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Xenografting of testicular tissue pieces: twelve years of an *in vivo* spermatogenesis system

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Abstract

Spermatogenesis is a dynamic and complex process that involves endocrine and testicular factors. During xenotransplantation of testicular tissue fragments into immunodecifient mice a functional communication between host brain and donor testis is established. This interaction allows for the progression of spermatogenesis and recovery of fertilization-competent spermatozoa from a broad range of mammalian species. In the last years, significant progress has been achieved in testis tissue xenografting that improves our knowledge about factors determining the success of grafting. The goal of this review is to provide up to date information about the role of factors such as donor age, donor species, testis tissue preservation or type of recipient mouse on the efficiency of this technique. Applications are described and compared with other techniques with similar purposes. Recent work demonstrates that testicular tissue xenografting is used as a model to study gonadotoxicity of drugs and to obtain sperm from valuable young males.

Introduction

Transplantation of testicular tissue was performed for the first time in 1767 by John Hunter. During the 19th century first Arnold Berthold and then others used testis grafting as a tool to probe the relationship between testes and secondary sexual characteristics and, later, androgens and spermatogenesis. These experiments were performed by autotransplantation (grafts were introduced into the same animal) or homo- or allotransplantations (between genetically different individuals of the same species) in roosters, rats and mice (Setchell 1990). Also, the role of temperature and place of grafting was revealed as xenografts could be localized into the natural environment, the testis or the scrotum (known as homotopic or orthotopic grafting) or into a different part of the body; subcutaneous, intra-abdominal, etc (heterotopic or ectopic grafting).

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Declaration of interest
The authors declare that there is no conflict of interest.

Around 1920 testis tissue hetero- or xenotransplantation (grafts are introduced in recipients of different species) was performed as an organotherapy by Serge Voronoff, John R. Brinkley and others. Pieces of testes from young animals were transplanted to old animals for the rejuvenation of the host and soon this approach was applied to humans. Fragments of monkey and goat testes were xenografted into humans and surgeons claimed that it restored physical and intellectual abilities (Setchell 1990). However, Voronoff's statements where refuted and rejection of donor testicular tissue was demonstrated (Gunn & Seddon 1930, Setchell 1990).

Later, the availability of immunodeficient mice, which do not reject donor cells and tissues, entailed an important contribution as it allowed for successful xenotransplantation. The first testicular xenografting as a tool to preserve the tissue was published in 1974 (Povlsen *et al*. 1974, Skakkebaek *et al*. 1974). They transplanted subcutaneously fetal human organs into nude mice and found, in the case of the testis, acceptance of tissue, preservation of histological structure and presence of gonocytes. Also, they reported xenotransplantation of rat testicular tissue into mice and found pachytene spermatocytes 40 days after grafting. But this fascinating finding was not pursued for almost 30 years until 2002 when the first complete testicular xenotransplantation into nude mice to rescue fertility from immature pigs and goats was published, showing that it allowed for the recovery of functional sperm (Honaramooz *et al*. 2002b). The success shown in this study sparked interest in this approach and since then, close to 100 studies have been published related to testis tissue xenografting with more than 45% of them in the last five years.

The purpose of this review is to summarize the state-of-the-art and breakthroughs in this field since 2002 while highlighting also the limitations and gaps in knowledge. Finally, we will discuss the applications of this approach and compare it with other techniques of *in vitro* spermatogenesis.

An overview of testis tissue xenotransplantation

Testicular tissue xenografting is performed by implanting pieces of testicular tissue into immunocompromised mice. It can be considered a specialized form of tissue culture in which the host mouse acts as a bioincubator for the grafted tissue. Xenografts can be placed into the testis but most commonly are grafted under the skin of the mouse (Figure 1). A functional circulatory connection is established between host and grafts. It was shown in rat testicular xenografts that the connection takes place by growing of capillaries from the grafts that connect, outside the graft, with newly formed larger vessels from the host mice (Schlatt *et al*. 2010b). This is different from the situation in human endometrium grafts where it has been demonstrated that human vessel gradually regress and are replaced by mouse neovessels after xenografting (Eggermont *et al*. 2005). After revascularization, a functional feedback loop is established between the pituitary of the mouse host and the endocrine cells in grafts and, over time (weeks or months), the xenografts grow and produce sperm (Figure 1). At that time, testicular tissue can be harvested and sperm isolated from xenografts, lacking maturation in the epididymis, are functional for fertilization after intracytoplasmic sperm injection (ICSI). Spermatozoa obtained from goat xenografts were able to initiate the fertilization process after injection into mouse oocytes (Honaramooz *et al*. 2002b). Also,

when xenogeneic Rhesus monkey spermatozoa or cattle round spermatids were injected into homologous oocytes embryonic development was confirmed by reaching the blascocyst stage (Kaproth *et al*. 2002, Honaramooz *et al*. 2004). Similarly, male and female pronuclear formation took place after dog sperm injection into heterologous (sheep) oocytes (Shirazi *et al*. 2014). Additionally, production of viable offspring that grew up normally and were fertile has been reported in rabbits and pigs after the embryos were transferred into recipient females (Shinohara *et al*. 2002, Nakai *et al*. 2010, Kaneko *et al*. 2012).

Factors affecting the outcome of testis tissue xenografting

Effect of donor species on the success and efficiency of spermatogenesis

So far, to our knowledge, 23 species of mammals (3 primates, 2 rodents, 1 lagomorph, 4 carnivores and 13 ungulates) belonging to 15 families classified in 6 orders have been used as donor for testicular tissue xenografting (Table 1). Testis tissue of all species with the exception of the marmoset (Schlatt *et al*. 2002) supplied the castrated host with androgens indicating that not only the spermatogenic but also the steroidogenic function of the testicular tissue is restored in the grafts. When human testis was grafted only one report mentioned low production of testosterone by the grafts (Sato *et al*. 2010). Also, in most cases, germ cells survive and complete spermatogenesis showing applicability of this technique across a broad range of mammals. In 16 out of the 23 species used testicular xenografting lead to complete spermatogenesis. We have considered complete spermatogenesis when the authors mentioned to have found elongating spermatids, elongated spermatids, sperm or spermatozoa. However, possibly, in the remaining species timing of spermatogenesis plays an important role as round spermatids were observed as the most advanced germ cells in some cases (Arregui *et al*. 2013). This will be discussed in the next section.

Interestingly, the efficiency of spermatogenesis measured as percentage of tubules presenting complete spermatogenesis widely varied between species. This parameter is difficult to compare as most studies have been designed to find sperm. Although several studies have shown that percentage of tubules showing complete spermatogenesis increases with time (Rathi *et al*. 2005, Arregui *et al*. 2008a, Kaneko *et al*. 2008, Abbasi & Honaramooz 2012), few experiments recovered the testicular tissue at different time points after completion of spermatogenesis. Pig, goat or sheep xenografts presented 50–60% of seminiferous tubules with complete spermatogenesis or a high sperm concentration (60–100 $\times 10^6$ sperm/g) has been isolated from grafts (Honaramooz *et al*. 2002b, Zeng *et al*. 2006). Conversely, bull or horse testicular tissue showed less than 10% tubules with sperm after xenografting (Oatley *et al*. 2004, 2005, Rathi *et al*. 2005, 2006, Huang *et al*. 2008).

Induction and maintenance of spermatogenesis requires a continuous and controlled interaction of several hormones in the hypothalamic-pituitary-testis axis. Interestingly, a coordinated hormonal interaction is established between the host mouse's hypothalamus and pituitary and the transplanted gonad that induces and maintains active spermatogenesis in the transplanted tissue. In most species Sertoli cells and Leydig cells present in donor tissue were able to respond to mouse gonadotrophin stimulation as Sertoli cell proliferation, maturation (Rathi *et al*. 2008) and full spermatogenesis have been confirmed (Table 1). The

mechanism underlying the consistent progression of spermatogenesis in grafted testicular tissue from different species but species-specific differences in the efficiency of spermatogenesis after grafting remain unknown.

Differences in spermatogenic efficiency could be due to similarities or differences of gonadotrophins between species. Pituitary gonadotrophins (FSH, LH) as other gonadotrophins (TSH and CG) are glycoproteins composed of two dissimilar subunits non-covalently linked, named α and β subunits. Both subunits are similar between mammals. Alpha subunits present uniformity in primary and secondary structure and can be interchangeable between vertebrate species obtaining active hormones (with one exception in mammals: the equine β subunit needs an equine α subunit but the equine α subunit can combine with β subunits from other species). Beta subunits define the activity of the protein and, although not as highly conserved as α subunits, present a high degree of sequence homology among species (Bousfield *et al*. 2006). However, other differences not in amino acid sequence could modulate binding affinity between receptor and ligand such as pattern of glycosylation (Bousfield *et al*. 1996). It could be speculated that similarities among gonadotrophins allow interaction between mouse hormones and donor testicular tissue receptors but dissimilarities cause inefficiency in this interaction. On the other hand, it has been found that the Platyrrhini lineage (New World monkeys), to whom the common marmoset belongs, presents a unique type of LH receptor with exon 10 missing in the mRNA (Gromoll *et al*. 2003). This makes marmoset cells insensitive to mouse LH leading to blockage of germ cell differentiation during testicular xenografting (Wistuba *et al*. 2004). However, increasing testosterone concentration by cografting with hamster tissue, supplementation with human chorionic gonadotrophin or testicular tissue autotransplantation did not overcome spermatogenesis arrest in this species (Wistuba *et al*. 2004, 2006). Complete spermatogenesis in the Common marmoset has only been achieved after orthotopic (in the scrotum) autografting while higher temperature under the mouse skin might contribute to spermatogenic arrest in ectopic grafts (Luetjens *et al*. 2008).

In addition, the role of testosterone in the negative feedback should be considered although the sequence and function of this hormone is highly conserved among vertebrates. Bovine xenografts produce large amount of testosterone as recipient mice present larger seminal vesicles than control mice (Rathi *et al*. 2005). However, in bovine xenografts, hormone supplementation to decrease androgen production and delay Sertoli cell maturation did not increase the percentage of tubules with differentiated germ cells (Rodriguez-Sosa *et al*. 2012).

Among vertebrates, testicular xenografting has been published only in mammalian species. Since the organization and regulation of spermatogenesis is similar in all amniotes it might also work in birds and reptiles. Other tissues (such as skin) from birds and reptiles xenografted into mice survive (Reed & Manning 1978). However, at least in the case of birds, the role of temperature should be considered as spermatogenesis takes place at higher temperatures than in mammals (Beaupré *et al*. 1997). To our knowledge one attempt has been performed with avian testicular tissue. Spermatogonia were found in chicken testis xenografts two months after grafting but spermatogenesis did not proceed further (G Avelar 2014, personal communication).

Effect of donor age on the success and efficiency of spermatogenesis

A clear effect of donor age at the time of grafting has been shown to occur with immature testis tissue having much better developmental potential for xenografting than adult testis tissue. Xenografting has been performed with adult testicular tissue with 11 species and most studies indicate that grafted tissue degenerated (Table 2). We have considered testicular degeneration when authors found hyalinized or sclerotic tissue or mentioned to observe degenerated tissue. However, germ cell and graft survival is less dependent on the absolute age of the tissue than on the state of spermatogenesis at the time of grafting. Progression of tissue degeneration has been related to the degree of presence of spermatozoa, intensity of sperm production for the species and on the efficiency of spermatogenesis of the tissue at the time of grafting. Xenografted tissue presenting a high percentage of spermatozoa and from a species with high sperm production degenerates faster. In addition, species-specific differences have been found but the reasons have not been resolved so far (Arregui *et al*. 2008b). Different hypotheses have been proposed to explain the different potential of immature versus mature testis tissue to survive after grafting with a different sensitivity to hypoxia the most likely (Schlatt *et al*. 2002, Yu *et al*. 2006, Arregui *et al*. 2008b, 2012). Degeneration of mature testicular tissue has been demonstrated not to be inherent to the adult tissue as suppression of spermatogenesis in adult mouse testis prior to allografting supports resurgence of spermatogenesis (Arregui *et al*. 2012).

Immature tissue at different ages has been used as donor tissue showing that neonatal, infant, juvenile and prepubertal tissue complete spermatogenesis when xenografted (Table 1). Young, sexually immature donors seem to present higher efficiency of spermatogenesis after xenografting than newborn testis tissue in bulls, cats and pigs (Oatley *et al*. 2005, Kim *et al*. 2007, Caires *et al*. 2008). It has been proposed that the closer the tissue is to puberty the highest concentration of spermatogonia is found (Orwig & Schlatt 2005). Thus, more spermatogonia survive the grafting procedure allowing the presence of stem cells in more seminiferous tubules and finally, showing a higher efficiency of spermatogenesis. Also, it has been suggested that a different responsiveness to mouse gonodatrophins exist among infant, juvenile and prepuberal monkey testis (Rathi *et al*. 2008). Finally, differential gene expression could be modulating graft growth and germ cells differentiation between bull calves of different ages (Schmidt *et al*. 2007).

Results published so far on xenografting of foetal testicular tissue showed presence of spermatogonia in humans and spermatocytes in bovine and Cuvier's gazelle (Povlsen *et al*. 1974, Skakkebaek *et al*. 1974, Yu *et al*. 2006, Rodriguez-Sosa *et al*. 2011, Arregui *et al*. 2013). It could be speculated that spermatogenesis will proceed further with longer incubation post-grafting but haploid germ cells have not been found in prenatal testicular grafts so far to our knowledge.

Differences in timing of spermatogenesis related to species and age of donor

Early in puberty gonocytes migrate to the periphery of the seminiferous tubules, contact the basal lamina and differentiate into spermatogonia which will enter into the first wave of spermatogenesis. It has been suggested that, after grafting, differentiated germ cells present

in the tissue degenerated and spermatogenesis restarts from surviving spermatogonia (Rathi *et al*. 2006, Kim *et al*. 2007). Time required for the completion of the first wave of spermatogenesis in the transplanted testicular tissue is called the timing of spermatogenesis and it is has been compared between grafts and the donor testis *in situ*.

Spermatogenesis in xenografted tissue appears to occur according to the kinetics of the donor as has been proposed in rabbit, bull, bison, ferret and alpaca (Shinohara *et al*. 2002, Oatley *et al*. 2004, 2005, Rathi *et al*. 2005, Abbasi & Honaramooz 2011b, Gourdon & Travis 2011, Elzawan 2013). Nevertheless, results have been inconsistent between different studies with some other species namely cat, pig and sheep. While some studies found similar timing to controls in cat and pig (Snedaker *et al*. 2004, Kaneko *et al*. 2008) others proposed it to be longer in cat (Kim *et al*. 2007) and shorter in the case of pig (Honaramooz *et al*. 2002b). In addition, it has been suggested to be shorter in Rhesus monkey, humans and Collared peccary (Honaramooz *et al*. 2004, Sato *et al*. 2010, Campos-Junior *et al*. 2013). In sheep, grafting of immature testicular tissue appeared to accelerate the onset of spermatogenesis (Dobrinski *et al*. 2003, Zeng *et al*. 2006) but further studies demonstrated that the observed effects were due to differences in the source of control tissue and not in the time needed to reach complete spermatogenesis (Arregui *et al*. 2008a). Similarly, studies with boar xenografts showed similar timing for complete spermatogenesis although different studies reached different conclusions (Honaramooz *et al*. 2004, Kaneko *et al*. 2008), Table 1). Also, spermatogenesis takes place more uniformly in the testis *in situ* than in xenografts. Control tissue is histologically more homogeneous among seminiferous tubules while graft tissue showed asynchronous development with seminiferous tubules with sperm next to tubules without differentiated germ cells (Oatley *et al*. 2004, 2005, Kim *et al*. 2007, Arregui *et al*. 2008a, Rathi *et al*. 2008). By contrast, in Rhesus monkey testis tissue xenografting clearly accelerated the maturation of the testicular tissue. Grafts from juvenile monkeys (13 months old) presented sperm 7 month post-transplantation while spermatogenesis *in situ* usually initiates at 3–4 years of age in this species (Honaramooz *et al*. 2004). Correspondingly, hormone stimulation of juvenile Rhesus monkeys (15–16 months old) to mimic adulthood induces the onset of precocious puberty and initiation of spermatogenesis at 19–22 months of age (Plant *et al*. 1989). Early maturation of immature tissue when exposed to a mature hormonal milieu could be responsible for the proposed shorter timing of spermatogenesis in tissue grafts from humans and Collared peccary (Sato *et al*. 2010, Campos-Junior *et al*. 2013) but this has not been critically analyzed in any species other than Rhesus monkey. Primate species are good models to study the effect of modulation of the hormone microenvironment as they reach sexual maturity later than other species.

Accordingly, it has been proposed that manipulation of the endocrine environment in the mouse host could aid to shorten the time span required for sperm production (Dobrinski 2005). Host mouse treatment with exogenous gonadotrophins stimulated tissue maturation in grafts from infant Rhesus monkeys and accelerated spermatogenesis in horse testis tissue grafts (Rathi *et al*. 2006, 2008) but administration of FSH to mice carrying human testis xenografts did not shorten the timing to spermatogenesis (Van Saen *et al*. 2013). Likewise, the use of sexually intact recipient mice maintaining high testosterone levels resulted in earlier onset of spermatogenesis in Water buffalo testis grafts (Reddy *et al*. 2012) but did not

accelerate germ cell differentiation in bull or pig testis grafts (Huang *et al*. 2008, Abbasi & Honaramooz 2010).

When considering the effect of donor species on the timing of spermatogenesis, the maturation status of the donor tissue should also be taken into account. Similar timing has been found with neonatal and prepubertal testicular tissue in cats and cattle (Snedaker *et al*. 2004, Oatley *et al*. 2005, Rathi *et al*. 2005, Kim *et al*. 2007, Huang *et al*. 2008). However, usually, grafts from different aged donors were recovered at the same time points without considering these differences of the tissues at the time of grafting.

Spermatogenesis in transplanted tissue has been proposed to reinitiate from remaining spermatogonial stem cells (Rathi *et al*. 2006, Kim *et al*. 2007). Also, the duration of the cycle of the seminiferous epithelium in xenografts has been shown to be similar to the duration *in situ* (Zeng *et al*. 2006). Therefore, differences between species appear to be inherent to the germ cell genotype and could result from different sensitivity to endogenous and exogenous hormones but further studies are needed.

To our knowledge, spermatocytes are the most advanced germ cells found in prenatal testicular grafts but they appeared later than in post-natal tissue grafts. Bovine and gazelle fresh foetal tissue survived and spermatogenesis proceeded to the spermatocyte stage 10 and 15 months post-grafting respectively (Rodriguez-Sosa *et al*. 2011, Arregui *et al*. 2013) while haploid germ cell are found at similar time point with young bovine testicular xenografts (Rathi *et al*. 2005). An initial loss of germ cells was noticed after grafting (Rathi *et al*. 2005, 2006, Arregui *et al*. 2008a). This loss could be more pronounced in foetal tissue as prenatal testes presented a lower number of spermatogonia per seminiferous tubule causing a delay in the timing of spermatogenesis (Arregui *et al*. 2013).

Most studies have focused on the earliest time point when sperm can be retrieved. However, the percentage of seminiferous tubules of host mice presenting complete spermatogenesis increased with time in cattle, sheep, pig and deer (Rathi *et al*. 2005, Arregui *et al*. 2008a, Kaneko *et al*. 2008, Abbasi & Honaramooz 2012). Interestingly, it has been suggested that fertility competence decreased in porcine xenogeneic spermatozoa obtained from old grafts probably due to a negative effect of accumulated sperm that suffer senescence due to the lack of excurrent ducts in grafted tissue (Honaramooz *et al*. 2008). Therefore, the optimal time window to recover the grafted tissue has to be determined as a tradeoff between the highest amount of mature sperm and the highest fertilizing ability.

Influence of recipient mice

Mainly immunodeficient nude mice have been used as host mice for testis tissue xenografting (for instances in studies show in Table 1 only Kim *et al*. 2007 and Campos-Junior *et al*. 2013 do not use nude mice). Nude mice lack a functional thymus and therefore are deficient in T-lymphocytes (Pelleitier & Montplaisir 1975). Similarly, severe combined immunodeficient mice (SCID) are athymic but they also lack B-lymphocytes (Bosma *et al*. 1983). Some experiments proclaimed that either recipient strain is suitable for this technique (Snedaker *et al*. 2004, Rathi *et al*. 2005, 2006, Schlatt *et al*. 2006) but it was hypothesized that SCID mice may provide a more suitable host environment for the development of

xenografts since they present a higher severity of immunodeficiency. No differences between the two strains were found when mouse prepuberal testicular tissue was allografted (Geens *et al*. 2006). However, the possible difference due to different subcutaneous temperature when using a naked (nude) or haired (SCID) mouse as host have not been kept in mind. Only one recent study had systematically compared the outcome of porcine testis tissue xenografting using both strains of mice. The results showed that the percentage of recovered grafts, graft weight, seminiferous tubular density and percentage of tubules cross sections presenting germ cells was higher when SCID mice were used as hosts while no differences were found in the percentage of tubules with elongated or round spermatids (Abbasi & Honaramooz 2010). It could be interesting to analyze whether these results are maintained when using another species as donor for the testicular tissue. But also, it has to be considered that the percentage of tubules presenting haploid germ cells did not differ between strains and, therefore, when the objective is to obtain sperm, nude and SCID are equally valid as host mice.

One of the most examined issues related to the recipient mice is the role of host castration. It has been demonstrated that following castration FSH and LH release from the pituitary increased markedly (Swerdloff *et al*. 1971) due to the loss of inhibition from testicular hormones. After revascularization, exposure to elevated levels of FSH has been suggested to stimulate Sertoli cells proliferation in the grafted tissue and LH secretion supports Leydig cell maturation and subsequent testosterone production needed for spermatogenesis to occur. Subsequently, pituitary hormone levels decrease to pre-castration levels. Therefore, using a gonadectomized male mouse has been proposed to be essential for the success of testicular transplantation but differences between species have been found. While immature tissue from Rhesus monkey did not develop in non-castrated recipients (Honaramooz *et al*. 2004), testis grafts from other species completed spermatogenesis (Shinohara *et al*. 2002, Huang *et al*. 2008, Abbasi & Honaramooz 2010, Schlatt *et al*. 2010a, Reddy *et al*. 2012). The percentage of recovered grafts from water buffalo tissue was lower and more degenerated seminiferous tubules were found but the onset of spermatogenesis appeared to be shorter when using intact host mice (Reddy *et al*. 2012). However, no difference in outcome has been observed between using castrated or intact mice for cattle, pig and hamster testis xenografts (Huang *et al*. 2008, Abbasi & Honaramooz 2010, Schlatt *et al*. 2010a).

In addition, female and male nude mice have been compared as recipients for testicular xenografts. With female recipients grafts were smaller, the percentage of recovered grafts was lower and less seminiferous tubules with haploid germ cells could be found (Abbasi & Honaramooz 2010). Also, the effect of single and group housing of nude host mice has been analyzed as social hierarchy between males correlates with serum androgen levels and reproductive parameters. However, no effect was found in the percentage of recovered grafts, percentage of tubules with differentiated germ cells, nor in the weights of grafts and seminal vesicle from host mice (Arregui *et al*. 2008a). Finally, aged nude host mice transplanted with testis tissue from newborn hamster showed more tubules with germ cells and haploid germ cells than young recipients probably due to immunosenescence in older recipients (Ehmcke *et al*. 2008). But, using 1-year-old mice may not be suitable if the

objective is to obtain sperm, as the remaining lifespan could be insufficient for complete tissue maturation and germ cell differentiation.

Taking all these results together, male castrated nude or SCID mice appear to be suitable hosts for testicular tissue xenografting.

The maximal lifespan of nude mice is approximately 1.5 years (Honaramooz *et al*. 2004) and shorter for SCID (JAX 2000).This fact could preclude reaching complete spermatogenesis in some species (Sato *et al*. 2010) when timing of spermatogenesis is conserved. However, as discussed in previous sections onset of spermatogenesis is shortened in grafts from some species and it could be accelerated by hormonal supplementation in others. Further studies are required to test whether complete spermatogenesis can be achieve when using species with long periods until sexual maturity as has been shown with Rhesus monkey (Honaramooz *et al*. 2004, Rathi *et al*. 2008). Also, survival is lower in immunocompromised mice than in wild type and although some studies kept mice for more than a year (Honaramooz *et al*. 2002b, Arregui *et al*. 2013) most of them commented that some mice became ill or died before the selected time point for sacrifice (Snedaker *et al*. 2004, Arregui *et al*. 2008a, Abbasi & Honaramooz 2010, 2011a, 2011b, 2012, Abrishami *et al*. 2010a, Sato *et al*. 2010,). SCID mice have a high lymphoma incidence and both strains present increased susceptibility to opportunistic organism (JAX 2000).

Does donor tissue preservation affect tissue survival and differentiation?

Xenografting has been mainly performed with fresh donor tissue but refrigerated and cryopreserved testicular tissue has also been used showing comparable graft survival and sperm production. Pig, cat and Rhesus monkey testicular xenografts retained high cell viability and similar developmental potential after refrigeration of whole testes at 4°C for one, two or more days (Honaramooz *et al*. 2002b, Jahnukainen *et al*. 2007, Zeng *et al*. 2009, Abrishami *et al*. 2010b, Mota *et al*. 2012). Interestingly refrigeration appeared to increase percentage of seminiferous tubules with differentiated germ cells and the authors hypothesized that cooling decreased metabolism rate, thereby decreasing damage due to the hypoxic environment during xenografting (Jahnukainen *et al*. 2007, Abrishami *et al*. 2010b, Abbasi & Honaramooz 2011b). In addition, different protocols for slow-freezing have been tested with pig, rabbit, monkey, human, cat, lynx and gazelle testes showing, in most cases, preservation of germ cells and differentiation potential (Honaramooz *et al*. 2002b, Shinohara *et al*. 2002, Jahnukainen *et al*. 2007, Wyns *et al*. 2008, Zeng *et al*. 2009, Abrishami *et al*. 2010b, Van Saen *et al*. 2011, Arregui *et al*. 2013). However, cat testis tissue cryopreserved using DMSO 1.4M failed to produce grafts with germ cells (Mota *et al*. 2012). Also, cryopreservation has been proposed to delay the initiation of spermatogenesis and increase the percentage of seminiferous tubules presenting only Sertoli cells suggesting that germ cells are highly susceptible to cryoprotective agents and freezing (Jahnukainen *et al*. 2007, Zeng *et al*. 2009). Finally, vitrification protocols for testicular tissue cryopreservation have been applied resulting in tissue survival and differentiation in porcine and maintenance of spermatogonia proliferation capability in Rhesus monkey testis grafts (Zeng *et al*. 2009, Abrishami *et al*. 2010b, Poels *et al*. 2012).

Adequate storage of testicular tissue prior to xenografting is of interest for the application of this technique for fertility recovery as recipient animals may not be immediately available or donor derived spermatozoa may be desired at a later time (Zeng *et al*. 2009). Results showed that protocols for tissue storage could be species specific and vary with tissue maturation (Abrishami *et al*. 2010b, Mota *et al*. 2012, Arregui *et al*. 2013). Also, fresh and frozen tissue presenting similar viability develop differently after grafting demonstrating that germ cell differentiation should be evaluated in addition to cell viability when optimizing freezing protocols. Grafting is currently the only functional assay to assess the developmental potential of testis tissue *in vivo* (Zeng *et al*. 2009). Further, spermatozoa collected from xenografts can be successfully cryopreserved (Zeng *et al*. 2009) and therefore fertilization capability of sperm obtained after xenografting of frozen tissue and cryopreserved spermatozoa harvested after xenografting of fresh tissue should be compared to select an adequate long-term storage protocol.

Current status: Where to?

In the last five years, significant progress has been made in testicular tissue xenografting. However, gaps in knowledge remain as outlined above and further studies are required for understanding the determinants of xenograft success and failure.

One of the main problems when using this approach is the low percentage of tubules showing complete spermatogenesis. A high heterogeneity of tubular development has been observed in all species studied to date (Oatley *et al*. 2004, 2005, Kim *et al*. 2007, Arregui *et al*. 2008a, Rathi *et al*. 2008). Seminiferous tubules presenting sperm could be found adjacent to others without differentiated germ cells. Also, the lumen of some tubules becomes over expanded and the seminiferous epithelium is reduced precluding spermatogonial differentiation (see Figure 1 in Arregui *et al*. 2008a). In addition, the percentage of tubules with every type of germ cell is not consistent among grafts in the same host.

The period of hypoxia following the transplantation procedure is critical for the success of the technique. The lack of uniformity in diffusion and new vessel development could be responsible for differential availability of factors supporting cell maturation and survival. Maturation of Sertoli cells and spermatogonia/gonocyte survival could play a major role in this asynchronous development and low efficiency of spermatogenesis. Therefore, protocols that reduce tissue stress induced by grafting should be developed and this lack of uniformity inside the tissue should be studied for further improvement of testis tissue xenografting. As previously mentioned testis tissue refrigeration seams to increase the percentage of tubules with differentiated germ cells (Jahnukainen *et al*. 2007, Abrishami *et al*. 2010b, Abbasi & Honaramooz 2011b). In addition, tissue treatment with vascular endothelial growth factor (VEGF) before grafting increased the percentage of seminiferous tubules presenting complete spermatogenesis (Schmidt *et al*. 2006).

Another key and only slightly studied topic is the viability and normality of offspring using sperm derived from xenografts. Fertilizing capability after ICSI of spermatozoa retrieved from xenografts has been compared with testicular, epididymal or ejaculated sperm in pigs, monkeys or dogs showing some differences (Honaramooz *et al*. 2004, Honaramooz *et al*.

2008, Nakai *et al*. 2009, Shirazi *et al*. 2014) that need to be explained. Moreover, although some studies claimed that progeny are fertile or showed normal reproductive ability (Shinohara *et al*. 2002, Kaneko *et al*. 2012) results are still limited and other parameters such as phenotype, behavior, health or senescence should be considered.

Applications of testis tissue xenografting

Different applications have been described previously and suggested for testicular tissue transplantation (Orwig & Schlatt 2005, Rodriguez-Sosa & Dobrinski 2009, Wyns *et al*. 2010) and have been achieved in the last years.

System to study testis function and pathology

Xenografts are representative of functional testis tissue in the donor. The duration of the cycle of the seminiferous epithelium was compared by labeling with bromodeoxyuridine (BrdU) showing that the kinetics of spermatogenesis is conserved in porcine and ovine xenografts (Zeng *et al*. 2006). Also gene expression has been examined with microarray analysis in pig testis xenografts and was comparable to testis tissue *in situ* (Zeng *et al*. 2007).Therefore, grafting can be considered a valid strategy to explore testicular development and physiology. Ectopic xenografting of hamster testicular tissue into aged or young recipients has been used to assess whether testicular aging is related to senescence of somatic or germ cells (Ehmcke *et al*. 2008) . Similarly, testicular xenotransplantation was used to elucidated whether spermatogenesis and steroidogenic activity is controlled by intra- or extratesticular factors by modulating the number of hamster grafts and castration of recipient mice (Schlatt *et al*. 2010a). Also, exogenous spermatogonial stem cells transplanted into a lamb testis prior tissue xenografting survived and colonized the niche confirming that xenografts could be used to study the effect of altered gene expression on testis function (Rodriguez-Sosa *et al*. 2010). For this reason, testicular xenografting constitutes a model to study (1) the gonadotoxicity of drugs and other agents on testicular function and it has been applied to humans or other primates (as a model for humans). Doses of busulfan are clinically used for marrow ablation before bone marrow transplantation or peripheral stem cell rescue. Busulfan treatment of host mice eliminates differentiating germ cells and decreases the percentage of seminiferous tubules presenting type B spermatogonia in testicular grafts from juvenile Rhesus monkeys. These findings are comparable with the pattern found after testicular irradiation in monkeys suggesting that the effect on xenografts would correspond well to the testicular effect expected after busulfan doses used clinically (Jahnukainen *et al*. 2006). Mice carrying rat foetal testis xenografts treated with phthalates exhibited suppressed steroidogenesis similar to what was shown after exposure of foetal rats *in utero* whereas there was no evidence of endocrine disruption by phthalates in human fetal testis xenografts (Heger *et al*. 2012, Mitchell *et al*. 2012). Similarly, using a xenograft approach it has been demonstrated that exposure of human foetal testis to diethylstilbestrol does not reduce testosterone production (Mitchell *et al*. 2013). Also, testis xenografting could be used to analyzed the (2) pathophysiology of testicular tissue. In horses testicular xenografting has been use to examine cryptorchidism and idiopathic testicular degeneration (ITD). Grafts showed that suppressed spermatogenesis due to cryptorchidism could be reversed as it involves an extra testicular cause while the defect in ITD lies within the testis

and not with a primary endocrine deficiency (Turner *et al*. 2010). Additionally, testis tissue allotransplantation is a novel experimental approach to study germ cell development or recover functional spermatozoa from animals with (3) poor viability or neonatally lethal phenotypes (Ohta & Wakayama 2005, Zeng *et al*. 2011). In addition, this approach allows accessibility to the tissue to manipulate spermatogenesis and steroidogenesis. Ectopic xenografting has been used to analyze whether infant monkey testes respond to high levels of gonadotropins and spermatogenic differentiation can be accelerated without a juvenile phase of low levels of gonadotrophins (Rathi *et al*. 2008). Also, manipulation of the mouse endocrine milieu was performed to try to increase spermatogenic efficiency of bovine (Rodriguez-Sosa *et al*. 2012) and Rhesus monkey xenografts (Ehmcke *et al*. 2011).

Fertility recovery

As show in previous sections testis tissue xenografting is a powerful tool for the recovery of fertility in young individuals and testicular cryopreservation offers a strong complement for the application of this technique. Mainly this approach has two potential applications: the fertility preservation of human childhood cancer patients and of endangered species or valuable livestock. Prepubertal male cancer patients undergo treatments that can prove toxic to the gonads and could lead to permanent infertility of the patient. The role of testicular xenografting for fertility preservation in boys have been widely reviewed (Wyns *et al*. 2010, Valli *et al*. 2014) and therefore will not be discuss in this review.

Survival of young animals of wild and captive populations is often low and this represents a significant problem for endangered species and valuable livestock conservation. In small populations, each individual genotype is highly valuable. Testicular tissue xenografting could provide an opportunity to rescue the genetic information of an immature male that died accidentally. Successful generation of offspring using xenogenic spermatozoa from that male could have a significant impact on the genetic diversity within a population. So far, testes from four endangered species have been xenotransplanted. Spermatogonia but no differentiated germ cells were found in Iberian lynx testis grafts more than one year post-grafting, spermatocytes were present in Javan Banteng and Cuvier´s gazelle grafts while xenografted tissue from Mohor gazelles contained round spermatids (Honaramooz *et al*. 2005, Arregui *et al*. 2013). Tissue handling and time from tissue collection to grafting could be critical for successful xenografting in endangered species. On the other hand, it is crucial that the development of other assisted reproductive techniques, such as *in vitro* maturation of oocytes, ICSI and embryo transfer for the target species are in place for the applicability of this strategy in endangered species and breeds.

Also xenografting of testicular tissue could offer an option for fertility recovery in cryptorchid animals. When testes do not descent into the scrotum and are therefore kept at core body temperature spermatogenesis is abolished. Testis tissue from cryptorchid horses was restored to full spermatogenesis when grafted into immunocompromised mice even after years (1–3 yr) of exposure to core body temperature. This experiment demonstrated that, at least in horses, germ cells present in cryptorchid testes retain their developmental potential (Turner *et al*. 2010).

To conclude, testicular tissue banking must be considered a priority and all the information compiled here should stimulate oncologists and people working with rare and endangered species (in zoos, captive breeding centers, farms) to consider the cryopreservation of testicular tissue from young patients or animals when possible.

Comparison to other techniques

Currently, different methods of *in vivo* and *in vitro* spermatogenesis are being developed in mammals. So far, two *in vitro* culture systems have been successful in obtaining spermatozoa very recently: the culture of testicular tissue fragments (Sato *et al*. 2011) and the testicular three-dimensional cell culture (Stukenborg *et al*. 2009, Abu Elhija *et al*. 2012). Conventional testicular cell culture in cell suspensions has not been able to provide complete spermatogenesis demonstrating the need for maintaining the spatial structure of this complex organ (Dores *et al*. 2012). When a single-cell suspension is allowed to reaggregate in a three-dimensional extracellular matrix mimicking the culture of testis pieces complete differentiation into spermatozoa has been found. Both systems support the testicular architecture and the paracrine environment but eliminate the endocrine signaling. These approaches could become appropriate for production of spermatozoa as they allow for a better control of spermatogenesis than testicular xenografting eliminating the possible effect of the mouse host. However, as novel techniques, the development of protocols for translation of these technologies to mammalian species other than mouse might need to be accomplished. In addition, the physiology of testicular tissue in culture, outside its natural environment and lacking blood supply, should be compared to the testes *in vivo* to demonstrate whether these approaches could be used as models to study testicular function. Probably these systems will not be appropriate for that purpose.

Another technique of *in vivo* spermatogenesis could be used for the same applications as testicular tissue xenotranslantation. Subcutaneous xenografting of rat, pig, sheep and peccary testicular cell suspensions formed functional testicular tissue. This procedure could be used to study testicular morphogenesis and cell interactions but the onset of spermatogenesis is delayed when compared with testicular fragment xenotransplantation (Gassei *et al*. 2006, Honaramooz *et al*. 2007, Arregui *et al*. 2008a, Campos-Junior *et al*. 2013). Finally germ cell transplantation into the seminiferous tubules could be used for the production of sperm from genetically close species (Dores *et al*. 2012). For large mammals, germ cell transplantation has been only performed between individuals of the same species (Honaramooz *et al*. 2002a, 2003, Kim *et al*. 2006). Autotransplantation of germ cell in humans after cancer treatment could be a possibility in the future once cell-sorting methods guarantee to be efficient enough to avoid the risk of reintroducing cancer cells into the patient. For endangered species, the phylogenetic distance to a domesticated or available model could preclude the success of germ cell transplantation. However, for rare or valuable breeds germ cell transplantation could be an option for fertility recovery.

Conclusions

Currently, testis tissue xenografting is a unique system that allows study of testicular maturation and function and, at the same time, obtaining fertilization competent

spermatozoa from immature testicular tissue from several mammalian species. Different factors have been shown to influence the outcome of testicular tissue xenotransplantation. The percentage of tubules presenting differentiated germ cells in the grafted testicular tissue is affected by: (1) the donor tissue species (2) the donor tissue age, (3) the time postgrafting, (4) the method for tissue preservation prior to xenografting and (5) the characteristics of the recipient mice.

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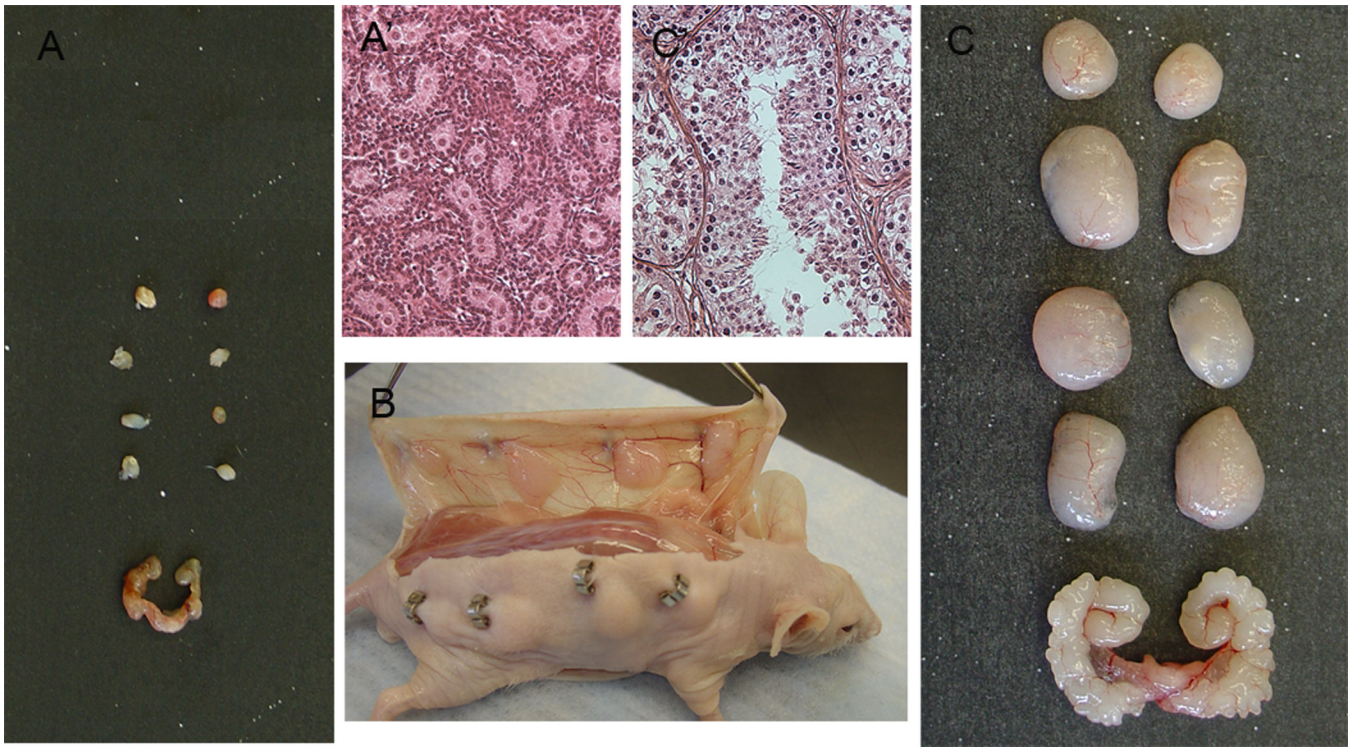

Figure 1.

Xenografting of testis tissue from lambs under the skin of nude mice. A) The size of lamb testis grafts before grafting and seminal vesicle of castrated host mouse and A') histological appearance of donor tissue showing gonocytes. B) Dorsal and ventral view of the skin of mouse with xenografts 12 weeks postgrafting. C) The size of lamb testis grafts and seminal vesicle of host mouse 16 weeks after grafting and C') histological appearance of graft tissue showing elongated spermatids. Scale bar, A and C 1cm, A' and C' 100μm.

Table 1

Xenografting of mammalian testis tissue from immature donors.

O[1]	F[2]	SF[3]	Donor species (Common name)	Donor age[4]	Most advanced germ cell found[5]	Time postgrafting[6]	Reference
RODENTIA							
	MURIDAE						
			Rattus norvegicus (Laboratory rat)	Neonatal (3 dy)	Spermatocytes	6 wk	Skakkebaek *et al.* 1974
	CRICETIDAE						
			Phodopus sungorus (Djungarian hamster)	Neonatal	Complete spgenesis	7 wk	Schlatt *et al.* 2002
LAGOMORPHA							
	LEPORIDAE						
			Oryctolagus cuniculus (Common rabbit)	Prepubertal (1–6 wk)	Complete spgenesis	8.4–12.6 wk	Shinohara *et al*. 2002
CARNIVORA							
	CANIDAE						
			Canis lupus familiaris (Domestic dog)	Immature (< 4 mo) Young (4–6 mo)	Complete spgenesis Complete spgenesis	16.8 wk	Abrishami *et al*. 2010a
	MUSTELIDAE						
			Mustela putorius furo (Domestic ferret)	Immature (8 wk)	Complete spgenesis	20 wk	Gourdon & Travis 2011
	FELIDAE						
			Felis Catus (Domestic cat)	Neonatal (2.5 wk)	Complete spgenesis	35 wk	Snedaker *et al*. 2004
				Early juvenile (8 wk)	Complete spgenesis	50 wk	
				Prepubertal (10–16 wk)	Complete spgenesis	30 wk	Kim *et al.* 2007
				Pubertal (5–7 mo)	Complete spgenesis	50 wk	
			Lynx pardinus (Iberian Lynx)	Foetal Juvenile (6 mo)	Degenerated Spermatogonia	36 wk 66 wk	Arregui *et al*. 2013
PERISSODACTYLA							

O[1]	F[2]	SF[3]	Donor species (Common name)	Donor age[4]	Most advanced germ cell found[5]	Time postgrafting[6]	Reference
	EQUIDAE						
			Equus ferus caballus	Immature (2 wk)	Spermatogonia		
			(Domestic horse)	Immature (5 mo)	Spermatocytes		
				Immature (8 mo)	Complete spgenesis *		
				Immature (8 mo)	Spermatocytes	34–35 wk	Rathi *et al*. 2006
				Immature (10 mo)	Complete spgenesis		
				Immature (12 mo)	Spermatocytes*		
				Immature (12 mo)	Spermatogonia		
CETARTIODACTYLA							
	CAMELIDAE						
			Vicugna pacos	Prepubertal (6–10 mo)	Complete spgenesis	24 wk	Elzawam 2013
			(Domestic alpaca)				
	SUIDAE						
			Sus scrofa domesticus	Neonatal (5–10 wk)	Complete spgenesis	17–18 wk	Honaramooz *et al*. 2002b, Kaneko *et al.* 2008
			(Domestic pig)				
	TAYASSUIDAE						
			Tayassu tajacu	Immature (3 mo)	Complete spgenesis	25.2 wk	Campos-Junior *et al*. 2013
			(Collared peccary)				
	CERVIDAE						
			Odocoileus virginianus	Immature (2 mo)	Complete spgenesis	33.6 wk	Abbasi & Honaramooz 2012
			(White-tailed deer)				
	BOVIDAE						
		BOVINAE					
			Bos taurus	Foetus (125–145 dy)	Spermatocytes	42 wk	Rodriguez-Sosa *et al.* 2011
			(Domestic cattle)	Neonatal (1–2 wk)	Complete spgenesis	25.2–33.6 wk	Rathi *et al*. 2005
				Neonatal (4 wk)	Complete spgenesis	24 wk	Oatley *et al*. 2004, 2005
				Immature (8 −28wk)	Complete spgenesis	20–25 wk	Kaproth *et al.* 2002; Oatley *et al*. 2005; Huang *et al*. 2008
			Bos javanicus	Neonatal (1 wk)	Spermatocytes	12.6 wk	Honaramooz *et al*. 2005
			(Banteng)				

O[1]	F[2]	SF[3]	Donor species (Common name)	Donor age[4]	Most advanced germ cell found[5]	Time postgrafting[6]	Reference
			Bison bison bison (Plains bison)	Neonatal (1dy)	Complete spgenesis	67.2 wk	Abbasi & Honaramooz 2011b
			Bubalus bubalis (Water buffalo)	Neonatal (8 wk) Neonatal (8 wk)	Spermatocytes Complete spgenesis[Θ]	24wk	Reddy *et al*. 2012
		ANTILOPINAE					
			Nanger dama mohorr (Mohor gazelle)	Immature (8 mo)	Round spermatids	16 wk	Arregui *et al*. 2013
			Gazella cuvieri (Cuvier's gazelle)	Foetus	Spermatocytes	58–67 wk	Arregui *et al*. 2013
		CAPRINAE					
			Capra hircus (Domestic goat)	Immature (4 wk)	Complete spgenesis	10 wk	Honaramooz *et al*. 2002b
			Ovis aries (Domestic sheep)	Immature (1–2 wk)	Complete spgenesis	12 wk	Zeng *et al*. 2006; Arregui *et al*. 2008a
PRIMATES							
		CERCOPITHECIDAE					
			Macaca mulatta (Rhesus monkey)	Infant (3 mo) Infant (3 mo) Infant (6 mo) Infant (6 mo)	Spermatocytes* Spermatogonia Complete spgenesis* Spermatogonia	28 wk	Rathi *et al*. 2008
				Juvenile (8–11 mo)	Spermatocytes	12.6 wk	Ehmcke *et al*. 2011
				Juvenile (13 mo)	Complete spgenesis	29.4 wk	Honaramooz *et al*. 2004
				Juvenile (16 month)	Complete spgenesis	21 wk	Ehmcke *et al*. 2011
		CALLITRICHIDAE					
			Callithrix jacchus (Common marmoset)	Neonatal	Spermatocytes*	22 wk	Schlatt *et al*. 2002
		HOMINIDAE					
			Homo sapiens (Human being)	Foetus (14–26 wk) Infant (3 mo)	Spermatogonia Spermatocytes	4–22 wk 54 wk	Skakkebaek *et al*. 1974; Yu *et al*. 2006 Sato *et al*. 2010

O[1]	F[2]	SF[3]	Donor species (Common name)	Donor age[4]	Most advanced germ cell found[5]	Time postgrafting[6]	Reference
				Infant (3–5 ye)	Spermatogonia	37.8 wk	Van Sean *et al*. 2011
				Prepubertal (7–12–13 ye)	Spermatocytes	25.2–37.8 wk	Wyns *et al*. 2008; Van Sean *et al*. 2011
				Pubertal (14 ye)	Mainly degenerated	25.2 wk	Wyns *et al*. 2008

[1] Order.

[2] Family.

[3] Subfamily.

[4] Dy: days, wk: weeks, mo: months and ye: years.

[5] Complete spermatogenesis when papers refers to elongating spermatids, elongated spermatids, sperm, or spermatozoa,

[*] with hormone supplementation,

[ϴ] using non-castrated host.

[6] Corresponds to the earliest time point when most advanced germ cell was found, time has been expresed in weeks (wk) by dividing by 7 when it was given in days and multiplied by 4.2 when it was shown in months.

Table 2

Xenografting of mammalian testis tissue from mature donors.

O[1]	F[2]	SF[3]	Donor species (Common name)	Donor age[4]	Most advanced germ cell in donor tissue (Reason)	Outcome	Time postgrafting[5]	Reference
RODENTIA								
	CRICETIDAE							
			Phodopus sungorus (Djungarian hasmter)		Azospermia (Photoregressed)	Spermatocytes	7 wk	Schlatt *et al.* 2002
CARNIVORA								
	CANIDAE							
			Canis lupus familiaris (Domestic dog)	7–22 mo	Sperm 5–80%	Degenerated	16.8 wk	Abrishami *et al.* 2010a
	FELIDAE							
			Felis Catus (Domestic cat)	8–15 mo	Sperm	Degenerated	10 wk	Kim *et al.* 2007
			Lynx pardinus (Iberian Lynx)	2 yr	Spermatogonia (Feline leukaemia)	Degenerated	22 wk	Arregui *et al*. 2013
PERISSODACTYLA								
	EQUIDAE							
			Equus ferus caballus	2 yr	Round spermatids	Spermatocytes	15 wk	Arregui *et al*. 2008b
			(Domestic horse)	1–4 yr	Sperm 38–69%	Spermatocytes	16.8 wk	Rathi *et al.* 2006
				1–4 yr	Spermatogonia (Cryptorchidism)	Spermatocytes	16.8 wk	
				5–25 yr	Sperm	Degenerated	16.8 wk	Turner *et al.* 2010
				9–28 yr	Azoospermia (ITD)	Degenerated	29.4 wk	
			Equus africanus (Domestic donkey)	13 mo	Sperm 33%	Sperm	15.2 wk	Arregui *et al*. 2008b
CETARTIODACTYLA								
	SUIDAE							
			Sus scrofa domesticus (Domestic pig)	8–11 mo	Sperm 86–88%	Degenerated	5 wk	Arregui *et al*. 2008b

O[1]	F[2]	SF[3]	Donor species (Common name)	Donor age[4]	Most advanced germ cell in donor tissue (Reason)	Outcome	Time postgrafting[5]	Reference
	BOVIDAE							
		BOVINAE						
			Bos taurus	7 mo	Sperm 9%	Sertoli cells	27 wk	Arregui *et al*. 2008b
			(Domestic cattle)	8 mo	Sperm	Spermatocytes	11 wk	Huang *et al.* 2008
				3 yr	Sperm 73%	Degenerated	8 wk	Arregui *et al*. 2008b
		CAPRINAE						
			Capra hircus	1.5–4 yr	Sperm 75–87%	Degenerated	9 wk	Arregui *et al*. 2008b
			(Domestic goat)					
PRIMATES								
	CERCOPITHECIDAE							
			Macaca mulatta	3 yr	Sperm 67%	Sperm	21 wk	Arregui *et al*. 2008b
			(Rhesus monkey)	6 yr	Sperm 76%	Spermatocytes	4 wk	
				11 yr	Sperm 86%	Sertoli cells	3.8 wk	
				12 yr	Sperm 78%	Degenerated	21 wk	
	HOMINIDAE							
			Homo sapiens	32–40 yr	Sperm (Obstructive azoospermia)	Degenerated	2–19 wk	
			(Human being)	32–40 yr	Azoospermia (Chemotherapy)	Sertoli cells only	2–19 wk	Schlatt *et al.* 2006
				-	Spermatogonia (Horm. suppression)	Spermatogonia	6–10 wk	
				-	Sperm	Spermatogonia	5–15 wk	Schlatt *et al.* 2006; Geens *et al*. 2006

[1] Order.

[2] Family.

[3] Subfamily.

[4] Dy: days, wk: weeks, mo: months and ye: years.

[*] Percentage refers to the percentage of seminiferous tubules presenting sperm,

[5] Time has been expresed in weeks (wk) by dividing by 7 when it was given in days and multiplied by 4.2 when it was shown in months.