CryoLetters 44(5), 249-257 (2023)

https://doi.org/10.54680/fr23510110112

PERSPECTIVE

POTENTIAL AND REALITY OF CRYOPRESERVING SOMATIC CELLS OF WILD FELIDS FOR CONSERVATION

Luanna Lorenna Vieira Rodrigues and Alexsandra Fernandes Pereira*
Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid (UFERSA), Mossoró, RN, Brazil.
* Corresponding author's E-mail: alexsandra.pereira@ufersa.edu.br

Abstract

The loss of biodiversity caused by anthropogenic actions is also a reality for the members of the Felidae family. Except for the domestic cat, all felid species have some degree of threat of extinction in their natural habitat. For this reason, felids have been included in conservation-related studies. This scenario has aroused increasing interest in the formation of somatic cell banks, which when efficiently implemented can be used in preservation strategies for the species. Nevertheless, one of the important steps in the formation of these banks is the understanding of the technical principles and variations involved in cryopreservation techniques, especially because cryopreservation increases the possibilities for Assisted Reproduction Technologies (ARTs) by making the use of biological materials independent of time and space. In wild felids, several species already have promising results in the formation of somatic cell banks, and studies aimed at better viability rates have been constantly proposed, as well as new species have been studied. In some species, aspects involved in successful cryopreservation are already well defined, and slow freezing associated with cryoprotectant solutions composed of intra- and extracellular substances is the most useful approach. The aim of this review was to present the main parameters involved in the elaboration of a somatic cell cryopreservation protocol and their effects, as well as to address the main results achieved for different wild felids.

Keywords: biological banks; conservation tools; slow freezing; cryoprotectants

INTRODUCTION

Felids are the top predators in many ecosystems (1) and their disappearance affects community structure through meso-predator release, resulting in an increase in the abundance of small predators, a decline in prey populations, and species extinctions (2). The Felidae family is divided into two groups: big cats and small cats. This division is due to the ability of these animals to roar or not; the large felids have the ability to roar, being the entire genus *Panthera*; the small felids represent all other genera that do not have that ability (3).

The Felidae family consists of 40 species, among which 28 have decreasing populations. Most of these declining species belong to the genera *Leopardus* and *Panthera*. Among eight species of the genus *Leopardus*, seven have declining populations. For the genus *Panthera*, all five existing species are in population decline, mainly due to illegal hunting, habitat loss and degradation (5). This fact has aroused interest in public policies aimed at the conservation of endangered species. As an example, the leopard (*Panthera pardus*) is legally protected in South Africa. However, the panther continues to be persecuted, along with meso-predators, such as caracal (*Caracal*

caracal). These conflicts can have negative impacts on biodiversity, and the continual and rapid decline of biodiversity at local and global scales requires informed and effective responses by policy makers, conservationists, and society to change the course of survival for species (5)..

In this context, somatic cell banks provide a viable and expandable source of genetic material and living cells that offer multiple possibilities for molecular and basic research (6), such as somatic cells as a source of cloning by somatic cell nuclear transfer [SCNT; (7)] and cells induced to pluripotency (8). Cell cryopreservation is a crucial step for these banks to be functional, being a key point responsible for the quality and viability of the samples after thawing (9).

In general, slow freezing at programmed or un-programmed cooling rates is the commonly used technique for somatic cell cryopreservation, in which the temperature is reduced in a gradual and controlled manner (10). In addition to the technique employed, the quality of the samples after thawing depends on the cryoprotectants (CPAs) used, which varies between intracellular, extracellular and their combinations (11, 12, 13). Slow freezing consists of the following steps: cells are resuspended and transferred to cryovials in the presence of cryoprotectant solution. The cryovials are transferred to the freezing container, which is subsequently taken by programmable freezing to −80 ºC for a period of 12 h, and then deposited in liquid nitrogen (9).

Cryo-banks of endangered animal species represent an extremely valuable backup of current biodiversity (6), and over the years somatic cells of small and big wild felids have been established and evaluated under different culture and preservation conditions. Our aim was to examine state-of-art cryopreservation for somatic cells, as well as the use of these cells in different conservation strategies. In addition, the preservation of wildlife genetic material, mainly derived from zoos, includes many endangered, extinct or completely extinct species (6). We also examine the biobanks around the world.

SLOW FREEZING OF SOMATIC CELLS FOR WILD FELIDS

Controlled rate or slow freezing methods have been developed over the last 40 years for the preservation of different types of samples. These samples are cooled in a controlled way (for mammalian cells −1 °C/min) using lower concentrations of CPAs, and thus producing ice crystals (14). However, intracellular ice formation is reduced with the use of slow cooling rates and dehydration of cells, and even if ice crystals are nucleated in the samples, cell viability and function are preserved in many cells (15). The considerations to achieve a successful slow freezing cryopreservation are: choice of CPA solution, sample preparation for freezing, controlled rate cooling protocol, storage, thawing and CPA removal (14). Cell based systems must be prepared to undergo freezing, and an appropriate CPA solution must be chosen avoiding the mechanisms that could impair the cell-based products function and integrity, i.e., osmotic stress and toxicity. Moreover, samples should be preserved at an appropriate low temperature and the thawing conditions optimised (14).

Cells of wild felids have been cryopreserved through slow freezing for applications in different ARTs (Table 1). Initially in small felids, Gómez et al (16, 17) cryopreserved African wild cat (*Felis silva lybica*) cells for performing SCNT of synchronized somatic cells into enucleated oocytes of domestic cats. Thongphakdee et al (4) established the culture and cryopreservation of fibroblasts from the marbled cat (*Pardofelis marmorata*). Gómez et al. (18) and Tovar et al. (19) carried out cryopreservation of cells from desert cat (*Felis margarita*) and Chilean cat (*Leopardus guigna*), respectively, both obtaining high rates (94% and 90.7%) of viability after thawing. Moro et al (20) cryopreserved and cultured cheetah cells producing blastocysts using domestic cat oocytes by SCNT. Moulavi et al (7) cryopreserved and cultured Asiatic cheetah cells. Veraguas et al (21) used cryopreserved domestic cat and kodkod cells by slow freezing in cell cycle synchronization studies and defined fetal bovine serum starvation as the method of choice for fibroblasts of both species, having been obtained from embryos by SCNT. More recently, a comparison of slow freezing and the efficiency of different cryopreservation solutions (2.5% and 10% dimethylsulfoxide (DMSO)) obtained viabilty > 80% for Northern tiger cat (*L tigrinus*) and pampas cat (*L. colocolo*) (11). Also, in Fishing cat (*Prionailurus viverrinus*), Sukaparangsi et al. (22) used slow freezing, DMSO and a commercially used medium (Recovery™ Cell

Table 1. Use of slow freezing for cell cryopreservation of small and big felids.

Species	Threat degree	Main result	Ref
		Small felids	
African wild cat (*Felis silvestres lybica*)	Endangered	Obtained SCNT embryos/Birth of cloned kittens from domestic cats	(16), (17)
Marbled car (*Pardofelis marmorata*)	Near threatened	Obtained SCNT embryos up to the blastocyst	(4)
Sand cat (*Felis margarita*)	Near threatened	Birth of cloned kittens born from domestic cats	(18)
Kodkod (*Leopardus guigna*)	Vulnerable	Method of cell culture	(19)
Cheetah (*Acinonyx jubatus*)	Vulnerable	Blastocysts produced using domestic cat oocytes	(20)
Asian Cheetah (*Acinonyx jubatus venaticus*)	Critically endangered	First report of iSCNT in Cheetah using non-viable frozen cells	(7)
Domestic cat (*Felis silvestris catus*), Kodkod (*Leopardus guigna*)	Domestic, vulnerable	Synchronization of cell cycle	(21)
Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*)	Vulnerable, near threatened	Establishment of cryoprotectant solution	(11)
Fishing cat (*Prionailurus viverrinus*)	Vulnerable	Cryopreservation of somatic cells from living and post-mortem samples	(22)
Pallas's cat (*Otocolobus manul*; *Felis manul*)	Least concern	Synchronization of cell cycle	(41)
		Big felids	
Siberian tiger (*Panthera tigres altaica*)	Endangered	Synchronization of cell cycle	(23)
Bengal tiger (*Panthera tigris tigris*)	Endangered	Establishment and cryopreservation of a cell line	(24)
Siberian tiger (*Panthera tigris altaica*)	Endangered	Establishment, characterization, and cryopreservation of a cell line	(25)
Iberian Lynx (*Lynx pardinus*)	Endangered	Cryobanking from skin biopsies, cryopreservation and culture of explants and cells	(26)
Snow leopard (*Panthera uncia*)	Vulnerable	Inducing pluripotency in somatic cells	(27)
Jaguar (*Panthera onca*)	Near threatened	Establishment, isolation, and cryopreservation of primary fibroblast culture	(28)
Jaguar (*Panthera onca*)	Near threatened	Establishment of cryoprotectant solution	(11)
Jaguar (*Panthera onca*)	Near threatened	Establishment of cryoprotectant solution	(9)
Jaguar (*Panthera onca*)	Near threatened	Establishment of cryoprotectant solution	(35)
Puma (*Puma concolor*)	Least concern	Isolation, characterization, and cryopreservation	(29)
Jaguarundi (*Puma yagouaroundi*; *Harpailurus yagouaroundi*).	Least concern	Synchronization of cell cycle	(41)

Culture Freezing Medium (ThermoFisher) as the basis for establishing a cryobank.

For big cats, Song et al (23) cultured and cryopreserved cells from Siberian tiger (*Panthera tigres altaica*). Guan et al (24) and Liu et al (25) cryopreserved a line of fibroblasts derived from the Bengal tiger (*Panthera tigris tigris*) and the Siberian tiger by slow freezing, respectively. León-Quinto et al (26) established somatic tissue banks for the Iberian lynx (*Lynx pardinus*), the most endangered felid in the world. In 2012, Verma et al (27), performed the cryopreservation of snow leopard *(Panthera uncia*) fibroblasts for pluripotency induction studies. Subsequently, Mestre-Citrinovitz et al (28) described a protocol for obtaining and cryopreserving fibroblasts from ear samples of jaguar (*Panthera onca*). More recently, studies carried out with the same species compared the efficiency of different cryoprotectant solutions during the cryopreservation of fibroblasts from these animals (9, 11). Another large felid is the puma, where data on somatic cell cryopreservation and establishment of fibroblast lines are already available (29).

In all the studies cited, both in small and big cats, slow freezing was the cryopreservation technique used, demonstrating its wide applicability to cells. Over the years, it has been established as the technique of choice for cell cryopreservation. Consequently, when there are variations between protocols, often this relates to the composition of the cryoprotectant solution.

VARIABLES OF SLOW FREEZING IN SOMATIC CELLS

Regardless of cell type, the success of any cryopreservation protocol is dictated by careful selection of a few common variables: type of CPA including permeant and non-permeant agents or a combination of both, as well as appropriate cooling and thawing rates (30). Two main issues are the cooling rate and ice crystal formation. Slow cooling rates (<1 °C/min) allow cells ample time to dehydrate in the presence of extracellular ice, thus preventing excessive intracellular ice formation.

Cells are exposed to high concentrations of solutes as well as any CPAs that have been added for a long period of time. Disproportionate dehydration can be irreversible and is one of the causes of damage induced by cryopreservation, because as ice crystals continue to grow in the extracellular medium, the solutes in the solution become concentrated in the wastewater channels between ice crystals, leading to osmotic shock and increased toxicity (31). The optimal cooling rate for cell survival is outlined by the hypothesis that the highest cell viability will be achieved by an intermediate cooling rate, which will provide a balance between these two scenarios, and it is important to mention that different cell types will have different optimal cooling rates (32).

Cooling devices

The choice of equipment to deliver the chosen cooling rate rests between passive cooling devices (PCDs) and controlled rate freezers (CRFs). PCDs, using an external cold source (often a -80°C freezer) and an insulated container to hold the samples, are the simplest and cheapest solution. Thus, varying the insulating material and the temperature of the cold source will allow the control of cooling rate and temperature range over which cooling may be approximately linear (33). Among the commercially available PCDs is Mr. Frosty® (Nalgene, Rochester, NY) that's been designed to offer a cooling rate of ~ 1 °C/min between -10 °C and −40 °C with isopropanol as the conductive medium (33).

Different studies have used Mr. Frosty® for slow freezing of cells. Gómez et al (16, 17) resuspended African wild cat cells in the CPA solution and cooled at 1.0 ºC/min to -80 ºC before storage in liquid nitrogen. Tovar et al. (19) cryopreserved kodkod (*Leopardus guigna*) cells using this device. In that work, the authors refer to this system as a mechanical freezer, where the freezing rate was around 1° C/min and then the sample is placed inside an □80°C freezer. In 2014, León-Quinto et al (34) used the same system for fetal and adult Iberian lynx fibroblasts. Cryovials containing 1.0 mL cryo-solution were cooled in a Mr. Frosty® container at a cooling rate of 1°C/min. Subsequently, when the sample reached -70°C, it was plunged into liquid nitrogen.

Subsequently, Veraguas et al (21) employed Mr. Frosty® in cryopreserving kodkod and domestic cat cells. The pelleted fibroblasts were resuspended in frozen medium and placed in cryogenic vials. Vials were frozen at 1°C/min using a freezing container placed inside a -80 °C freezer for 3 days and were then transferred to liquid nitrogen. More recently, two works used Mr. Frosty® for controlled temperature reduction in puma fibroblasts. Cell suspension in cryovials were maintained at 4°C for 10 min, and transferred to a -80 °C freezer in this PCD system for 12 h using a cooling rate of 1 °C/min before the cryovials were stored in liquid nitrogen (9, 35). The same methodology has been employed for puma fibroblasts (29).

In addition to the Mr. Frosty® system, there are other systems. Arantes et al (11) worked with cells from three different wild felids: northern tiger cat, pampas cat and puma. In their work, they used straws. Six straws were frozen for each concentration of cryoprotectant tested. The 0.25 mL straws were submitted to freezing at -80°C for 24 h before placing in liquid nitrogen and remained there until further evaluation. In addition to being a device for slow freezing, straws are devices for filling samples (11).

From these data, it is possible to observe the importance of cooling devices as a parameter of success when using slow freezing. Mr. Frosty® appears to be the most used due to its low cost and efficiency in gradually reducing temperature.

Table 2. Cryoprotectant solutions used for the slow freezing of somatic cells derived from wild felids.

Species	Cryo-solution	Main result	Ref
	Small felids		
African wild cat (*Felis silvestres lybica*)	10% DMSO + 10% DMSO	85-95% of cell viability / 1.0-3.5% of embryo survival	(16), (17)
Marbled car (*Pardofelis marmorata*)	10% DMSO + FBS	Reprogrammed fibroblast cells in domestic cat and rabbit, obtained SCNT embryos to the blastocyst	(4), (43)
Sand cat (*F. margarita*)	10% DMSO + 10% FBS	94% of cell viability	(18)
Kodkod (*Leopardus guigna*)	8% DMSO + 22% FBS	79.6% of cell viability	(19)
Cheetah (*Acinonyx jubatus*)	10% DMSO + 10% FBS	47.7% of rate blastocyst	(20)
Asian cheetah (*Acinonyx jubatus venaticus*)	10% DMSO + 50% FBS	First iSCNT report in Cheetah using nonviable frozen cells	(7)
Domestic cat (*F. silvestris catus*), Kodkod (*L guigna*)	8% DMSO + 22% FBS	74.7-95.9%/ 83.5-97.3% of cell viability	(21)
Northern tiger cat (*L. tigrinus*), pampas cat (*L. colocolo*)	2.5% DMSO + 10% FBS, 10% DMSO + 10% FBS, CryoSOfree™	82.2%-98% of cell survival	(11)
Fishing cat (*Prionailurus viverrinus*)	10% DMSO, Recovery™	Preserved somatic cells from living and *post-mortem* samples	(22)
Pallas's cat (*Otocolobus manul*; *Felis manul*)	10% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	Above 80% of cell viability	(41)
	Big felids		
Siberian tiger (*Panthera tigres altaica*)	10% DMSO + 40% FBS	90%~95% of cell viability	(23), (25)
Bengal tiger (*Panthera tigris tigris*)	10% DMSO + 90% FBS	Above 90% of cell viability	(24)
Iberian Lynx (*Lynx pardinus*)	5-15% DMSO + 0.1-0.2 M sucrose + 35% FBS	90% of cell viability	(26)
Snow leopard (*Panthera uncia*)	10% DMSO + 90% FBS	Inducing pluripotency in somatic cells	(27)
Jaguar (*Panthera onca*)	10% DMSO + 10% FBS	Establishment of efficient culture and cryopreservation	(28)
Jaguar (*Panthera onca*)	10% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	61.9-84.4% of cell viability	(11)
Jaguar (*Panthera onca*)	10% DMSO or 10% EG w/ or w/o sucrose	45.8-58.6% of cell viability	(9)
Jaguar (*Panthera onca*)	1.5 M DMSO + 10% FBS + 0.2 M sucrose	Above 95.7% of cell viability	(35)
Puma (*Puma concolor*)	10% DMSO + 10% FBS + 0.2 M sucrose	Above 92% of cell viability	(29)
Jaguarundi (*Puma yagouaroundi*; *H. yagouaroundi*)	10% DMSO + 10% FBS, CellBanker2®, CryoDefend Cell Lines®	Above 80% of cell viability	(41)

Cryoprotective solutions

Cryopreservation processes inflict damage to cells in several ways, and to moderate the damage, CPAs are employed (31). In general, the protective solution acts from a combination of factors, including hydrogen bonding, protection of cell membrane, solute dilution and increased solution viscosity, among others (31). Permeating agents, also knowns as intracellular CPAs, include glycerol, DMSO, ethylene glycol (EG), and propanediol (PG). The ability of these compounds to protect cells from mechanical and osmotic effects depends on several properties (30). One mechanism is the strong interaction of permeating CPAs with water through hydrogen bonding, resulting in freezing point depression (36).

Some CPAs like DMSO are thought to increase cell permeability by altering membrane dynamics. At low concentrations (5%) DMSO decreases membrane thickness and in turn increases membrane permeability. At commonly used concentrations (10%), water pore formation in biological membranes is induced, making intracellular water more readily replaced by CPAs (30). In order to reduce cell injury, intracellular CPAs plays a significant role in the cryo-banks. For these reasons, it is necessary to evaluate and compare the effects of intracellular cryoprotectants on cell viability (Table 2).

Silva et al. (35) reported 73.2% viability for jaguar (*Panthera onca*) fibroblasts with 10% DMSO. When evaluating 2.5% and 10% DMSO for fibroblasts of Northern tiger cat (*Leopardus tigrinus*), pampas cat (*Leopardus colocolo*) and jaguar, Arantes et al. (11) reported no differences in cell viability of three species after thawing, obtaining 82.2–98% viable cells. Both works corroborate previous studies in fibroblasts from different wild felids, which showed good cell viability (>80%) after thawing with 10% DMSO as cryoprotectant (9, 26, 27). The use of 10% EG was evaluated in cryopreservation of jaguar somatic cells in the presence or absence of sucrose; a lower cell viability (45.8%) was noted when used alone and higher viability (52.4%) in combination with sucrose (9).

Non-permeating agents (NPAs) do not enter into the intracellular spaces and exert their protective influence outside of the cells. They are typically larger and covalently linked as either polymers, dimers, or trimers. Some commonly-used agents in this class are sucrose and fetal bovine serum (FBS) (30). Several studies point to the efficiency of sucrose in association with intracellular cryoprotectants for cell cryopreservation in wild felids. For example, León-Quinto et al. (26) observed that the combination of 10% DMSO with 0.1 M or 0.2 M sucrose was more efficient as compared to the absence of sucrose. Subsequently, León-Quinto et al (34) observed that sucrose has a positive effect on cell viability, as it acted by promoting a decrease in osmotic pressure through cell dehydration, resulting in increased cell viability (36, 37).

Silva et al (35) suggested that the efficiency of maintaining cell viability of jaguar fibroblasts after cryopreservation is linked to combining 10% DMSO with 0.25M sucrose. They reported that, corroborating Oliveira et al. (9), the use of FBS also increased cell protection capacity when an extracellular cryoprotectant is used.

Several studies successfully used FBS in cell cryopreservation at different concentrations. Song et al (23) used 10% DMSO in conjunction with 40% FBS, obtaining 95% viability for Siberian tiger cells after warming. Subsequently, Liu et al (25) used 50% FBS in association with 10% DMSO to cryopreserve Siberian tiger cells and reported a cell viability greater than 90% after freezing. Verma et al (27) used 10% DMSO + 90% FBS, and obtained an 80% survival in snow leopard cells. Moulavi et al (7) used 50% FBS + 10% DMSO for cryopreserving Asiatic Cheetah cells, and obtained 85% cell viability. Lower concentrations of FBS have also been reported to be successfully applied, as in the work by Silva et al. (35), who used 10% FBS, DMSO and sucrose and obtained a cell viability of 73.2%.

These works demonstrate the variability of CPA concentrations and combinations that can be successfully used in different felids, denoting the need to compare different concentrations to define an optimized protocol according to the species of interest.

THE USE OF CRYOPRESERVED CELLS IN CRYO-BANKS

The establishment and use of wildlife bio-banks is crucial to the development of basic and applied scientific research and is indispensable for the long-term storage of somatic cells (10, 38). Zoos and zoological research institutions are key players in the conservation of genetic variability and provide reliable access to

Table 3. Wild felids somatic resource biobanks around the world.

Species	Threat	Biobank	Sample	Country	Ref
		Small felids			
Sand cat (*Felis margarita*)	Near threatened	Birmingham Zoo	Tissue	USA	(18)
Marbled cat (*P. marmorata*), flat-headed cats (*P. planiceps*)	Near threatened, Endangered	Genome Resource Bank	Somatic cell and tissue	Thailand	(4)
Asian golden cat (*C. temminckii*), Marbled cat (*P. marmorata*), Siamese cat (*F. catus*)	Near threatened, near threatened, domestic	Khao Kheow Open Zoo	Tissue	Thailand	(39)
Fishing cat (*Prionailurus viverrinus*)	Vulnerable	Biobank Liquid Nitrogen Tank Facility	Cell	Thailand	(22)
Pallas's cat (*Otocolobus manul*; *Felis manul*)	Least concern	Zoological Garden	Cells	Poland	(41)
		Big felids			
Siberian tiger (*Panthera tigres altaica*)	Endangered	Xuzhou Zoo	Tissue	China	(23)
Iberian Lynx (*Lynx pardinus*)	Endangered	Iberian lynx Biological Resource Bank	Tissue	Spain	(26)
Snow leopard (*Panthera uncia*)	Vulnerable	Mogo Wildlife Park	Tissue	Australia	(27)
Leopard (*Panthera pardus*)	Vulnerable	Khao Kheow Open Zoo	Tissue	Thailand	(39)
Jaguar (*Panthera onca*)	Near threatened	Biobank Buenos Aires Zoo	Tissue	Argentina	(28)
Leopard (*Panthera pardus*), tiger (*P. tigris*), lion (*P. leo*)	Vulnerable, endangered, vulnerable	Nehru Zoological Park	Tissue	India	(40)
Northern tiger cat (*Leopardus tigrinus*), pampas cat (*L. colocolo*) and jaguar (*P. onca*)	Vulnerable, near threatened, near threatened	Brasília Zoo	Cells	Brazil	(11)
Jaguarundi (*Puma yagouaroundi*; *H. yagouaroundi*).	Least concern	Zoological Garden	Cells	Poland	(41)

valuable materials. Sampling is optimally implemented in routine zoo veterinary work (6). Studies for conservation and recovery of cells from endangered wild felids from several continents were carried out in cooperation with zoos and cryo-banks around the world (Table 3). Thus, in studies with small felids in North America, Gómez et al (18) used samples from *Felis margarita* in the Birmingham Zoo to obtain cells for SCNT cloning and three kittens from frozen/thawed cells were born from cloned embryos. Thongphakdee et al (4) used somatic cells from epithelial and muscular tissues of male and female marbled cats and flat-headed cats (*Prionailurus planiceps*) and demonstrated that donor cell line affects the developmental success up to the morula stage of the embryos. They reported that the genomes of both species have been preserved since 2003 in the Genome Resource Bank, which was jointly developed by the Zoological Park Organization of Thailand, on the Asian continent. Another source of somatic resources in the same country is the Khao Kheow Open Zoo, from where Wittayarat et al (39) recovered skin samples from Asian golden cat (Catopuma temminckii), Marbled cat (*Pardofelis marmorata*), Siamese cat (*Felis catus*), and established fibroblasts which were used for cell cycle synchronization in G_0/G_1, an essential step for SCNT cloning.

For big cats, Song et al. (23) performed cell culture and cell cycle synchronization of Siberian tiger fibroblasts where ear tissue from a 9-month-old male animal was used to generate

cell lines. In Europe, León-Quinto et al (26) carried out culture and cryopreservation of Iberian lynx cells obtained from a somatic bank established by the same group in 2009, where somatic tissues from different parts of the body (muscle, oral mucosa, bone marrow, spinal cord, and intestines) were recovered. In Oceania, Verma et al. (27) used snow leopard tissue samples allocated at Mogo Wildlife Park, Australia, to obtain fibroblasts, which were used in pluripotency induction studies.

Another work carried out by Mestre-Citrinovitz et al. (28) described the collection, isolation, and culture of jaguar somatic tissues by the Biobank of the Buenos Aires Zoo, Argentina. The Biobank has a collection of 570 samples of 45 autochthonous and endangered species, including the jaguar. The fibroblasts generated were part of 6,700 samples, including tissues such as muscle, ovary, testis, blood, fibroblast, sperm, hair and fluids, and cells from 450 individuals from 87 different species. In India, Yeliseti et al (40) performed interspecies nuclear transfer using fibroblasts derived from ear skin that was collected post-mortem from three large felids (leopard, tiger, and lion). These were successfully synchronized and used for the development of blastocysts using rabbit oocytes as recipient cytoplasm. In South America, the collection, establishment and cryopreservation have been successfully carried out based on samples of big felids obtained from zoos in the northeast region (9, 29, 35) and the central west of Brazil (11).

More recently, in Poland, skin biopsies were obtained from jaguarundi and Pallas's cat that were sourced from the Zoological Garden in Kraków to study effects of serum starvation and contact inhibition on cell cycle synchronization and survival of fibroblasts. The research finds application in preparing donor karyoplasts for SCNT in felids (41). Also, Sukparangsi et al (22) provide a conservation plan using cell technology for fishing cats and recommend tissue collection and culture procedures for zoo to facilitate the preservation of cells from post-mortem animals and living animals. All cells in this study are currently stored in the Biobank Liquid Nitrogen Tank Facility of the Zoological Park Organization (ZPO) of Thailand.

These intensively managed programs allow zoos to maintain genetically diverse populations over time, but with very few exceptions, these populations are not currently sustainable through natural breeding alone (42). There is a great variety of somatic resource biobanks spread around the world from which samples can be used for cloning, reproduction and conservation.

FINAL CONSIDERATIONS

In view of the results of the work carried out with wild felids over the years, one can see the importance of forming somatic banks for these species, with the aim of conserving them using ARTs. It is evident that knowledge and technical tools for cell cryopreservation are necessary for the efficient use of these somatic banks, such as filling devices and optimised cryoprotectants. In some species these aspects are already well defined, and slow freezing associated with cryoprotectant solutions composed of intra and extracellular cryoprotectants is the most used methodology. Therefore, the next step is the improvement of techniques such as cloning and inducing cells to pluripotency.

REFERENCES

1. Gross M (2012) *Current Biology* **22**, R893–R895.
2. Prugh LR, Stoner CJ, Epps CW, Bean WT, Ripple WJ, Laliberte AS & Brashares JS (2009) *Bioscience* **59**, 779-791.
3. Hast MH (1989) *Journal of Anatomy* **163**, 117-121.
4. Thongphakdee A, Siriaroonrat B, Manee-In S, Klincumhom N, Kamolnorranath, S, Chatdarong K & Techakumphu M (2010) *Theriogenology* **73**, 120-128.
5. Tshabalala T, McManus J, Treves A, Masocha V, Faulconbridge S, Schurch M, Goets S & Smuts B (2021) *Ecological Indicators* **121**, 107201.
6. Hildebrandt TB, Hermes R, Goeritz F, Appeltant R, Colleoni S, de Mori B, Diecke S, Drukker M, Galli C, Hayashi K, Lazzari G, Pasqualino L, Payne J, Renfree M, Seet S, Stejskal J, Swengen A, Williams SA, Zainuddin ZZ & Holtze S (2021) *Theriogenology* **169**, 76-88.
7. Moulavi F, Hosseini SM, Tanhaie-Vash N, Ostadhoseini S, Hosseini SH et al (2017) *Theriogenology* **90**, 197-203.
8. Verma R, Liu J, Holland MK, Temple-Smith P, Williamson M & Verma PJ (2013) *BioResearch* **2**, 72-76.

9. Oliveira LRM, Praxedes ÉA, Silva MB, Ribeiro LR, Silva HVR & Pereira AF (2021) *Anais da Academia Brasileira de Ciências* **93**, e20190314.
10. Praxedes ÉA, Borges AA, Santos MVO & Pereira AF (2018) *Zoo Biology* **37**, 258-263.
11. Arantes LG, Tonelli GS, Martins CF & Báo SN (2021) *Biopreservation and Biobanking* **19**, 11-18.
12. Weng L & Beauchesne PR (2020) *Cryobiology* **94**, 9-17.
13. Elliott GD, Wang S & Fuller BJ (2017) *Cryobiology* **76**, 74-91.
14. Gurruchaga H, Del Burgo LS, Hernandez RM, Orive G, Selden C, Fuller B, Ciriza J & Pedraz JL (2018) *Journal of Controlled Release* **281**, 119-138.
15. Massie I, Selden C, Hodgson H, Fuller B, Gibbons S & Morris GJ (2014) *Tissue Engineering Part C: Methods* **20**, 693-702.
16. Gómez MC, Jenkins JA, Giraldo A, Harris RF, King A, Dresser BL & Pope CE (2003) *Biology of Reproduction* **69**, 1032-1041.
17. Gómez MC, Pope CE, Giraldo A, Lyons LA, Harris RF, King A & Dresser BL (2004) *Cloning and Stem Cells* **6**, 247-258.
18. Gómez MC, Pope CE, Kutner RH, Ricks DM, Lyons LA, Ruhe M & Reiser J (2008) *Cloning and Stem Cells* **10**, 469-484.
19. Tovar H, Navarrete F, Rodríguez L, Skewes O & Castr F O (2008) *In Vitro Cellular & Developmental Biology - Animal* **44**, 309–320.
20. Moro LN, Hiriart MI, Buemo C, Jarazo J, Sestelo A, Veraguas D, Rodriguez-Alvarez L & Salamone DF (2015) *Reproduction* **150(1)**, 1-10.
21. Veraguas D, Gallegos PF, Castro FO & Rodriguez-Alvarez L (2017) *Reproduction in Domestic Animal*s **52**, 881-889.
22. Sukparangsi W, Thongphakdee A, Karoon S, Ayuttay NSN, Hengkhunthod I, Parkongkeaw R, Bootsri R & Sikaeo W (2022) *Frontiers in Veterinary Science* **9**, 989670.
23. Song J, Hua S, Song K & Zhang Y (2007) *In Vitro Cellular & Developmental Biology - Animal* **43**, 203-209.
24. Guan WJ, Liu CQ, Li CY, Liu D, Zhang WX & Ma YH (2010) *CryoLetters* **31**, 130-138.
25. Liu C, Guo Y, Liu D, Guan W & Ma Y (2010) *Biopreservation and Biobanking* **8**, 99-105.
26. León-Quinto T, Simón MA, Sánchez A, Martín F & Soria BN (2011) *Cryobiology* **62**, 145-151.
27. Verma R, Holland MK, Temple-Smith P & Verma PJ (2012) *Theriogenology* **77**, 220-228.
28. Mestre-Citrinovitz AC, Sestelo AJ, Ceballos MB, Barañao JL & Saragueta P (2016) *Current Protocols in Molecular Biology* **116**, 28-7.
29. Lira GPO, Borges AA, Nascimento MB, Aquino LVC, Moura LFMP, Silva HVR, Ribeiro LR, Silva AR & Pereira AF (2022) *Biopreservation and Biobanking* **20**, 557-566.
30. Whaley D, Damyar K, Witek RP, Mendoza A, Alexander M & Lakey JR (2021) *Cell Transplantation* **30**, 1-12.
31. Murray KA & Gibson MI (2022) *Nature Reviews Chemistry* **6**, 579-593.
32. Mazur P (1970) *Science* **168**, 939-949.
33. Hunt CJ (2017) in *Stem Cell Banking*, (ed) Crook J & Ludwig T, New York, pp 41-77.
34. León-Quinto T, Simón MA, Cadenas R, Martínez A & Serna A (2014) *Cryobiology* **68**, 227-233.
35. Silva MB, Praxedes ÉA, Borges AA, Oliveira LRM, Nascimento MB, Silva HVR, Silva AR & Pereira AF (2021) *Zoo Biology* **40**, 288-296.
36. Mandumpal JB, Kreck CA & Mancera RL (2011) *Physical Chemistry Chemical Physics* **13**, 3839-3842.
37. Cetinkaya G & Arat S (2011) *Cryobiology* **63**, 292-297.
38. Comizzoli P (2017) *Animal Reproduction* **14**, 30-33.
39. Wittayarat M, Thongphakdee A, Saikhun K, Chatdarong K, Otoi T & Techakumphu M (2013) *Reproduction in Domestic Animals* **48**, 305-310.
40. Yelisetti UM, Komjeti S, Katari VC, Sisinthy S & Brahmasani SR (2016) *In Vitro Cellular & Developmental Biology - Animal* **52**, 632-645.
41. Młodawska W, Mrowiec P, Bochenek M, Wnęk K, Kochan J, et al (2022) *Annals of Animal Science* **22**, 1245-1255.
42. Swanson WF (2023) *Theriogenology* **197**, 133-138.
43. Thongphakhee A, Numchaisrika P, Omsongkram S, Chatdarong K, Kamolnorranath S, Dumnui S & Techakumphu M (2006) *Reproduction in Domestic Animals* **41(3),** 219-226.