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Possibilities for preserving genetic resources in birds

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This review discusses various techniques of storage of avian genetic resources. Semen cryopreservation still seems to represent one of the most effective and feasible methods for preservation of genetic resources. Various cryoprotective agents, cooling rates and semen packaging methods are discussed. The use of ovarian tissue, embryonic cells, such as primordial germ cells (PGCs) or blastodermal cells, is the most promising approach because both male and female genetic information can be preserved. A relatively innovative technique; transplantation of cryopreserved testicular cells into adult animals with subsequent production of donor-derived progeny is also mentioned. This transplantation method may contribute to the preservation of endangered avian species and to maintaining their genetic variability.

Keywords: genetic resources; semen; spermatogonial cells; testes; embryonic cells; ovarian tissue

Introduction

Progress in reproduction biotechnology and greater requirements for yield in all farm animal species has resulted in highly productive hybrid breeds in the poultry. The populations of original breeds are reared in small numbers, and therefore may suffer from inbreeding and a decrease in genetic diversity. Continual selection for meat productivity in most poultry species decreases the efficiency of natural mating, semen quality and reproduction potential (Lukaszewicz and Kruszynski, 2003). Habitat destruction, air pollution and increased density of natural predators have resulted in a decline of wild avian species (Kowalczyk *et al.*, 2012). Currently, more than 13% of avian species are threatened by extinction (IUCN, 2015). The possibilities of preserving the genetic diversity in avian species are discussed below.

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Semen

Semen cryopreservation is probably one of the most effective and feasible methods for preservation of genetic resources. The greatest advantage of this method lies in the fact that it is non-invasive for both donors and recipients and enables storage of a very large number of samples in a relatively short time. Experiments with long-term semen storage started with the discovery of the cryoprotective effect of glycerol. Polge *et al.* (1949) revealed that cockerel's spermatozoa can survive temperatures of -79°C with 20% glycerol and their motility after thawing is as high as that of fresh spermatozoa. Since then, many laboratories have devoted their efforts to long-term storage of avian spermatozoa. Despite numerous attempts to develop an effective and reliable procedure for cryopreserving avian semen, a high fertility level was only achieved at the end of the 20th century in cockerels (Chalah *et al.*, 1999; Tselutin *et al.*, 1999); however, the same procedure applied to other species has either failed or exhibited low efficiency. Semen cryopreservation in birds is a method with varying levels of success depending on several factors such as species, breed, line, and even the individual within the flock (Blanco *et al.*, 2000; Massip *et al.*, 2004; Fulton, 2006; Long, 2006; Blesbois *et al.*, 2007; Blesbois, 2011; 2012). There are specific differences among species in sperm morphology, metabolism, acrosome structure, stability and toleration to osmotic changes (Blanco *et al.*, 2000; 2008). For example, the morphology of guinea fowl sperm is similar to that of chicken sperm; however, differences in biochemical composition and biophysical properties of the cell membrane makes guinea fowl sperm less tolerant of freezing and thawing processes (Blesbois *et al.*, 2005). The most critical points affecting the cell structure and sperm metabolism during freezing and thawing are the type of cryoprotective agent used and its concentration, cooling rate, and type of semen packaging.

Cryoprotective agents

A cryoprotective agent (CPA) is a substance with the ability to protect cells against damage caused by a temperature decrease during cooling and freezing and by a temperature increase during thawing. Penetrating CPAs enter the cells and avoid intracellular water crystal formation and prevent damage caused by excessive extracellular concentration of solutes (Holt, 2000; Pereira *et al.*, 2008). However, CPAs can have a damaging effect when used at high concentrations. Non-penetrating CPAs act outside the sperm cell in the extracellular space and protect cells by dehydrating the intracellular space and by limiting the osmotic swelling during thawing.

The most widely used CPA, not only in avian cells, is glycerol. Glycerol is probably the most effective and the least toxic CPA for chicken spermatozoa (Hammerstedt and Graham, 1992; Chalah *et al.*, 1999; Tselutin *et al.*, 1999). Glycerol-treated spermatozoa display great motility after thawing, but unfortunately this CPA has a contraceptive effect after intravaginal insemination in the chicken. More than 2% glycerol in an insemination dose causes complete absence of fertility (Neville *et al.*, 1971; Lake, 1986). This concentration is insufficient for sperm protection during cryoconservation. The problem may be solved by dialysis or centrifugation of the insemination dose after thawing to remove glycerol. However, dialysis is a relatively time-consuming procedure and centrifugation has deleterious effects on the motility and fertilising ability of spermatozoa (Lake and Stewart, 1978; Hammerstedt and Graham, 1992; Long and Kulkarni, 2004; Mocé *et al.*, 2010). The other most widely used penetrating CPAs are dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA),

dimethyl formamide (DMF), and ethylene glycol (EG). Semen samples can be thawed without further processing and high levels of fertility have been obtained with these CPAs depending on the cooling rate and type of semen packaging (Santiago-Moreno *et al.*, 2011). There are some studies reporting higher fertility rates with N-methyl acetamide (MA) than with DMSO, DMA or DMF (Sasaki *et al.*, 2010). Ehling *et al.* (2012) obtained very high fertilisation results with combination of MA and DMF.

The non-penetrating CPAs, also known as osmoprotectants, are low molecular weight, hydrophilic, nontoxic molecules that help stabilise internal solutes under osmotic stress in cells (Cleland *et al.*, 2004). The non-penetrating CPAs include polyvinyl pyrrolidone (PVP) and sugars such as raffinose, trehalose, glucose, sucrose or lactose. These CPA have often been used in combination with penetrating CPA. Sexton (1975) used glucose, sucrose and PVP to determine the effect on the viability of chicken spermatozoa. Herrera *et al.* (2005) compared DMSO and PVP in roosters, pheasants and hawks and no significant differences were found between these two CPAs. Blanco *et al.* (2011) reported experiments with sucrose, trehalose and betaine-hydrochloride in a DMA-based cryodiluent on turkey and crane spermatozoa cryosurvival and motility. Inclusion of sugars in the cryodiluent improved post-thaw motility, but not viability in turkey. The use of betaine hydrochloride improved the post-thaw viability of turkey sperm, although motility of crane sperm was not improved using sugars or betaine-hydrochloride.

Cooling rate

The cooling and thawing rate is another important factor in cryopreservation. It is dependent on the type of CPA and its concentration, diluents and dilution, and semen packaging. Optimal conditions are provided by programmable freezers enabling controlled and precise decrease of the temperature and cooling rate. The first studies of preserving fowl semen used a slow cooling rate (Lake and Stewart, 1978; Sexton; 1981), *e.g.* 1-10°C/min to -35°C. Slow cooling rates are usually employed with glycerol or DMSO but are not recommended with DMA (Santiago-Moreno *et al.*, 2011). Currently, fast cooling rates are preferable (Woelders *et al.*, 2006; Blesbois *et al.*, 2007), *e.g.* 20-100°C/min. In the case of DMA, a rapid cooling rate has been used with success. Small pellets are dropped directly into liquid nitrogen at -196°C (Chalah *et al.*, 1999; Tselutin *et al.*, 1999; Váradi *et al.*, 2013). Long *et al.* (2014) used a combination of DMA, fast freezing rate and straws in turkey semen.

Recently, the very rapid cooling rate vitrification has been employed. Vitrification is the solidification of a liquid without crystallisation and the growth of ice crystal. This is achieved when solutes are sufficiently concentrated or when the cooling rate is sufficiently rapid. The disadvantage of this rapid method is the use of high concentration of CPAs that could be toxic to cells (Dinnyes *et al.*, 2007). The use of vitrification of spermatozoa in avian species is sporadic (Mphaphathi *et al.*, 2012) with poor success. Another option for semen freezing is gradual cooling by exposing the semen sample to liquid nitrogen vapour (Purdy *et al.*, 2009; Santiago-Moreno *et al.*, 2011; Ehling *et al.*, 2012; Váradi *et al.*, 2013) or on the surface of dry ice. Another easy and inexpensive method is the use of a small freezing container containing propyl alcohol, which guarantees a decrease of temperature by 1°C/min.

Type of semen packaging

Semen may be stored in glass vials, plastic straws or tubes, or in pellets. Currently, glass vials have been replaced with plastic straws (volume 0.25 or 0.5 ml), which are widely used for semen cryopreservation both in mammals, including humans, and in birds. Plastic straws are practical and provide clear identification of samples. Distribution of temperature during freezing and thawing is even, unlike in plastic tubes. Pellets, whereby small drops of diluted semen are dropped directly into liquid nitrogen or on the surface of dry ice, are extensively used in cryopreservation especially with DMA (Chalah *et al.*, 1999; Tselutin *et al.*, 1999). This method provides a very good fertility level, but it is not suitable for purposes of cryobanking because there is a danger of sample substitution.

Testicular cells

Another option for preserving genetic resources is transplantation of male germ cells. Isolation of testicular cells from one male and their transfer into the testes of another male was first demonstrated in mice (Brinster and Zimmermann, 1994). These authors isolated testicular cells from fertile donor males and transplanted them into the testes of sterile recipient males. Donor cells colonised the seminiferous epithelia of recipient males and restored spermatogenesis. This experiment resulted in production of morphologically normal spermatozoa with the donor genotype in one third of experimental animals. Since then, the transplantation of male germ cells has been used experimentally in many mammalian and several avian species (Brinster and Avarbock, 1994; Nagano *et al.*, 2001; Honaramooz *et al.*, 2002; Izadyar *et al.*, 2003; Trefil *et al.*, 2006; 2010; Song and Silversides, 2007a; Herrid *et al.*, 2009; Mucksova *et al.*, 2013; Pereira *et al.*, 2013) with differing success in the restoration of spermatogenesis in recipient males. Several studies demonstrated not only restoration of spermatogenesis in the recipient testes, but also production of fully developed mature spermatozoa and subsequent production of healthy progeny with the donor genotype (Brinster and Avarbock, 1994; Lee *et al.*, 2006; Trefil *et al.*, 2006; 2010; Herrid *et al.*, 2009; Benesova *et al.*, 2014). This transplantation method together with cryopreservation of male germ cells represents a unique and valuable tool for preservation of genetic resources.

The first attempts to cryopreserve testicular tissue were done in rats (Parkes and Smith, 1954). Since then, freezing protocols for male germ cells have been described in mammals (Avarbock *et al.*, 1996; Dobrinski *et al.*, 1999; 2000; Ogawa *et al.*, 1999; Nagano *et al.*, 2001; Izadyar *et al.*, 2002; 2003; Shinohara *et al.*, 2003), but progeny has only been obtained in rodents and sheep. In birds, cryopreservation and transplantation of testicular tissue grafts (Song and Silversides, 2007b) and testicular cells (Benesova *et al.*, 2014) have been described. Song and Silversides (2007b) isolated the testicular tissue from newly hatched chicks, froze it in DMSO, and, after thawing, transplanted it into the abdominal cavity of castrated one-day-old chicks. The adult cockerels were killed, and testicular fluid was collected from the transplanted testes. Spermatozoa obtained from this fluid were used in intramagnal insemination, resulting in the production of donor-derived offspring. This experiment contributed to avian testicular tissue cryopreservation methods; however, it was not suitable for preservation of genetic resources as it was based on newly hatched chicks. Another disadvantage was the fact that the spermatozoa for insemination were collected after removing the transplants from the body and after the death of the recipient. The number of mature spermatozoa was thus limited, and relatively few chicks could be obtained from one male. Benesova *et al.* (2014) first described successful transplantation of cryopreserved testicular cells in adult chickens, from a

fertile cockerel to an infertile cockerel, with the subsequent production of donor-derived progeny. This transplantation model uses adult mature donors and recipients and is less time-consuming, with spermatogenesis starting to resume within two months. The successfully recolonised recipients' testes were able to produce mature, fully developed spermatozoa. Although donor cockerels were sacrificed in this study, in the case of endangered or rare species, or breeds with low population size, biopsy of testicular tissue may be done without donor sacrifice.

The efficiency of cryoconservation of male germ cells performed by the freezing procedures commonly used for somatic cells, and the viability of these cells after thawing, were surprisingly high compared to the difficulties in semen cryopreservation (Brinster and Nagano, 1998). Stem cells resemble somatic cells in structure and biology, whereas spermatozoa have a unique morphology, significant nuclear DNA condensation and almost no cytoplasm, which may make the cells sensitive to physical and biochemical changes during the freezing and thawing procedures. Testicular or spermatogonial cells of endangered species or breeds could be preserved indefinitely in liquid nitrogen and after thawing and transplantation into suitable recipients could restore spermatogenesis. Subsequently, mature spermatozoa could be collected and used in natural mating or in artificial insemination to renew the original species or breed.

Embryonic cells

Birds belong to the heterogametic sex and males are the homogametic sex with ZZ chromosomes, therefore the loss of mitochondrial DNA and genes on the W chromosome represents a significant disadvantage to spermatozoa and spermatogonial cell cryopreservation. The long-term preservation of oocytes and embryos represents another approach to the preservation of genetic resources; however, the use of these techniques in birds is limited because of the oocyte size, high lipid content and polarity (Massip *et al.*, 2004; Hiemstra *et al.*, 2005), and arduous access to the zygote or oocyte (Sang, 2004). The alternative for preserving both male and female genetic information is the long-term preservation of embryonic cells such as blastodermal cells or primordial germ cells.

At the time of oviposition the embryo is in the X blastoderm stage (Eyal-Giladi and Kochav, 1976). This is a single layer of cells that consists of two visibly distinct regions; the *area pellucida* and *area opaca*. Upon incubation, the *area pellucida* differentiates into two layers, an upper epiblast and a lower hypoblast. Only the epiblast will give rise to the embryo and the hypoblast contributes to extraembryonic tissues (Sang, 2004; Petitte, 2006). The stage X blastoderm contains primordial germ cells (PGCs) or their immediate precursors in the blastodermal cell population (Naito, 2003a). PGCs are progenitors of oocytes and spermatozoa. The PGCs translocate gradually from the ventral surface of the epiblast to the dorsal side of the hypoblast. As the vascular system develops, the PGCs enter the circulation and begin to circulate through the blood of the embryo and finally colonise into the germinal ridges, the location of future gonads (Kuwana, 1993; Sang, 2004). In the male embryo, after 13 days of incubation, PGCs that enter into the testes start to divide and differentiate into spermatogonia. At around 10 weeks after hatching, the spermatogonia start to proliferate by mitotic division and some of them differentiate into primary spermatocytes. During the first meiotic division the primary spermatocytes differentiate into secondary spermatocytes, and then during the second meiotic division give rise to spermatids. Finally, they differentiate into spermatozoa. The

development of eggs in the female is similar, but only the left ovary is functional (Naito, 2003b).

For preservation of endangered species and for cryopreservation, cells (blastodermal cells or PGCs) must first be obtained from embryos. These cells can then be transferred into the recipient embryos at the appropriate stage of development. The manipulated eggs would need to hatch, and the resulting chimeras could then be mated to each other to provide identifiable offspring that would reconstitute the original line, breed or species (Petitte, 2006). The techniques for producing germline chimeric chickens using both blastodermal (Petitte *et al.*, 1990; Carsience *et al.*, 1993; Kino *et al.*, 1997) and primordial germ cells (Tajima *et al.*, 1993; Naito *et al.*, 1994; Chang *et al.*, 1997; Park *et al.*, 2003) have been established. Several attempts have been made to produce live offspring derived from xenografted PGCs via germline chimeric chickens. Kang *et al.* (2008) successfully produced Korean pheasant-chicken chimera by intravascular injection of pheasant PGCs into recipient chicken embryos. Duck-chicken germline chimeras have been produced by transfer of blastodermal cells from stage X embryos of Maya duck into the subgerminal cavity of recipient chicken embryos at stage X (Li *et al.*, 2002). A live Houbara bustard chick was obtained from houbara PGCs transplanted into a chicken recipient embryo (Wernery *et al.,* 2010). These results show that the chicken seminiferous tubule provides a suitable environment for germ cells of many avian species.

The first experiment with transfer of blastodermal cells from one embryo to another was described in 1990. Petitte *et al.* (1990) isolated cells from stage X embryos and injected them into the subgerminal cavity of unincubated embryos. The authors demonstrated that isolated cells from stage X embryo are able to give rise to functional spermatozoa in the recipient embryo and subsequently to viable chimeric offspring, although only one chimeric chicken was obtained. This rooster was both somatic and germline chimera. The efficiency of germ line transmission was very low - about 0.3%. The advantage of using blastodermal cells for germline chimera production appears to lie in easier collection and transplantation compared to methods using primordial germ cells, but the number of obtained cells is a limiting factor.

There are three sources of PGCs; the germinal crescent, the embryonic blood (circulating PGCs; cPGCs), and the embryonic gonad (gonadal PGCs; gPGCs). The germinal crescent is the least useful source of PGCs for cryopreservation given the low numbers of germ cells (Petitte, 2006). Blood collected from stage 14-16 embryos appears to be the most appropriate because the concentration of cPGCs reaches a peak in the bloodstream at this stage (Tajima *et al.*, 1999; D'Costa *et al.*, 2001). The number of PGCs is the highest in the gonads compared with that in two other collection sites (Nakamura *et al.*, 2013). Cells could be retrieved from the gonads of five-day-old embryos (Kim *et al.*, 2005) or from the gonads of seven-day-old embryos (Nakajima *et al.*, 2011).

To increase the efficiency of producing germline chimeric chickens, several methods of elimination or reduction of recipient germ cells prior to transplantation of donor cells have been developed. The first approach consists of the removal of blastodermal cells from the centre of the germinal crescent at stage X (Kagami *et al.*, 1997) or the removal of blood from recipient embryos (Naito *et al.*, 1994). The second approach is based on the irradiation of the embryo by ultraviolet, gamma and X-rays (Carsience *et al.*, 1993; Kino *et al.*, 1997). This intervention could induce DNA damage, cause abnormalities and embryo mortalities, and decrease hatchability (Aige-Gil and Simkiss, 1991a; Park *et al.*, 2010; Nakamura *et al.*, 2012). The third approach consists of the application of alkylating agent busulphan or Concanavalin A (Aige-Gil and Simkiss, 1991b; Al-Thani and Simkiss 1991; Vick *et al.*, 1993; Song *et al.*, 2005). However, these methods may affect

embryonic development. To increase the number of donor cells, several techniques have been developed for purification of PGCs; Ficoll density gradient centrifugation (Yasuda *et al.*, 1992; Tajima *et al.*, 1993; Ono *et al.*, 1998) or Nycodenz density gradient centrifugation (Zhao and Kuwana, 2003). Another method is based on antigen-antibody reaction; immunomagnetic cell separation (Ono and Machida, 1999; Wei *et al.*, 2001) or fluorescence-activated cell sorting purification (Mozdziak *et al.*, 2005). Rather than increasing the concentration of germ cells, *in vitro* culture techniques for proliferating PGCs could be helpful. Allioli *et al.* (1994) were the first to report that gPGCs from five-day-old embryos could proliferate *in vitro*, and these cultured cells had the ability to migrate to the germinal ridge of recipient embryos. Chang *et al.* (1995a; 1995b) developed a method for cultivating cPGCs for about five days and produced germline chimeras (Chang *et al.*, 1997). Han *et al.* (2002) reported a method for the long-term culture of gPGCs for up to two months that maintained germ line competence. Kim *et al.* (2005) reported the production of quail germline chimeras by the transfer of gPGCs into recipient embryos. These cells were cultured *in vitro* for up to three days. No changes in gPGCs morphology were apparent during this period and viability was not affected. Van de Lavoir *et al.* (2006; 2012) and Naito *et al.* (2015) developed a novel method for the long-term culture of chicken PGCs. PGCs are a type of cells suitable for transgenesis, and this culture system represents great progress in biotechnology and is a powerful tool both for transgenesis and preservation of genetic resources.

Methods for the collection, manipulation, culture and transplantation of embryonic cells are very helpful in the preservation of genetic resources. PGCs or blastodermal cells from the donor embryo could be cryopreserved and stored for an unlimited time in liquid nitrogen. After thawing, cells injected into the blood of the recipient embryo could colonise the developing gonads. The resulting chimeric embryo could be used for the production of donor-derived offspring. The advantage of using embryonic cells is that both male and female fertility can be preserved. Naito *et al.* (1994) reported the first successful preservation of avian PGCs in liquid nitrogen and hatching of viable offspring. No apparent difference was observed in the morphology of PGCs before and after the freezing-thawing treatment under the light microscope. The PGCs were collected from the blood of embryos at stage 13-15. DMSO (10%) was used as the CPA. The viability of the PGCs after thawing was 94.2%. The PGCs were transferred into recipient embryos, where they differentiated normally into functional gametes (Naito *et al.*, 1994). Kino *et al.* (1997) reported the production of chicken chimeras from the injection of frozen-thawed blastodermal cells with 10% DMSO as a cryoprotectant. Cryopreservation of gPGCs in liquid nitrogen has been described in the chicken (Tajima *et al.*, 1998), as well as in the quail (Chang *et al.*, 1998). DMSO is one of the most common CPAs. Moore *et al.* (2006) compared DMSO with ethylene glycol. DMSO was selected due to its wide use as a cryoprotectant and because it was previously used to freeze PGCs (Naito *et al.*, 1994; Tajima *et al.*, 1998; 2003; 2004). Ethylene glycol was selected because it was reported to be a superior CPA to DMSO (Kobayashi *et al.*, 2003). The 10% EG treatment produced favourable results and a viability level very similar to 10% DMSO. Kuwana *et al.* (2006) and Nakamura *et al.* (2010) have described preservation of PGCs in endangered domestic fowl.

Ovarian tissue

The other option for preserving female genetic information is long-term preservation and transplantation of ovarian tissue. The first step in the transplantation of ovarian tissue in birds was made in 2006, when Song and Silversides (2006) developed a surgical

technique of ovarian tissue transplantation in newly hatched chickens. Pieces of ovarian tissue from donor chickens were transplanted in the position of the removed left ovary into the newly hatched recipient chicken. Song and Silversides (2007c) subsequently demonstrated that donor-derived offspring could be obtained using this transplantation technique in chickens, quail (Song and Silversides, 2008) and in ducks (Song *et al.*, 2012). Experiments with chickens and ducks suggested the period just after hatch could be the best time for transplantation experiments, but one-day-old quails showed high mortality at this age. The experiments with one-week-old quails provided better results. Liu *et al.* (2015) demonstrated that ovarian tissue from adult Japanese quail could restore fertility by transplantation into week-old quail chicken. Song *et al.* (2012) described ovarian transplantation between Muscovy and Pekin ducks and obtained donor-derived progeny. Although these two species are closely related, it represents the first step in interspecies transplantation which could be very helpful in the preservation of endangered species.

Methods of ovarian tissue transplantation have shown great progress and challenges in the preservation of genetic resources. Cryopreservation of ovarian tissue has been used in mammals including humans. The first successful experiment was performed in 1960, when Parrot (1960) obtained normal offspring from mice with orthotopic ovarian grafts of tissue that had been frozen and stored at -79°C with glycerol as the CPA (Parrot, 1960). Since then many experiments have been carried out in mammals and offspring have been obtained, *e.g.* in rodents (Wang *et al.*, 2002; Liu *et al.*, 2008); rabbits (Neto *et al.*, 2007) and sheep (Bordes *et al.*, 2005; Campbell *et al.*, 2014). In birds, the cryopreservation of ovarian tissue in combination with the transplantation technique has been used to restore reproductive potential in quail (Liu *et al.*, 2010; 2012), using both slow-freezing and vitrification. Pieces of ovarian tissue were frozen in straws at 0.5° C/min in a programmable freezer using DMSO. In the case of vitrification, pieces of ovarian tissue were put on acupuncture needles and subsequently into the cryovials (Liu *et al.*, 2010) or into the straws (Liu *et al.*, 2012). A mixture of DMSO, EG and sucrose were used as the CPAs. Donor-derived offspring were obtained from the transplanted ovarian tissue that had been cryopreserved by either the slow-freezing or vitrification method, with the latter having have greater efficiency and better cell viability (Liu *et al.*, 2010).

Conclusions

Although sperm cryopreservation is one of the most effective and feasible methods for the preservation of genetic resources, in birds there is a high variability in the efficiency and sperm viability after cryopreservation even in closely related species. Most of the freezing protocols have been developed for individuals with high fertility. However, endangered species and breeds usually display a poor fertility level. Therefore, focus should be placed on other approaches to the conservation of endangered species, such as the use of spermatogonial and embryonic germ cells or ovarian tissue. These approaches represent a promising alternative for the conservation of endangered species. Embryonic cells and ovarian tissue are the most interesting options because both male and female genetic information can be preserved.

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