Focus on Fertility Preservation

Animal models for fertility preservation in the male

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Abstract

Fertility preservation in the male is routinely focused on sperm. In clinical and veterinary settings, cryopreservation of sperm is a widely used tool. However, the goals for male fertility preservation differ between experimental models, maintenance of livestock, conservation of rare species, and fertility protection in men. Therefore very different approaches exist, which are adapted to the specialized needs for each discipline. Novel tools for male fertility preservation are explored targeting immature germ cells in embryonic or immature testes. Many options might be developed to combine germline preservation and generation of sperm \textit{ex vivo} leading to interesting new perspectives. This review highlights current and future options for male fertility preservation with a special focus on animal models and a consideration of the various disciplines in need of novel tools.


Traditional and novel options for male fertility preservation

Fertility preservation in the male usually focuses on cryopreservation of sperm as the routine technique in human and veterinary medicine (Kliesch \textit{et al.} 1997, Li \textit{et al.} 2007, Maxwell \textit{et al.} 2007, Sancho \textit{et al.} 2007, Saragusty \textit{et al.} 2007) (Fig. 1; Table 1). This method has become the standard operating procedure, both in attending issues of male infertility in adult patients and in the routine management of livestock populations. In patients, sperm is banked before the onset of potentially gonadotoxic chemotherapeutical regimes in oncological and autoimmune diseases (Quinn & Kelly 2000, Ginsberg \textit{et al.} 2008). In livestock management, sperm from donor breeding males presenting a series of desirable characteristics are used to artificially inseminate females in order to ensure optimum revenue from the resulting offspring. Furthermore, sperm cryopreservation is being employed in order to establish fertility reserves from endangered species, although the successful derivation of offspring is yet to be demonstrated in most species. One of the major limitations of sperm cryopreservation for male fertility preservation is the fact that mature sperm cannot be retrieved from prepubertal donors; thus, this approach is restricted to adult donor populations.

Modern strategies may open different and versatile approaches to generate male gametes with full fertilization ability. Methods currently under investigation include the xenologous and autologous transplantation of testicular tissues and germline (stem) cells, the development of procedures to enable \textit{in vitro} spermatogenesis, and the optimization of cryopreservation protocols for testicular cells and tissues. If tools become available to generate male gametes from immature germline precursors, fertility preservation can also focus on the maintenance of germline cells at any stage of development. In this scenario, preservation of cells is performed on precursors of sperm (e.g. spermatogonial cryopreservation). The target population is made up from any germline cell starting with primordial germ cells in the epiblast of the early embryo to spermatogonia in the adult testis. The preservation of immature germline cells is only valuable when strategies become available to differentiate immature germ cells into sperm with fertilizing ability. In this regard, various breakthrough studies have opened fascinating and promising perspectives. Figure 1 lists many approaches which can be categorized into tools providing germline preservation, fertility preservation, or both aspects. Certainly, a creative combination of the various approaches opens many potential novel pathways to facilitate long-term preservation of the male germline and generation of sperm.

In this review, we will present cryopreservation strategies using sperm from adult males and the subsequent use in assisted reproduction techniques (ART). We will pay particular attention to animal models that have attempted culture or xenotransplantation of...
germ cells that have no fertilization potential under natural or in vitro conditions. Immature elongating and elongated spermatids can be retrieved from testicular biopsies by testicular sperm extraction (TESE). TESE has been originally developed to retrieve sperm from testicular tissue of patients who have no sperm in the ejaculate, but show some degree of spermatogenesis in testicular biopsies. Although this strategy has quickly been implemented in infertility clinics in many countries after its original description (Palermo et al. 1992), it is illegal in some countries. Interestingly, this approach had never been tested in animal models prior to its human application and has – in contrast to its widespread use in ART clinics – found very limited use as an experimental tool in research and veterinary medicine.

Astounding results were achieved in mouse studies after injection of spermatocytes into eggs (Kimura & Yanagimachi 1995). The microinjected secondary spermatocytes were not only able to undergo the two meiotic divisions in the oocyte cytoplasm but were also able to provide a haploid genome for generation of normal offspring. Similarly, surprising is the finding that freeze-dried preparations of sperm have the full developmental potential to generate offspring when injected into mouse eggs (Wakayama & Yanagimachi 1998). Freeze-dried sperm must be considered dead. The integrity of the genome in these sperm was maintained for several months at room temperature. These findings indicate that the eggs have an enormous fertilization potential even when injected with immature germ cells at a tetraploid stage or a haploid genome from dead sperm. Whenever the male genome can be reconstructed, an egg can initiate the fertilization and developmental process. Other cells have been used for fertilization trials on oocytes. Although somatic cell nuclear transfer is not a natural act of fertilization, since no gametes are involved it can be regarded as an experimental approach for male fertility preservation, at least when a male somatic cell like Sertoli cell (Wakayama 1995) is used for the transfer. In cloning a diploid nucleus is reprogrammed and the egg is stripped of its own genome. This emphasizes the great potential of eggs to generate offspring from whatever genome it was obtained from. Egg quality seems to be a major parameter for success of fertilization in both veterinary and human-assisted...
reproduction approaches (Krisher 2004, Sirard et al. 2006). A controversial but highly relevant research field would be created if strategies can be developed to haploidize somatic cells and to create artificial gametes. In recent years strategies for in vitro haploidization have been explored, however, as yet with limited success (Tesarik & Mendoza 2003, Nagy & Chang 2007). Although highly speculative, for the future it may be possible that routes for transfer of haploidized somatic cells or in vitro matured artificial gametes are developed, which may include the injection of naked DNA into eggs. In addition, it has been shown that embryonic stem cells can differentiate into male gametes in vitro (Toyooka et al. 2003, Geijsen et al. 2004), indicating the possibility to derive functional sperm from embryonic stem cells (for review, see Daley 2007). Figure 2 depicts schematically the potential scenarios to induce fertilization.

Animal models for sperm collection and long-term preservation

In laboratory rodents, collection of sperm samples is usually performed after killing the animal. However, alternative strategies are available for collection of sperm and long-term storage opening up various pathways to explore experimentally novel options for male fertility preservation. In mice and rats, sperm can be collected either by electroejaculation (Tecirlioglu et al. 2002a, 2002b) or by the collection of ejaculated sperm from the uteri of females (Yamauchi & Ward 2007, Yamauchi et al. 2007). As indicated, the most commonly used procedure is the retrieval of sperm from the cauda epididymis of the deeply anesthetized or dead male animal (Klinefelter et al. 1991, Yamuchi et al. 2007). Long-term preservation of mouse sperm can either be achieved by standard cryopreservation protocols (Kaneko et al. 2006), by evaporative drying of sperm before freezing (Li et al. 2007) or freeze-drying procedures and maintenance of the freeze-dried sample at room temperature (Wakayama & Yanagimachi 1998).

A whole industry exists in respect to semen collection and cryostorage in farm animals. Semen collection and artificial insemination have become routine procedures in livestock reproduction. Semen is usually collected employing an artificial vagina (Curry 2007). Only under experimental conditions, the collection of epididymal sperm has been attempted (Martins et al. 2007). In many livestock species (e.g. cattle, horse), sperm from desired breeding males are cryopreserved, shipped, and used to inseminate large numbers of females (Morrell 2006, Haugan et al. 2007, Metcalf 2007, Saragusty et al. 2007). In some species (e.g. pig), no satisfactory cryopreservation procedure for sperm have been developed yet, so samples of fresh semen are routinely shipped for artificial insemination of sows (Gerrits et al. 2005).

In nonhuman primates, sperm is routinely collected by electroejaculation (Amboka & Mwethera 2003, Leibo et al. 2007) and research has focused on developing adequate long-term cryopreservation methods (Morrell & Hodges 1998). On some occasions, penile vibratory stimulation has also been proposed as a less invasive procedure (Yeoman et al. 1998). Other authors have recently retrieved epididymal sperm for cryopreservation (Dong et al. 2008). Nonhuman primate sperm have been used for fertilization of oocytes employing ICSI, and live birth from the resulting pregnancies have been reported (Nusser et al. 2001, Ng et al. 2002).

Testicular tissue as a target for male fertility preservation

Compared with the widespread routine use of sperm cryopreservation and artificial insemination in both the livestock industry and the medical field, the cryopreservation of gonadal tissues for fertility preservation has as yet only entered an experimental stage. Concerning female reproductive tissues, some clinical studies involving pregnancies after autotransplantation of cryopreserved ovarian tissues (Demeestere et al. 2006) and the derivation of embryos in vitro from cryopreserved and allografted ovarian tissues (Donnez et al. 2007) have caught recent public attention. Regarding the successful use of germ cells from cryopreserved male reproductive tissues, clinical studies have shown success with the derivation of sperm via TESE from cryopreserved biopsies obtained from adult infertility patients (Zitzmann et al. 2006), but many additional approaches have been explored in animal models.

The retrieval and cryopreservation of testicular tissue has been successfully attempted in animal models, both targeting basic research and preclinical issues. This approach allows the retrieval and long-term storage of germline stem cells within their natural niche even from

Figure 2 Potential scenarios to induce fertilization.
adult and sexually immature donors (Paris & Schlatt 2007, Milazzo et al. 2008). Recently, the derivation of functional sperm has been attempted from those cryopreserved immature tissues employing a variety of ex situ culture approaches in animal models, indicating that these cryopreserved tissues hold the potential to produce sperm long after the testicular tissues have been removed from the immature donors (Schlatt et al. 2002, Jahnukainen et al. 2007). This approach would be of high interest to prepubertal patients where no other form of fertility preservation is possible (Wyns et al. 2007). Xenografting will be extremely interesting for livestock management since xenografting accelerates the production of sperm from any desired immature donor, especially in species with long periods from birth to puberty as is the case in many farm animals but also in primates where this advantage has already been described. A significant acceleration of spermatogenic induction was already shown in monkeys (Honaramooz et al. 2004). The acceleration of sperm production has high significance for livestock management as it could decrease the turnover time from one generation to the next. In the following, we will explore in more detail potential options for the generation of sperm from adult or immature testicular tissue.

Successful TESE has been reported from fresh testicular tissues from men and nonhuman primates (Fig. 3; Hewitson et al. 2002), and the retrieved sperm has been employed to produce offspring. Whereas TESE has been widely used both in the reproductive clinic and in several animal models, little use has apparently been seen in this technique in domestic animals, as studies addressing this approach in livestock species have not been reported.

Cryopreserved testicular tissue from adult donors can be maintained for indefinite periods of time. After thawing, the tissue can be used as a source for sperm. Adult murine testicular tissue can routinely be cryopreserved (Milazzo et al. 2008). Sperm can be retrieved from cryopreserved adult mouse testicular tissue and used for production of offspring (Ogonuki et al. 2006). As yet, no studies describe the successful retrieval of sperm or spermatids from cryopreserved testicular tissues in either farm animals or monkey species. Human testicular tissue, however, has been cryopreserved after retrieval of biopsies from patients with non-obstructive azoospermia (Dafopoulos et al. 2005a, 2005b). Sperm derived by TESE from cryopreserved biopsies was used for assisted fertilization (Zitzmann et al. 2006). The clinical application revealed that this strategy can in principle be applied as an experimental tool or for livestock management.

The successful extraction of mature sperm from cryopreserved adult testicular tissue could be expected in consideration of the high resistance of ejaculated sperm to cryopreservation-induced damage. It was interesting to explore experimentally whether normal sperm could also be retrieved from immature testicular tissue, which was cryopreserved and then allowed to mature ex situ. Many studies in rodents focused on extracting functional sperm from testicular tissue, which was dissected from immature animals and underwent sexual maturation either in vitro or after xenografting into a suitable host. Whereas all attempts to culture immature testicular tissues in vitro until sexual maturation of the seminiferous epithelium have failed so far, very promising results have been obtained by xenografting immature testicular tissues into immunodeficient mouse hosts (Fig. 4).

We showed that ectopically xenografted immature testicular tissue matures in a nude mouse host and contains differentiated germ cells up to the stage of spermatids and sperm after several weeks (Honaramooz et al. 2002). Mouse offspring could be generated using sperm from xenografted tissue dissected from neonatal donors (Schlatt et al. 2003). Others have shown that live offspring can be produced using sperm retrieved from cryopreserved and subsequently xenografted immature mouse testis tissues using TESE and in vitro insemination (Shinohara et al. 2002). Initiation of complete spermatogenesis as well as active steroidogenesis has also been shown in xenografted immature testicular tissues derived from the Djungarian Hamster (Schlatt et al. 2002). By contrast, as yet all attempts to xenograft adult testicular tissue have resulted in poor development and cessation of spermatogenesis (Fig. 5).

Cryopreservation and xenografting of immature testicular tissues were experimentally attempted in some farm animals, with one recent study showing the production of live offspring after xenografting of immature donor testis tissue in chicken (Song & Silversides 2007). In several other livestock species, it has been shown that haploid germ cells up to the stage of mature spermatids develop in xenografted immature testicular tissue, but no live offspring has been reported to date using xenografted sperm (Oatley et al. 2005, Rathi et al. 2006, Zeng et al. 2006, Kim et al. 2007).

We have performed preclinical studies in nonhuman primates to assess the feasibility of cryopreservation of testicular tissue as a potentially relevant clinical tool. Since xenografting of immature testicular tissues appears

Figure 3 Live offspring using sperm derived from fresh or cryopreserved testicular tissues have been produced in rodents, nonhuman primates, and man, but not in livestock species.
much more promising compared with xenografting of adult tissue and since the target patient group for testicular tissue preservation in a clinical setting would be prepubertal patients we performed these studies using neonatal and juvenile rhesus macaques. As a prerequisite for additional studies we compared various strategies in order to optimize the cryopreservation protocol for immature rhesus monkey testicular tissue (Jahnukainen et al. 2007). We determined that cryomedia containing 1.4 M dimethyl sulfoxide (DMSO) showed a better outcome in terms of recovery after xenografting than testicular tissue cryopreserved with ethylene glycol or lower concentrations of DMSO. We did use a simple, uncontrolled freezing protocol that worked well in this study but might be open for additional optimization after systematic experiments are performed. We also attempted to show if nonhuman primate testicular tissues can be maintained on ice for 24 h prior to xenografting (Jahnukainen et al. 2007). The rationale behind these studies was to explore if a time window exists in which immature testicular tissue – like many other organs prior to transplantation – can be transported on ice prior to processing without noticeable deterioration of the tissue. If the tissue allows short-term storage on ice without significant deterioration, it could be retrieved at any location and be shipped to a centralized facility that is specialized on the more complex procedures required for testicular tissue xenografting. Here, the tissue could then be processed and xenografted under optimum conditions. Our study revealed that a storage for 24 h on ice did not reduce the potential of immature rhesus monkey testes to survive as a xenograft and to initiate spermatogenic differentiation.

Cryopreservation of human testicular tissue has attained increasing attention as a novel approach for fertility preservation in patients prior to cancer therapy (Gosden 2002, Hovatta 2003, Orwig & Schlatt 2005, van den Berg et al. 2007, Keros et al. 2007). This strategy is also applicable to prepubertal patients whose testes are surgically removed due to cryptorchidism ( Kvist et al. 2006). Attempts have been made to optimize the cryopreservation procedure in order to improve germ cell survival in these tissues (Keros et al. 2005). The xenografting procedure has been used with limited success to mature prepubertal testicular tissues for subsequent sperm extraction (Wyns et al. 2007).

Outlook and perspective

Cryopreservation of sperm, germ cells, and testicular tissue has become a standard operating procedure in the propagation of livestock species and in assisted reproduction clinics. In livestock management, the strategies for cryopreservation of sperm are constantly improved. Novel approaches might target species (e.g. pigs) for which currently no useful large-scale procedures for industrial use are available. In regard to the protection of endangered species, many recent studies focus on felids (Zambelli et al. 2006, Swanson et al. 2007). The intention of this work is to enable wildlife reserves and zoos the exchange of male gametes from the limited numbers of captive males. Furthermore, a cryogenic repository is established for those highly endangered species for which only few individuals remain in the wild and/or captivity.

Xenografting of testicular tissue has become a valuable experimental tool to explore testicular physiology and male germ cell development. This tool may open novel scenarios for preservation of the male germ lineage and may create useful strategies also for conservation of valuable livestock and rare animals (Paris & Schlatt 2007). Instead of focusing on preservation of mature sperm, these strategies will focus on preservation of tissue from immature donors. It is to be expected that the recent and ongoing studies on the cryopreservation of

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**Figure 4** The production of live offspring by employing sperm retrieved from xenografted immature testicular tissues has so far only been successful in a limited number of species, and has not been shown in other species (shaded grey).

**Figure 5** Micrographs of rhesus monkey testicular tissue recovered 4 months after xenografting into nude mouse host. Note that advanced spermatogenesis is established in the tissue obtained from a juvenile rhesus monkey (a), and that no spermatogenic activity is seen in testicular tissue obtained from an adult rhesus monkey (b). Scales = 50 μm.
nonhuman primate testicular tissues will eventually lead to novel technologies in fertility preservation, with a focus on the current studies on establishing a fertility reserve in prepubertal primates. These clinically relevant studies might also lead to new approaches for fertility preservation in boys undergoing oncological treatments or other gonadotoxic therapeutic regimens. In addition to studies in nonhuman primates, many of the technologies developed in a variety of animal models will eventually lead to safe and efficient methods to avoid long-term reproductive failure in patients undergoing gonadotoxic treatments in adult life as well as during childhood. Thus, exciting results can be expected in the near future, leading to both advances in basic reproductive sciences and to the development of immense interest to the agroindustrial community, wildlife protection agency, and the field of reproductive medicine.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


Klinefelter GR, Gray LE Jr & Suarez JD 1991 The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. Reproductive Toxicology 5 39–44.


Kinesler GR, Gray LE Jr & Suarez JD 1991 The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. Reproductive Toxicology 5 39–44.


