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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*)

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Running head: Testis xenografting in endangered species

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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 foetus, two perinatal cubs, two 6 month-old, and one 2 year-old), two Cuvier's gazelle foetuses and one 8 month-old Mohor gazelle were transplanted ectopically into nude mice. Tissue from the lynx foetus, perinatal cubs and 2-year old donors degenerated while in transplanted testis tissue from 6 month-old donors, spermatogonia were present in 15% of seminiferous tubules more than 70 weeks post-grafting; seminal vesicle weights (indicative of testosterone production) increased over time. Progression of spermatogenesis was observed in xenografts from gazelles and it was donor age-dependent. Tissue from Cuvier's gazelle foetuses contained spermatocytes 40 weeks post-grafting. Finally, round spermatids were found 28 weeks post-transplantation in grafts from the 8-month old Mohor gazelle. This is the first time that xenotransplantation of testicular tissue is 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.

Key words: testicular tissue, xenografting, threatened species, conservation

Introduction

The development of assisted reproductive techniques plays an important role in conservation and management of threatened species because they could benefit free and 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. Sperm can be recovered from live or recently deceased adult males (Garde *et al.* 1998, 2003; Martinez-Pastor *et al.* 2005; Gañan *et al.* 2009a, 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, sperm cannot be collected from immature males and their death represents the loss of their genetic resource forever. Relevant progress has been achieved in *in vitro* spermatogenesis and the entire spermatogenic cycle from spermatogonia to spermatozoa has been obtained in a 3D culture system (Stukenborg *et al.* 2009) and offspring obtained after culturing immature mouse testis (Sato *et al.* 2011). Alternatively, somatic cells could be employed 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 the opportunity to rescue the genetic information of a juvenile male from an endangered species (Paris and Schlatt 2007). Testis tissue xenografting involves 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 sperm from these tissue fragments, fertilize 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 successfully employed 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.* 2008a; Abrishami *et al.* 2010a; Abbasi and Honaramooz 2011, 2012; Gourdon and Travis 2011). But so far, there is only one short report on xenografting of testis tissue from an endangered species: testis from 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 the last decades and are still decreasing. The Iberian lynx is the most endangered felid in the world categorized as "critically endangered" by the IUCN since 2002 (IUCN 2012). It is an endemic species of the Iberian peninsula and current total population has been estimated to be around 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 is regarded as "endangered" since 1986 (IUCN 2012). It is an endemic species of the Atlas Mountains and has 1,700-3,000

individuals in fragmented populations in Morocco, Algeria and Tunisia but none of them had more than 250 mature individuals (Mallon and Cuzin 2008). The current population trend is unknown. Dama gazelle (*Gazella* (=*Nanger*) *dama*) is considered to be "critically endangered" since 2006 (IUCN 2012) with very small and fragmented subpopulations, and has 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 programmes 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 programmes, studies have been carried out for the characterization of sperm traits (Cassinello *et al.* 1998; Gañan *et al.* 2010), electrostimulation for sperm recovery (Cassinello *et al.* 1998; Garde *et al.* 2003; Gañan *et al.* 2009*b*) and sperm cryopreservation (Garde *et al.* 2003, 2008; Gañan *et al.* 2009b).

Premature death of young individuals is a significant problem in the conservation of these species as 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 two 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 month of age (Abaigar and Cano 2005; Barbosa and Espeso 2005). The development of a technique to recover germ cells of these individuals will be an important tool to maintain their alleles in the population genetic pool.

The aim of this study was, therefore, to test whether testis tissue xenografting could be an option to develop sperm 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 - 10ºC (Table 1). Donor tissue for xenografting was used from animals of different ages: one 6 week-old foetus, 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.

Gazelle testes

Testicular tissue was obtained from necropsies at Estación Experimental de Zonas Áridas (CSIC, Almeria, Spain) or at ZooAquarium Madrid (Madrid, Spain) and sent to the laboratory at 5 - 10ºC (Table 1). Testes from two species of gazelles were used as donor tissue for this study: two foetuses 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 (about 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 media was prepared with foetal bovine serum (FBS; Gibco, Madrid, Spain), Dulbecco's Modified Eagle Medium (DMEM; Gibco) and dimethylsulfoxide (DMSO; Sigma, Madrid, Spain) at a ratio of 1:3:1 (v/v/v). One to ten pieces of testicular tissue fragments were added to

0.5 ml freezing media 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 -1ºC min^{-1} when placed in a -80ºC freezer. The tissue fragments were left at -80ºC overnight and they were subsequently transferred to liquid nitrogen.

Cryopreserved testes were stored for at least one 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 25ºC for 1 min. Afterwards, 1.5 ml of DMEM at 25ºC were added to each vial and the content was transferred to a centrifuge tube. Then, the tissue fragments were washed twice with DMEM to remove cryoprotectant by centrifugation (300 x *g*, 2 min) and re-suspension.

Male immunodeficient mice (NCR-nude), 7-12 weeks old, were anaesthetized by inhalation of isofluorane, castrated and, during the same surgery, 2 - 8 fragments of donor testis tissue were implanted under their back skin. Recipient mice were sacrificed by CO_2 inhalation and recovered grafts were fixed in Bouin's solution and analyzed by histology. Four testicular tissue fragments from a lynx foetus were transplanted to 2 immunodeficient mice each, two pieces from perinatal cubs testes to 10 mice, six to eight fragments from 6 month-old males to 17 mice, and eight tissue pieces from a 2 year-old male were transplanted to 6 mice. Testis tissue from Cuvier's gazelles was grafted in 15 mice whereas testis tissue from Mohor gazelle was transplanted in 7 mice. Six to eight tissue pieces from gazelles were subcutaneously transplanted per host mouse. Number of grafted mice per donor and recovered grafts are shown 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.

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171 ***Analysis of testicular tissue***

172 Donor and graft tissue were examined using tissue sections stained with haematoxylin and eosin.
173 A graft was considered to be successful when seminiferous tubules could be identified. All
174 seminiferous tubules present in one section per sample were examined under 200X
175 magnification and the most advanced germ cell present was recorded. When gonocytes or
176 differentiated germ cells were not observed, the presence of spermatogonia was verified after
177 immunostaining for PGP 9.5 that is specifically expressed in germ cells of several mammalian
178 species (Wrobel *et al.* 1996; Luo *et al.* 2006). An antibody against PGP 9.5 was used as
179 described by Arregui *et al.* (2008*a*). Briefly, citrate antigen retrieval was used after
180 deparaffinization by boiling in Antigen Unmasking Solution (Vector Laboratories, Burlingame,
181 CA, USA) for 10 min. Then, slides were treated with 3% H_2O_2 (Sigma) in distilled water for 10
182 min and blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories,
183 Newmarket, Suffolk, UK) in PBS for 40 min at room temperature, avidin block for 10 min and
184 biotin block for 10 min (Zymed, Invitrogen, Alcobendas, Madrid, Spain). Subsequently, sections
185 were incubated overnight at 4ºC in a humidified chamber with the primary antibody (rabbit anti-
186 PGP 9.5; AbD Serotec, Kidlington, Oxford, UK) diluted 1:500 in PBS. The following day,
187 samples were treated for 30 min with the secondary antibody (biotinylated goat anti-rabbit IgG,
188 1.5 mg/ml, Vector) diluted to 6 µg/ml in PBS and exposed for 30 min to streptavidin horseradish
189 peroxidise (1 mg/ml, Vector) in a concentration of 3 µg/ml in PBS. Finally, peroxidise activity
190 was detected with VIP (Vector) for 2 min and samples were mounted.

191 Graft tissues from Iberian lynx and Cuvier's gazelle were analyzed at two time points:
192 before 40 weeks post-grafting (grafts recovered between 25 and 38 weeks) and more than 40
193 weeks after transplantation (range: 42 - 71 weeks). Data between these two groups were
194 compared using a *t*-test implemented in SPSS 12.0 . Mohor gazelle tissue was recovered and

analyzed from one mouse at 12 weeks and two mice each at 16, 20 and 28 weeks post-grafting.

Results

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

Lynx testes

Histological analysis of testes from the 6 week-old foetus showed cubic epithelia that did not correspond to seminiferous cords (Fig. 1A). This epithelium was, probably, excurrent duct such as epididymis that in very young testes occupied a high volume. Testicular tissue from 1-3 day-old lynxes showed formation of seminiferous cords (Fig. 1B). In 6 month-old testes, seminiferous tubules were observed in the testicular tissue and they were characterized by a lack of lumen formation and 56% of tubules had germ cells (Fig. 1C). The 2 year-old lynx presented no differentiated germ cells and some picnotic cells inside the seminiferous tubules but pre-meiotic germ cells were observed after PGP 9.5 immunocytochemistry staining in 89.7% of tubules (Fig. 1D).

Lynx xenografts

Only one and three grafts were recovered from the foetus and tissue from perinatal cubs, respectively, but none of them contained testicular tissue and recipient mouse seminal vesicles weighed less than 10 mg indicating that 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 foetus and perinatal cubs. Grafts were recovered from all mice with tissue from donor 1 (Tables 1 and 2). 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. 2A). Six mice hosting testicular tissue from 6 month-old donor 2 (Table 1 and 2) were kept for more than 40 weeks and grafts with seminiferous tubules were found in two of them. Histological appearance was similar to that of the other 6 month-old donor. PGP 9.5 staining of grafts recovered 28 weeks post-grafting showed spermatogonia in 10% of tubules whereas, after 66 weeks of transplantation, 15% of tubules contained spermatogonia (Fig. 2B).

Grafts from the older Iberian lynx (2 years old) were found in five out 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 foetus presented 36% of seminiferous tubules with no germ cells, while 21%, 27%, and 15% had one, two or more germ cells per round tubule section, respectively. Tissue from the full-term foetus 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. 1E). 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. 1F).

Gazelle xenografts

Grafts were recovered before and after 40 weeks from 5 out 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 ($p > 0.05$; 9.2 mg ± 0.3 *vs* 9.3 ± 0.7, mean ± SEM; n = 11 and n = 3, respectively). Grafts from Cuvier's gazelle fresh tissue showed no differentiated germ

cells when recovered less than 40 weeks post-grafting. When grafts were recovered after 40 weeks post-grafting (between 57 and 67 weeks) spermatocytes were the most advanced germ cells found and they were present in 82% of the tubules examined (Fig. 2C). 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 times 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 post-grafting, 8% of seminiferous tubules in graft tissue contained spermatocytes and 1% contained round spermatids (Fig. 2D). Seminal vesicles weighed ≥300 mg (Fig. 3).

Discussion

Testis tissue xenografting has been employed 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 this study, testis tissue and spermatogonia from 6-month old Iberian lynx survived more than 70 weeks post-grafting. Tissue from a Cuvier's gazelle foetus exhibited spermatocytes after 40 weeks post-grafting, while 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.* 2010a; 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 post-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 our study, xenografts from prepubertal Iberian lynx tissue showed spermatogonia, with the percentage of tubules containing spermatogonia increasing from 28 to 66 weeks post-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 one year after grafting and, also, that testosterone secretion increased 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 pre-pubertal donors than in that from the foetus. When testis tissue is grafted, an initial loss of germ cells takes place, probably due to a transient lack of blood supply (Rathi *et al.* 2005). This may affect foetal and pre-puberal tissues differently. Early in puberty spermatogonia experience a proliferative phase; hence, the number of spermatogonia per tubule, or per Sertoli cell, is higher in pre-pubertal than in foetal tissues (Vergouwen *et al.* 1991) as has been observed in this study. Therefore, the effect of the initial loss of spermatogonia will be more pronounced in foetal than in pre-pubertal testis and the onset of spermatogesis would be delayed in grafts from foetal testicular tissue.

Previous studies on xenografting foetal testicular tissue have focused mainly on humans

(Povlsen *et al.* 1974; Skakkebaek *et al.* 1974; Yu *et al.* 2006; Mitchell *et al.* 2010), whereas only one study has reported work on bovine foetal testis tissue (Rodriguez Sosa *et al.* 2011). Therefore, the present study is the first to use foetal testicular tissue from endangered species as donor material. Human and bovine fresh foetal 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 of 19 weeks) was not sufficient to reach the onset of spermatogenesis. In contrast, bovine foetal testis xenografts started spermatogenesis and spermatocytes at the pachytene stage were observed at 10 months post-grafting (Rodriguez Sosa *et al.* 2011). Similarly, in the present study, we observed that Cuvier's gazelle grafts from foetal testes survived and spermatogenesis progressed, but only with freshly grafted tissue, while cryopreserved tissue did not contain seminiferous tubules. Iberian lynx cryopreserved foetus 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 foetal human testes were applied to pre-puberal human tissue and different results were obtained, with more tissue damage observed when the protocol for foetal tissue was used (Keros *et al.* 2007). Therefore, specific protocols for foetal testicular tissue cryopreservation will need to be developed for endangered species.

Cryopreserved neonatal or prepubertal tissue used for xenotransplantation 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). On the other hand, no germ cells survived after cryopreservation and xenografting of pre-pubertal and pubertal cats (Mota *et al.* 2012). Hence, a species effect may underlie differences in survival.

In contrast to 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.* 2008b; Abrishami *et al.* 2010a). However, suppression of spermatogenesis prior to grafting enhances survival of spermatogonia 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 in comparison with that observed after grafting. Grafts have been found to shorten the time required to recover haploid spermatids in monkeys (Honaramooz *et al.* 2004) while, interestingly, in bull, sheep, bison, deer and ferret (Oatley *et al.* 2004; Arregui *et al.* 2008a; 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 post-grafting (Snedaker *et al.* 2004) corresponding to control cats that present complete spermatogenesis by 32 weeks of age (Sanchez *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 two 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, nude mice lifespan 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 gonadotrophin supplementation as was observed in monkey xenografts (Rathi *et al.* 2008) and in isolated cells co-grafted ectopically with testicular tissue (Arregui *et al.* 2008*a*), and 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). Mohor gazelle male donor for this experiment (8 months old) exhibited round spermatids 28 weeks (6 - 7 months approximately) 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.* 2008a; 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, sperm could be obtained after longer periods of time, nude mouse lifespan may limit applicability of this approach. Acceleration of testicular maturation by supplementation with gonadotrophins may potentially overcome this limitation. Progression of spermatogenesis in gazelle grafts was donor-age dependent. While spermatocytes were found 40 weeks after transplantation of fresh foetal 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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601

602 **FIGURE LEGENDS**

603

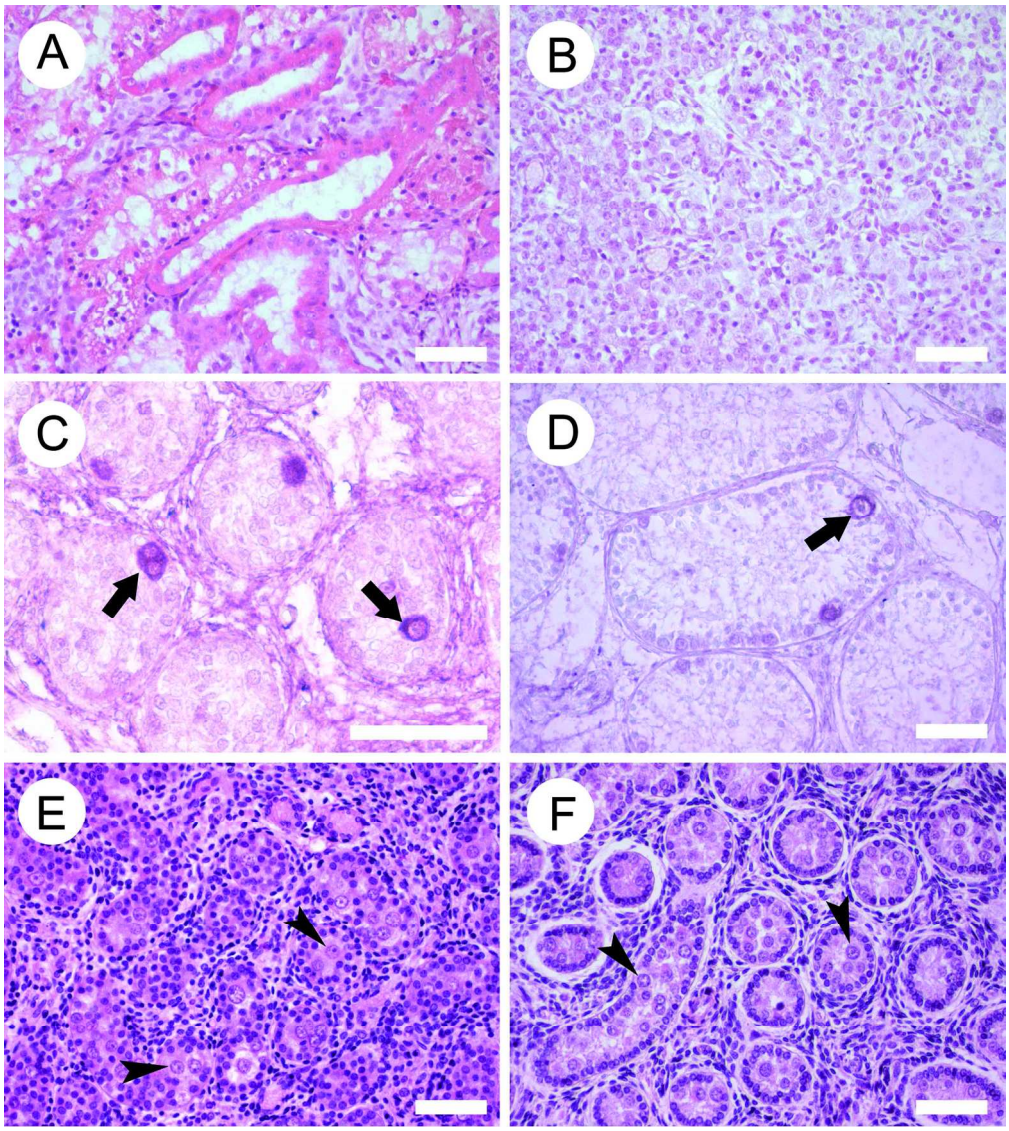
604 **Figure 1.** Histological appearance of donor testicular tissue. (A) Iberian lynx, 6 week-old foetus,
605 (B) Iberian lynx, 1.5 day-old cub, (C) germ cells labelled by PGP 9.5 immunostaining in a 6
606 month-old Iberian lynx testis tissue, (D) germ cells labelled by PGP 9.5 immunostaining in a 2
607 year-old Iberian lynx, (E) Cuvier's gazelle aborted foetus, and (F) Mohor gazelle, 8 month-old
608 male. Spermatogonia are indicated with arrows and gonocytes with arrowheads. Scale bar =
609 50μm

610

611 **Figure 2.** Histological appearance of grafted testicular tissue. (A) Iberian lynx, 28 weeks post-
612 grafting, (B) germ cells labelled by PGP 9.5 immunostaining in Iberian lynx testis graft, 66
613 weeks after transplantation, (C) Cuvier's gazelle testis graft after 58 weeks, and (D) Mohor
614 gazelle testis graft 28 weeks post-grafting. Spermatogonia are indicated with arrows, round
615 spermatids with arrowheads and Leydig cells with asterisks. Scale bar = 100μm.

616

617 **Figure 3.** Percentage of seminiferous tubules with most advanced germ cell type in grafts and
618 seminal vesicle weight (SVw) of mice hosting Mohor gazelle testis tissue. Round = round
619 spermatids; spcyt = spermatocytes; nodiffGC = with no differentiated germ cells.



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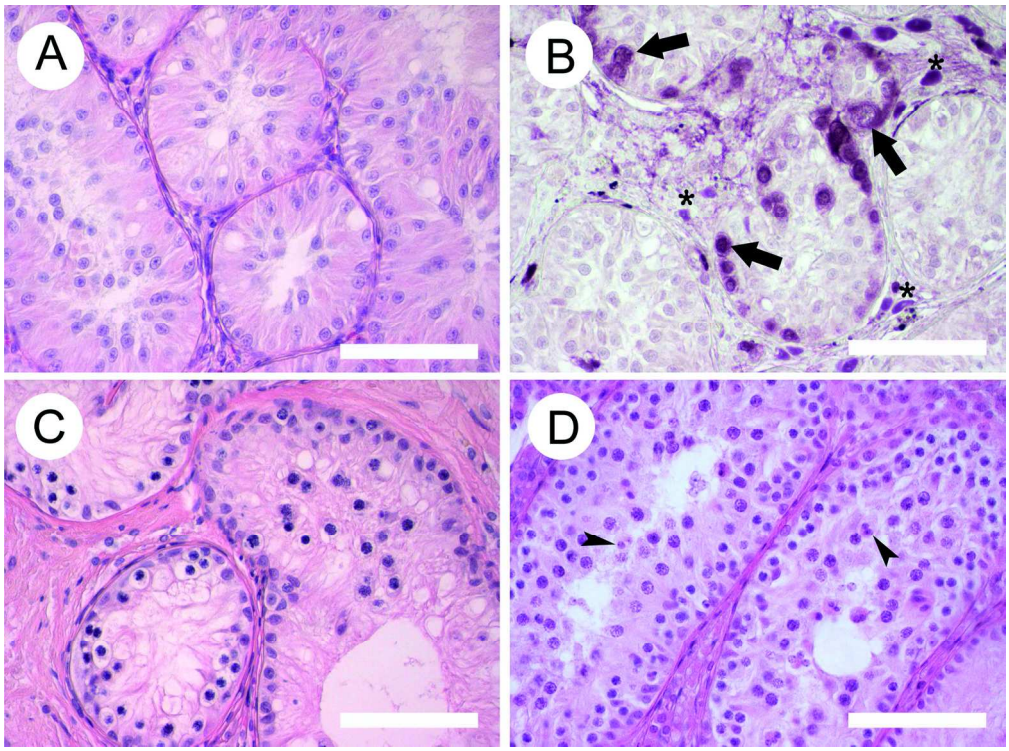

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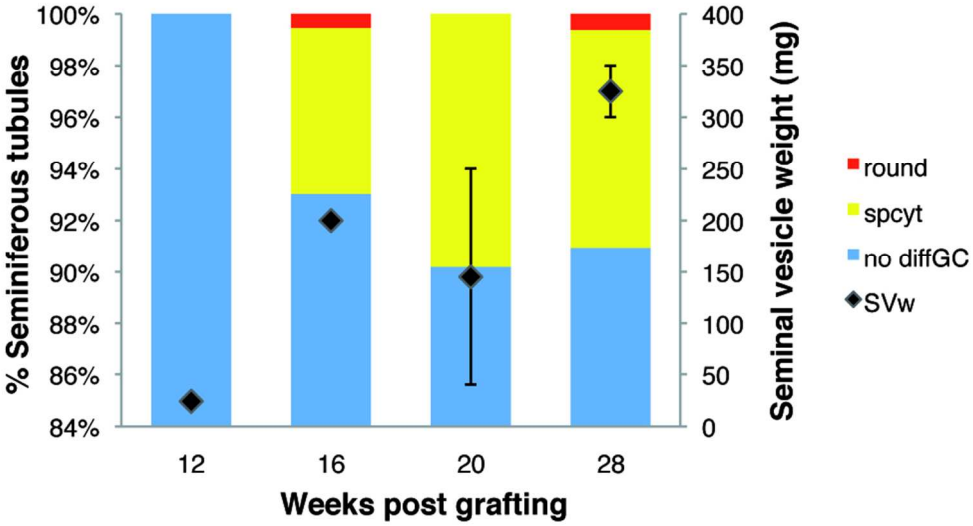

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Table 1 Age, phenotype, cause of death and origin of grafted tissues.

species	age	TW (g)[1]	TD (mm)[2]	cause of death	captivity/free[3]	date of death	time to lab (h)
Lynx pardinus	6 wk foetus	0.011	3.51x2.04	maternal stress	captivity EA	16 March 2007	36
Lynx pardinus	1.5 dy	0.013	3.99x1.97	hypothermia	captivity EA	31 March 2007	36
Lynx pardinus	3 dy	0.013	3.86x1.58	unknown	captivity EA	02 April 2007	12
Lynx pardinus(1)	6 mo	-	-	road kill	free SM	08 October 2005	24
Lynx pardinus(2)	6 mo	-	-	road kill	free DO	21 September 2006	24
Lynx pardinus	2 yr	1.32	15.21x12.33	feline leukemia	captivity LV	12 July 2008	48
Gazella cuvieri	Mid-term abortion	-	4.74x2.91	unknown	captivity EZ	19 February 2008	24
Gazella cuvieri	Full-term abortion	0.07	5.89x4.58	unknown	captivity EZ	16 October 2007	24
Gazella dama	8 mo	0.20	9.08x6.02	anaemia	captivity MZ	16 September 2008	12

[1]Testicular weight (TW) of one testicle in lynxes and average of both testes in gazelles.

[2]Testicular dimensions (TD) (length x width) of one testis in lynxes and average of both testes in gazelles.

[3]Lynxes kept in captivity were housed at El Acebuche (EA) and Los Villares (LV) and 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). Abbreviations: dy, days; wk, weeks; mo, months; yr, years.

Table 2 Tissue recovered after xenografting and weight of seminal vesicle (SVw) of grafted mice.

Species	Age	Testis	Mice <40[1]	Mice >40[2]	%Recovery[3] <40	%Recovery[3] >40	SVw[4] <40	SVw[4] >40
Lynx pardinus	6 wk foetus	cryopreserved	1/0/0	1/1/0	0%	0%	NA	NA
Lynx pardinus	1.5 dy	cryopreserved	2/2/0	2/1/0	0%	0%	NA	NA
Lynx pardinus	3 dy	cryopreserved	2/0/0	4/0/0	0%	0%	NA	NA
Lynx pardinus (1)	6 mo	cryopreserved	7/7/6	3/3/2	75.7 ± 8.5	56.3 ± 6.3	20.3 ± 10.3	158 ± 72
Lynx pardinus (2)	6 mo	cryopreserved	1/1/1	6/4/2	33.3	33.3 ± 10.5	NA[5]	57.5 ± 42.5
Lynx pardinus	2 yr	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
Gazella cuvieri	Full-term abortion	fresh	1/1/1	3/3/3	37.5	54.2 ± 20.8	10	64 ± 42.6
Gazella cuvieri	Full-term abortion	cryopreserved	NA	5/3/0	NA	0%	NA	NA
Gazella dama	8 mo	cryopreserved	7/7/7	NA	50 ± 4.7	NA	194.9 ± 46.7	NA

[1]Total grafted mice/grafted mice with recovered grafts/grafted mice with recovered grafts showing seminiferous tubules; before 40 weeks.

[2]Total grafted mice/grafted mice with recovered grafts/grafted mice with recovered grafts showing seminiferous tubules; after 40 weeks.

[3]Calculated as recovered grafts showing seminiferous tubules x 100/ total transplanted grafts, before (<40) or after (>40) 40 weeks.

[4]Seminal vesicle weight (mg) calculated from mice with recovered successful grafts before (<40) or after (>40) 40 weeks. Seminal vesicle weight of castrated mice (control): 9.3 ± 0.7 mg (n=3).

[5]Mouse was found dead and seminal vesicle weight could not been measured.

Abbreviations: dy, days; wk, weeks; mo, months; yr, years; NA, not available.