formation of seminiferous cords (Fig. 1*b*). In 6-month-old testes, seminiferous tubules were observed in the testicular tissue; they were characterised by a lack of lumen formation and 56% of tubules had germ cells (Fig. 1*c*). The 2-year-old lynx

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presented no differentiated germ cells and some picnotic cells inside the seminiferous tubules, but premeiotic germ cells were observed after PGP 9.5 immunocytochemistry staining in 89.7% of tubules (Fig. 1*d*).

Lynx xenografts

Only one and three grafts were recovered from the fetus and tissue from perinatal cubs, respectively, but none of them contained testicular tissue and recipient mouse seminal vesicles weighed ~10 mg, indicating that the grafts did not contain functional Leydig cells (Table 2).

Survival of testicular grafts from 6-month-old lynxes was different from that observed in grafts of fetus and perinatal cubs. Grafts were recovered from all mice with tissue from Donor 1 (Tables 1, 2). The percentage of recovered grafts presenting seminiferous tubules was lower after 40 weeks than before 40 weeks post-grafting ($P = 0.037$), but seminal vesicle weight increased with time ($P = 0.049$; Table 2). Seminiferous tubules with a small lumen could be observed in grafts, but no differentiated germ cells were found at any time point (Fig. 2*a*). Six mice hosting testicular tissue from 6-month-old Donor 2 (Tables 1, 2) were kept for more than 40 weeks and grafts with seminiferous tubules were found in two of them. The histological appearance was similar to that of the other 6-month-old donor. PGP 9.5 staining of grafts recovered 28 weeks after grafting showed spermatogonia in 10% of tubules, whereas 66 weeks after transplantation, 15% of tubules contained spermatogonia (Fig. 2*b*).

Grafts from the older Iberian lynx (2 years old) were found in five of six grafted mice. No seminiferous tubules were observed and seminal vesicle weight suggested that no testosterone was being produced (Table 2).

Gazelle testes

Testis from the mid-term Cuvier's gazelle fetus presented 36% of seminiferous tubules with no germ cells, whereas 21%, 27% and 15% had one, two or more germ cells per round tubule section, respectively. Tissue from the full-term fetus had a similar histological appearance and presented 50% of tubules without germ cells, 27% with one, 14% with two and 9% with three or four gonocytes per round tubule section (Fig. 1*e*). The testis from the 8-month-old Mohor gazelle had clearly defined seminiferous tubules and 3% of round sections contained no gonocytes, 15% had one or two, 22% had three and 59% had four or more gonocytes (Fig. 1*f*).

Gazelle xenografts

Grafts were recovered before and after 40 weeks from five of 11 mice grafted with cryopreserved Cuvier's gazelle testicular tissue, but seminiferous tubules were not found in any of them. Seminal vesicle weights of these mice were not different from seminal vesicles from castrated mice that received no grafts ($P > 0.05$; mean (±s.e.m.) 9.2 ± 0.3 vs 9.3 ± 0.7 mg, respectively; $n = 11$ and 3, respectively). Grafts from Cuvier's gazelle fresh tissue showed no differentiated germ cells when recovered less than 40 weeks after grafting. When grafts were recovered after 40 weeks post-grafting (between 57 and 67 weeks),

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spermatocytes were the most advanced germ cells found and they were present in 82% of tubules examined (Fig. 2*c*). At this time, the size of seminal vesicles from grafted mice had increased (Table 2).

Transplanted tissue recovered from Mohor gazelle after 12 weeks post-grafting presented no differentiated germ cells, but seminal vesicle weight was twice that recorded for seminal vesicles from castrated mice (Fig. 3). After 16 weeks post-grafting, 62% of grafts were recovered (Table 2) and they showed 7% of tubules with spermatocytes and 1% with round spermatids. Seminal vesicle weight increased 10-fold at this time point (Fig. 3). At 20 weeks after transplantation, round spermatids were not observed but 10% of tubules had spermatocytes. Finally, at 28 weeks after grafting, 8% of seminiferous tubules in graft tissue contained spermatocytes and 1% contained round spermatids (Fig. 2*d*). Seminal vesicles weighed 300 mg (Fig. 3).

Discussion

Testis tissue xenografting has been used in several species but, to our knowledge, this is the first successful testicular tissue xenotransplantation, where haploid germ cells have been found, in endangered species and the first attempt at xenotransplantation in an endangered felid. In the present study, testis tissue and spermatogonia from 6-month-old Iberian lynx survived more than 70 weeks after grafting. Tissue from a Cuvier's gazelle fetus exhibited spermatocytes after 40 weeks post-grafting, whereas round spermatids could be found 16 weeks after transplantation of 8-month-old Mohor gazelle testis tissue.

Xenografting of testis tissue from prepubertal mammals of different species has resulted in complete spermatogenesis (Honaramooz *et al.* 2002, 2004; Schlatt *et al.* 2002; Shinohara *et al.* 2002; Oatley *et al.* 2004; Snedaker *et al.* 2004; Rathi *et al.* 2006; Zeng *et al.* 2006; Abrishami *et al.* 2010*a*; Abbasi and Honaramooz 2011; Gourdon and Travis 2011). However, until now, there has been only a preliminary, unsuccessful attempt of xenografting in a threatened ungulate. Javan banteng testis tissue presented spermatocytes at 9 months after grafting and did not proceed further through meiosis; at 15 months after transplantation, spermatocytes were still the most advanced germ cell observed (Honaramooz *et al.* 2005). In the present study, xenografts from prepubertal Iberian lynx tissue showed spermatogonia, with the percentage of tubules containing spermatogonia increasing from 28 to 66 weeks after grafting. In addition, seminal vesicle weights in mice carrying Iberian lynx grafts increased after 40 weeks post-transplantation. These findings indicate that spermatogonial proliferation takes place 1 year after grafting and that testosterone secretion increases in that period of time. Based on this finding, it could be proposed that progression of spermatogenesis and sperm production could, potentially, be observed at a later sampling point. In xenotransplanted gazelle testis, we observed that spermatogenesis occurred and round spermatids were recorded in Mohor gazelle grafts after 16 weeks post-grafting.

In gazelle testicular grafts, the onset of spermatogenesis and androgen production occurred earlier in tissue from prepubertal donors than in that from the fetus. When testis tissue is grafted, an initial loss of germ cells takes place, probably due to a transient lack of blood

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supply (Rathi *et al.* 2005). This may affect fetal and prepubertal tissues differently. Early in puberty spermatogonia experience a proliferative phase; hence, the number of spermatogonia per tubule, or per Sertoli cell, is higher in prepubertal than in fetal tissues (Vergouwen *et al.* 1991), as was observed in the present study. Therefore, the effect of the initial loss of spermatogonia will be more pronounced in fetal than prepubertal testis and the onset of spermatogenesis would be delayed in grafts from fetal testicular tissue.

Previous studies on xenografting fetal testicular tissue have focused mainly on humans (Povlsen *et al.* 1974; Skakkebæk *et al.* 1974; Yu *et al.* 2006; Mitchell *et al.* 2010), whereas only one study has reported work on bovine fetal testis tissue (Rodriguez-Sosa *et al.* 2011). Therefore, the present study is the first to use fetal testicular tissue from endangered species as donor material. Human and bovine fresh fetal tissue survived after being xenografted into nude mice, and human tissue showed normal structure and function (Yu *et al.* 2006; Mitchell *et al.* 2010; Rodriguez-Sosa *et al.* 2011). However, in humans, differentiated germ cells were not found, although perhaps the recovery time (maximum 19 weeks) was not sufficient to reach the onset of spermatogenesis. In contrast, bovine fetal testis xenografts started spermatogenesis and spermatocytes at the pachytene stage were observed at 10 months after grafting (Rodriguez-Sosa *et al.* 2011). Similarly, in the present study we observed that Cuvier's gazelle grafts from fetal testes survived and spermatogenesis progressed, but only with freshly grafted tissue, whereas cryopreserved tissue did not contain seminiferous tubules. Iberian lynx cryopreserved fetus tissue was transplanted in two mice, but seminiferous tubules were not observed in grafts, although young lynx tissue cryopreserved by the same protocol showed survival of spermatogonia. In addition, protocols for the cryopreservation of adult or fetal human testes were applied to prepubertal human tissue and different results were obtained, with more tissue damage observed when the protocol for fetal tissue was used (Keros *et al.* 2007). Therefore, specific protocols for fetal testicular tissue cryopreservation will need to be developed for endangered species.

Cryopreserved neonatal or prepubertal tissue used for xeno-transplantation initiated spermatogenesis in pig, rabbit and rhesus monkey (Honaramooz *et al.* 2002; Shinohara *et al.* 2002; Orwig and Schlatt 2005; Jahnukainen *et al.* 2007; Abrishami *et al.* 2010*b*) and allowed survival of spermatogonia in humans (Wyns *et al.* 2007, 2008). Conversely, no germ cells survived after cryopreservation and xenografting of prepubertal and pubertal cats (Mota *et al.* 2012). Hence, a species effect may underlie differences in survival.

In contrast with the ability of young testis tissue to reinitiate spermatogenesis when grafted, transplantation of adult mammal testicular tissue does not result in germ cell differentiation and, usually, the tissue degenerates (Schlatt *et al.* 2002, 2006; Geens *et al.* 2006; Rathi *et al.* 2006; Kim *et al.* 2007; Arregui *et al.* 2008*b*; Abrishami *et al.* 2010*a*). However, suppression of spermatogenesis before grafting enhances survival of spermato-gonia in human adult testis tissue xenografts (Schlatt *et al.* 2006) and allows sperm recovery in adult mouse testis tissue allografts (Arregui *et al.* 2012). Sub-adult Iberian lynx testes without differentiated germ cells were grafted, but testicular tissue degenerated completely.

One of the issues to consider for testis tissue xenografting is the age at which full spermatogenesis is established in the intact animal compared with that observed after

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grafting. Grafts have been found to shorten the time required to recover haploid spermatids in monkeys (Honaramooz *et al.* 2004), whereas, interestingly, in bull, sheep, bison, deer and ferret (Oatley *et al.* 2004; Arregui *et al.* 2008*a*; Abbasi and Honaramooz 2011, 2012; Gourdon and Travis 2011) xenografts and intact tissues had shown similar timing of sperm production. For domestic cats, there have been discrepancies between studies (Snedaker *et al.* 2004; Kim *et al.* 2007), although donors of different ages have been used. Donors of 2.5 weeks of age showed elongated spermatids 35 weeks after grafting (Snedaker *et al.* 2004), corresponding to control cats that present complete spermatogenesis by 32 weeks of age (Sánchez *et al.* 1993). Spermatozoa in semen obtained by electroejaculation in Iberian lynx are first observed at 2 years of age (N. Gañan and E. R. S. Roldan, unpublished observations), in agreement with the presence of spermatozoa in Eurasian lynx of similar age (*Lynx lynx*; Axnér *et al.* 2009). After 70 weeks post-grafting (>1 year and 4 months) no differentiated germ cells were found in Iberian lynx testis tissue grafts. It is likely that at least 2 years will be needed for the establishment of full spermatogenesis, or longer if spermatogenesis is delayed in felid xenografts, as proposed by some authors (Kim *et al.* 2007). Hence, the lifespan of nude mice would be shorter than the period of time required to ensure complete germ cell differentiation in Iberian lynx grafted tissue. In addition, mouse health may deteriorate over time, reducing the number of grafts available (Schlatt *et al.* 2002; Snedaker *et al.* 2004; Abrishami *et al.* 2010*a*). Nevertheless, testicular maturation could be accelerated by gonadotropin supplementation, as was observed in monkey xenografts (Rathi *et al.* 2008) and in isolated cells cografted ectopically with testicular tissue (Arregui *et al.* 2008*a*); further studies are required to test this possibility.

The youngest males of Cuvier's and Mohor gazelles fathering offspring have been recorded at 1–1.5 years of age (Espeso 2007). The Mohor gazelle male donor for this experiment (8 months old) exhibited round spermatids 28 weeks (~6–7 months) post-transplantation. Therefore, it could be speculated that full spermatogenesis would occur after 8–9 months post-grafting, at the same time as in the intact animal, in agreement with results in other ungulates (Oatley *et al.* 2004; Arregui *et al.* 2008*a*; Abbasi and Honaramooz 2011, 2012).

In conclusion, we found that spermatogonia survive in Iberian lynx grafts for more than 70 weeks post-grafting and although, theoretically, spermatozoa could be obtained after longer periods of time, the lifespan of nude mice may limit the applicability of this approach. Acceleration of testicular maturation by supplementation with gonadotropins may potentially overcome this limitation. Progression of spermatogenesis in gazelle grafts was dependent on donor age. Although spermatocytes were found 40 weeks after transplantation of fresh fetal Cuvier's gazelle testes, round spermatids were obtained from cryopreserved testicular tissue of prepubertal Mohor gazelle after 16 weeks post-grafting. These results represent an important step in the conservation of these three critically endangered species.

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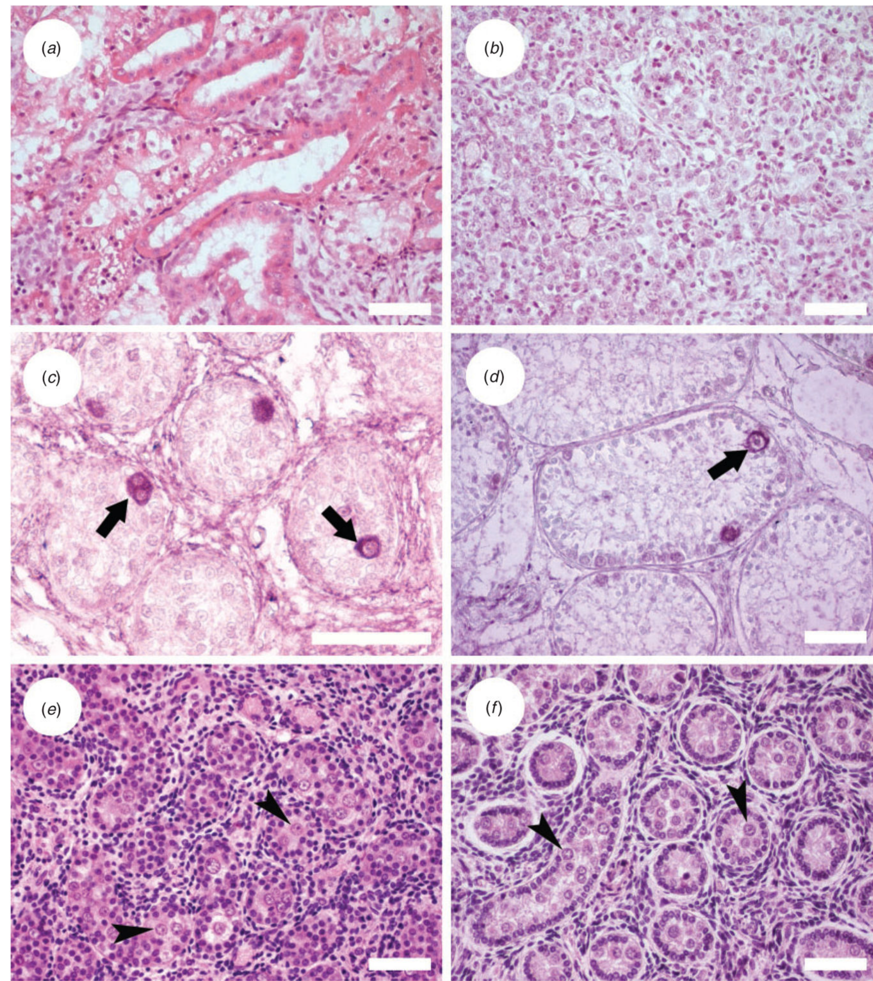

Fig. 1.

Histological appearance of donor testicular tissue. (*a*) Iberian lynx, 6-week-old fetus; (*b*) Iberian lynx, 1.5-day-old cub; (*c*) germ cells labelled by protein gene product (PGP) 9.5 immunostaining in a 6-month-old Iberian lynx testis tissue; (*d*) germ cells labelled by PGP 9.5 immunostaining in a 2-year-old Iberian lynx; (*e*) Cuvier's gazelle aborted fetus; and (*f*) Mohor gazelle, 8-month-old male. Arrows indicate spermatogonia; arrowheads indicate gonocytes. Scale bars = 50 μm.

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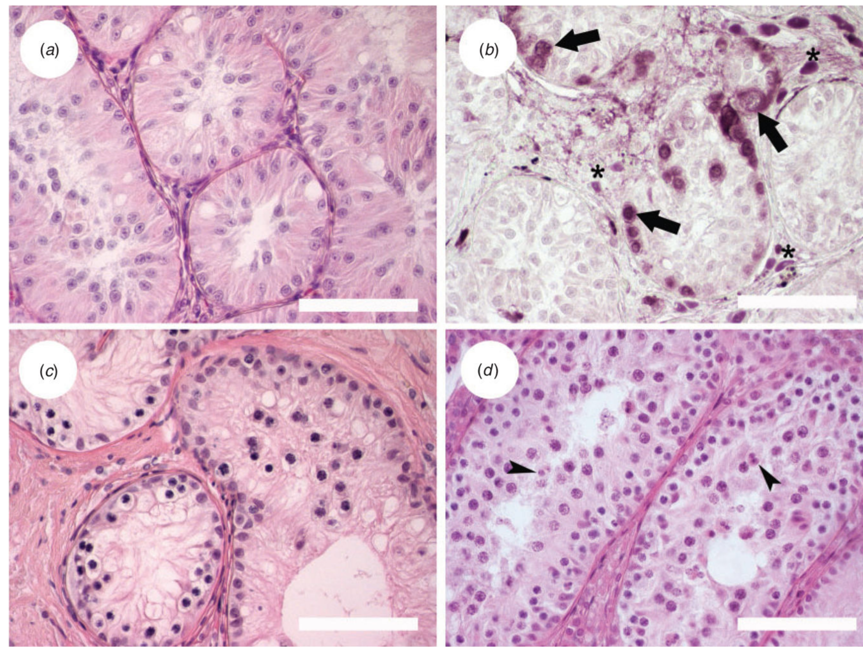

Fig. 2.

Histological appearance of grafted testicular tissue. (*a*) Iberian lynx, 28 weeks after grafting; (*b*) germ cells labelled by protein gene product (PGP) 9.5 immunostaining in Iberian lynx testis graft, 66 weeks after transplantation; (*c*) Cuvier's gazelle testis graft after 58 weeks; and (*d*) Mohor gazelle testis graft 28 weeks after grafting. Arrows indicate spermatogonia; arrowheads indicate round spermatids; asterisks indicate Leydig cells. Scale bars = 100 μm.

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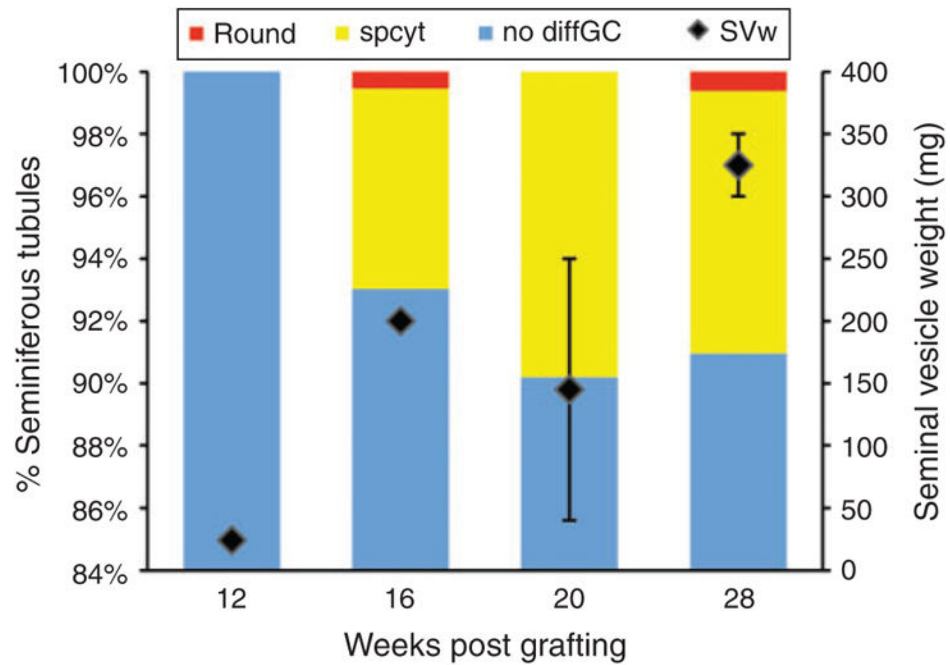

Fig. 3.

Percentage of seminiferous tubules with the most advanced germ cell type in grafts and seminal vesicle weight (SVw) of mice hosting Mohor gazelle testis tissue. Round, round spermatids; spcyt, spermatocytes; nodiffGC, with no differentiated germ cells.

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Table 1

Age, phenotype, cause of death and origin of grafted tissues

Testicular weight (TW) is for one testicle in lynxes and the average of both testes in gazelles. Testicular dimensions (TD; length × width) are for one testis in lynxes and the average of both testes in gazelles

Species	Age	TW (g)	TD (mm)	Cause of death	Captive/free[A]	Date of death	Time to laboratory (h)
Lynx pardinus	6 week fetus	0.011	3.51 × 2.04	Maternal stress	Captive (EA)	16 March 2007	36
L. pardinus	1.5 days	0.013	3.99 × 1.97	Hypothermia	Captive (EA)	31 March 2007	36
L. pardinus	3 days	0.013	3.86 × 1.58	Unknown	Captive (EA)	2 April 2007	12
L. pardinus (1)	6 months	–	–	Road kill	Free (SM)	8 October 2005	24
L. pardinus (2)	6 months	–	–	Road kill	Free (DO)	21 September 2006	24
L. pardinus	2 years	1.32	15.21 × 12.33	Feline leukaemia	Captive (LV)	12 July 2008	48
Gazella cuvieri	Mid-term abortion	–	4.74 × 2.91	Unknown	Captive (EZ)	19 February 2008	24
G. cuvieri	Full-term abortion	0.07	5.89 × 4.58	Unknown	Captive (EZ)	16 October 2007	24
G. dama	8 months	0.20	9.08 × 6.02	Anaemia	Captive (MZ)	16 September 2008	12

[A] Lynxes kept in captivity were housed at El Acebuche (EA) and Los Villares (LV), whereas samples from free-ranging animals were from two populations: Sierra Morena (SM) or Doñana (DO). Gazelles were kept in captivity at the Estación Experimental de Zonas Áridas (EZ) or Madrid Zoo (MZ).

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Table 2

Tissue recovered after xenografting and weight of seminal vesicle of grafted mice

Where appropriate, data are given as the mean ± s.e.m. NA, not available

Species	Age	Testis	Mice ~40 weeks[A]	Mice >40 weeks[B]	% Recovery[C]		Seminal vesicle weight[D] (mg)	
					~40 weeks	>40 weeks	~40 weeks	>40 weeks
Lynx pardinus	6 week fetus	Cryopreserved	1/0/0	1/1/0	0%	0%	NA	NA
L. pardinus	1.5 days	Cryopreserved	2/2/0	2/1/0	0%	0%	NA	NA
L. pardinus	3 days	Cryopreserved	2/0/0	4/0/0	0%	0%	NA	NA
L. pardinus (1)	6 months	Cryopreserved	7/7/6	3/3/2	75.7 ± 8.5	56.3 ± 6.3	20.3 ± 10.3	158 ± 72
L. pardinus (2)	6 months	Cryopreserved	1/1/1	6/4/2	33.3	33.3 ± 10.5	NA[E]	57.5 ± 42.5
L. pardinus	2 years	Fresh	2/2/0	4/3/0	0%	0%	NA	NA
Gazella cuvieri	Mid-term abortion	Cryopreserved	2/1/0	4/1/0	0%	0%	NA	NA
G. cuvieri	Full-term abortion	Fresh	1/1/1	3/3/3	37.5	54.2 ± 20.8	10	64 ± 42.6
G. cuvieri	Full-term abortion	Cryopreserved	NA	5/3/0	NA	0%	NA	NA
G. dama	8 months	Cryopreserved	7/7/7	NA	50 ± 4.7	NA	194.9 ± 46.7	NA

[A] Data show numbers of total grafted mice/grafted mice with recovered grafts/grafted mice with recovered grafts showing seminiferous tubules within 40 weeks of transplantation.

[B] Data show total grafted mice/grafted mice with recovered grafts/grafted mice with recovered grafts showing seminiferous tubules after 40 weeks post-transplantation.

[C] Percent recovery was calculated as (the number of recovered grafts showing seminiferous tubules/total transplanted grafts) ×100, before (~40) or after (>40) 40 weeks post-transplantation.

[D] Seminal vesicle weight was calculated from mice with recovered successful grafts before (~40) or after (>40) 40 weeks post-transplantation. The seminal vesicle weight of castrated mice (control) was 9.3 ± 0.7 mg ($n = 3$).

[E] Mouse was found dead and seminal vesicle weight could not be measured.