Preservation of wild feline semen by freeze-drying: experimental model

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Abstract

According to the Convention on International Trade in Endangered Species, 36 wild feline species are threatened by extinction or severely endangered, and to save them is the target of several conservation programs. This study aimed to assess the viability of the freeze-drying technique for domestic cat sperm cells, with the ultimate goal of transferring this technology to the wild feline species. The domestic cat is an excellent experimental model for wild felids. It is in this scenario that the freeze-drying process (low-temperature vacuum dehydration) of sperm cells shows its value in preserving male cats' germplasm. Results from membrane and DNA integrity analysis are promising and validates the use of frozen-dried sperm samples in intracytoplasmic sperm injections (ICSIs). Further studies are still necessary to evaluate the ICSI embryo production using domestic cat frozen-dried sperm and the possibility of using such technology with wild felines.

Keywords: cat, DNA integrity, frrozen-dried sperm, wild felines.

Introduction

For decades, major investments have been made in biotechnology to preserve sperm cells in order to perpetuate and multiply important genetic material. The most widespread technology to preserve sperm cells is the freezing process using liquid nitrogen to form a germplasm bank and preserve genetic material.

Assisted reproduction biotechnologies had a leaping development in species of economic interest. In carnivores such progress in biotechnologies occurred only when bonds between humans and domestic animals were strengthened (Soares *et al*., 2002).

As for the domestic cat, there has been an increasing interest in the collection, evaluation and cryopreservation of its semen. The ability of collecting and preserving feline semen in low temperatures provides not only an exchange of genetic diversity among domestic cat breeds (*Felis silvestris catus*), but also an extensive use of sperm of high-genetic-valued tomcats. It also reduces the transmission of infectious diseases (Siemieniuch and Dubiel, 2007) and may help the conservation of wild felids (e.g. *Leopardus pardalis*, *Leopardus tigrinus*, and *Leopardus geoffroyi*). The domestic cat is considered an excellent research model for wild felids. Thirty-six wild felid species are threatened by extinction or severely endangered (Convention on International Trade in Endangered Species of Wild Flora and Fauna - CITES, 1973) and to save them is the target of several conservation programs (Siemienicuh and Dubiel, 2007).

It is in this scenario that the freeze-drying process (low-temperature vacuum dehydration) of sperm cells shows its value in preserving male germplasm with several advantages (Martins, 2006).

The freeze-drying technique is useful because of the following reasons: 1) transportation becomes easy, thus facilitating the import and/or export of important gene pools; 2) it reduces storage costs, since there is no need to refill liquid nitrogen containers; 3) it helps assisted reproduction in animals and humans; 4) it preserves genetic lines of various species, rare breeds and transgenic lineages; 5) it facilitates the study of species and breeds characterization by using genetic markers (Martins, 2006).

Therefore, developing a technique that is not only cheaper, but also more practical to preserve and store wild felid sperm cells is a must. Our interest in freeze-drying domestic cat sperm is based in the possibility of using such technology with wild felids, especially the ones that are on the verge of extinction. The objective of our study was to evaluate the feasibility of such technique for domestic cat sperm.

Cryopreservation

There are at least two stress moments that the sperm cells undergo during the freezing and thawing processes. The first one is related to the effects of temperature change, and the second is the formation and dissolution of ice crystals.

Cold shock refers to the peculiar sensitivity of spermatozoa to the refrigeration process (Watson, 1981; Watson and Morris, 1987; White, 1993).

Sperm cells show membrane integrity loss and cell dysfunction when they are fast cooled in the interval between 20 and 0ºC. The severity of this effect depends on the cooling rate, temperature interval, and temperature amplitude. In general, it is more severe in the 12 to 2°C amplitude (Watson, 1981). It remains unclear the reason for this phenomenon, but it is probably correlated with the transitional phases of membrane lipids that lose their selective permeability

after going through changes (Watson and Morris, 1987).

However, after 60 yr of semen cryopreservation research, many aspects of the process are still unknown (Saragusty *et al.*, 2009a), and much of the progress in this field is empirical in nature (e.g. Saragusty *et al.*, 2009b). Different species may present spermatozoa of different shapes, sizes, membrane composition, osmotic pressure, sensitivity to chilling, and pH as well as other differences. Such items need to be characterized in order to design the most suitable cryopreservation protocol for each species.

There is evidence that frozen/thawed cells suffer injuries during thawing and this occurs due to the re-crystallization of microscopic ice crystals (Fiser and Fairfull, 1986).

Freeze-drying

Nowadays, the most common method to store mammal sperm cells for long periods of time is in liquid nitrogen (N_2). The period of time that these cells can be stored under this condition depends on storage costs, since the constant need of N_2 refilling may be costly. Thus, it is necessary to provide a storage technique for sperm cells that has low costs and that may be used in both *in vitro* and *in vivo* fertilization (Cabrera *et al.*, 1998).

The freeze-drying process is a technique that aims to preserve cells through the freezing of one substance followed by the reduction of one of the solvents, usually water, via sublimation and desorption to levels in which there is no chemical or biological reaction. The temperature and water vapor pressure must be lower than the triple point, which means to have 273.16 kelvin (0.01°C) and 611.73 pascal ($\cong$0.006 bar). Under these conditions and with a 2.84 MJ/kg latent heat, ice turns directly into vapor.

The advantage of the freeze-drying process is the fact that spermatozoa may be stored at room temperature for long periods and then be recovered with morphologically intact heads, containing normal DNA, and intact tails (Cabrera *et al.*, 1998).

After freeze-drying, the connection between spermatozoa head and tail becomes fragile and simple pipetting may easily disconnect head and tail. Moreover, many spermatozoa have their acrosome detached after rehydration (Magalhães, 2010). Despite all these physical changes in the sperm structure, the freeze-dried spermatozoa nucleus seems to be cytogenetically intact (Magalhães, 2010), producing normal offspring of murine (Wakayama and Yanagimachi, 1998), leporine (Liu *et al.*, 2004), bovine (Hamano *et al.*, 1999), and swine (Nakai *et al.*, 2003) species.

It is worth noting that all offspring were produced using Intracytoplasmic Sperm Injection (ICSI) because the freeze-drying process makes the sperm cells immotile and incapable of fertilizing oocytes both *in vivo* and *in vitro* (Liu *et al.*, 2004).

Sperm quality evaluation

The objective of the sperm evaluation is to investigate the sperm fecundating capacity in relation to the number of cells of normal structure and function (Watson, 1990). Currently, there is no isolated test or assessment that may predict the fecundating capacity of ejaculates (Magalhães, 2010; Peña, 1997).

Sperm membranes

The following sperm membranes are affected by cryopreservation: plasma membrane, outer acrosomal membrane, and mitochondrial membranes. The plasma membrane surrounds the whole spermatozoon and the evaluation of sperm membrane integrity is an important marker for the success of cryopreservation, since membranes are extremely sensitive to cryoinjuries.

Under cryopreservation stress, membranes may reorganize themselves thus allowing the formation of vulnerable sites leading to excessive permeability or even membrane collapse. In the sperm membrane such stress is related to the lipid transitional phase changes, in which the functional state of the membrane is altered (Holt *et al.*, 1992).

Fluorescent probes

To assess the membrane integrity, several staining methods have been developed. Staining with propidium iodide (PI) became a popular method because it is technically simple, stable and efficient in assessing plasma membrane integrity, alone or in association with other probes (Arruda *et al.*, 2007). PI specifically binds to DNA, but it does not cross the intact plasma membrane. Thus, the nucleus of ruptured plasma membrane cells present red color whereas intact membrane presents fluorescent green color (Garner *et al.*, 1986; Celeghini *et al*., 2004).

Pisum sativum agglutinin (PSA) specifically binds to the manosidase (Cross *et al.*, 1986), and when used together with fluorescein isotiocianate (FITC) it stains acrosome-reacted sperm in yellowish-green (Graham *et al.*, 1990; Celeghini *et al.*, 2004).

It is important to assess structural integrity of the sperm cell because the functionality of gametes is directly related to the plasma membrane integrity, which means that for every structural injury there is a corresponding functional change (Zuccari, 1998).

Nucleic proteins associated with DNA constitute the sperm chromatin, which is more compact than the chromatin of somatic cells (Evenson *et al.*, 1980). The high condensation of sperm chromatin helps maintaining DNA integrity and protection against

mutation or environmental stress, thus enabling normal oocyte fertilization and chromatin decondensation. Chromatin alterations may affect the decondensation rate, which seems to be a species-specific phenomenon and may be related to the kind of protamin present and the extension of disulphide links (Perreault *et al*., 1988).

Staining with the Acridine Orange probe (AO) allows the differentiation between double-stranded and single-stranded DNA (Ichimura, 1975). AO molecules permeate intact DNA (yellowish-green staining) or bind to denatured DNA phosphate groups (orangey-red staining; Mello, 1982). An important limitation of this technique is the subjectivity of the intepretation of results. Nevertheless, AO is recommended due to its practicality.

Comet assay

Another DNA analysis test is the Comet Assay (CA). In this technique a single cell is analyzed and DNA damage detected and quantified. The CA uses nucleus embedded in agarose and exposed to an electric field (Östling and Johanson, 1984). Singh *et al*. (1988) formulated its alkaline version to detect single-stranded DNA breaks. This version is being used to evaluate sperm cells that underwent cryopreservation (Magalhães, 2010).

The electric current pulls the DNA out of the nucleus so that damaged or loose DNA fragments migrate more than intact ones. Resulting images resemble the shape of a comet and their assessment enables the measurement of the extension of DNA damage (Fairbairn *et al*., 1995).

Together with other DNA integrity assessments, the Comet Assay may help predict whether a freeze-dried sample shall be used for the embryo production.

Conclusion

Taking into consideration the fact that the cryoinjuries that occur during the freeze-drying process do not cause damage to sperm DNA, domestic cat freeze-dried sperm samples might be used in the IVP of embryos. Nevertheless, there is a need for Intracytoplasmic Sperm Injection (ICSI) since spermatozoa are immotile after freeze-drying. The ICSI is already common practice in feline embryo production and thus can be recommended for wild felines.

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