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REVIEW

# Cryopreservation in mammalian conservation biology: current applications and potential utility



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**Abstract:** A rapid and continuous decline in wild mammalian species populations has been documented in recent decades. Although in situ conservation strategies such as habitat preservation are usually the best way to preserve biodiversity, other rescue strategies such as germplasm cryopreservation are sometimes necessary. Germplasm cryopreservation involves the freezing of gametes, embryos, gonadal tissues, or somatic tissues of species threatened with extinction. There is substantial diversity in the cryobiological requirements among cell types and tissues of each species. Research has focused on adapting techniques developed for the conservation of the genetic material of domestic animals for use with wild species about which relatively little is known. This review describes and discusses the current and potential use of cryobanking for the preservation of cells and tissues of threatened species.

**Keywords:** cryobanking, gametes, somatic cells, germplasm preservation

## Introduction

A rapid and continuous decline in wild mammalian species has been documented in the last decade, whereby one in every four mammalian species is threatened with extinction.[1] Although in situ conservation strategies such as habitat preservation are usually the best way to preserve biodiversity, other rescue strategies such as germplasm cryopreservation are sometimes necessary to facilitate the natural processes of evolution to continue.[2] However, this method alone is inefficient when a population is severely reduced or when the majority of remaining mammals are located in unprotected areas.[3] As a result, there has been a rise in the number of ex situ conservation programs involving both in vivo and in vitro preservation, including the establishment of germplasm banks.[4]

Conservation of genetic resources focuses on the cryopreservation of gametes, embryos, gonadal tissues, and somatic tissues.[2] Germplasm cryopreservation[3] represents a connection between in situ and ex situ conservation programs.[2] In the present review, we describe and discuss the current and potential uses of cryobanking of cells and tissues aimed at the prevention of the extinction of threatened mammalian species.

## The preservation of female gametes

Female gametes (ova) can be obtained through follicle puncture, ovarian tissue biopsies, unilateral or bilateral ovariectomy, or ovary collection immediately after an animal's death, irrespective of its age.[5] Ovarian tissue, isolated follicles, and mature or immature oocytes can all be stored by cryopreservation.[6] The option of cryopreserving the

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ovarian tissue avoids many limitations encountered in mature oocyte preservation, such as the low number of mature oocytes available in the ovaries, possible deleterious effects of its conservation under low temperatures, and the need for super-ovulation procedure.[7] The major limitation of its use is the difficulty in preserving ovarian tissue, given the diversity of cell types and tissue components.[8]

Oocyte preservation is more challenging, not least because oocytes tend to be large cells that have a low surface-to-volume ratio and a low permeability coefficient, both of which hinder the migration of water and cryoprotectants (CPAs) through the cell.[9] Immature oocytes at the germinal vesicle stage that have not yet formed the spindle lack cortical granules and have a higher membrane permeability, making them more resistant to chilling injury than mature metaphase II oocytes.[10] Two methods have been described for female gametes preservation: slow freezing (SF) and vitrification. SF or conventional freezing refers to the exposure of the tissues/cells to a low concentration of CPA and cooling them slowly in a programmable freezer.[11] CPA concentration and exposure time prior to freezing needs to be balanced in order to reach sufficient dehydration; however, it has to be low enough to avoid cytotoxic damages.[12] Although it is a widespread method, in general, sophisticated and expensive programmable freezers are required for the cooling procedure, but these freezing devices are not generally available when endangered animals are found dead on the field, making impossible the gamete preservation of wild animals in such conditions.[7] On the other hand, vitrification is considered a cheap method that can be performed under field conditions with no need for special equipment, making it a good alternative for use in various settings often encountered with wildlife species,[13] including after animal death.[14] This method involves the use of high concentrations of CPAs and rapid cooling (–20,000°C/min –40,000°C/min) to achieve a glass-like highly viscous solution without the formation of ice crystals.[15] Vitrification promotes a viscosity state to the solution, but without water crystallization.[16]

In recent years, several studies have demonstrated the possibility of adapting the techniques developed for cryopreserving domestic female genetic material to wild species. The chilling of ovarian tissue has been efficiently demonstrated in kangaroos (*Macropus giganteus*[17]), red deer (*Cervus elaphus hispanicus*[18]), and collared peccaries (*Pecari tajacu*[19]), in which ovarian tissue vitrification allowed the maintenance of the morphological integrity of more than 70% of the follicles after rewarming.[20]

Cryopreservation of the gametes of non-human primates has received a great deal of attention because these animals are used as experimental models for humans. In the baboon (*Papio anubis*), ovarian tissue vitrification followed by autografting resulted in follicle survival, growth, and ovulation (as indicated by the presence of corpora lutea).[21] Similar results have been reported in cynomolgus monkeys (*Macaca fascicularis*) using both vitrification[22] and SF followed by autografting.[23] The birth of offspring from freshly grafted ovarian tissue was first described in rhesus monkeys (*Macaca mulatta*).[24]

Wiedemann et al[25] reported the success of the SF method followed by the in vitro culture of ovarian tissue in the African lion (*Panthera leo*), Amur leopard (*Panthera pardus orientalis*), black-footed cat (*Felis nigripes*), oncilla (*Leopardus tigrinus*), Geoffroy's cat (*Leopardus geoffroyi*), Northern Chinese leopard (*Panthera pardus japonensis*), rusty-spotted cat (*Prionailurus rubiginosus*), serval (*Leptailurus serval*), and Sumatran tiger (*Panthera tigris sumatrae*). On the other hand, viable immature oocytes of wolves were obtained using the vitrification method.[26]

Studies carried out on carnivorous marsupials (*Sminthopsis crassicaudata*, *Sminthopsis harrisii*, *Dasyurus viverrinus*, and *Dasyurus hallucatus*) demonstrated that follicle viability could be maintained for 48 hours during ovarian tissue chilling using phosphate-buffered saline (PBS) as the base medium. In addition, almost 70% of isolated oocytes either survived vitrification[27] or the preservation of ovarian fragments after vitrification.[28]

Conventional freezing of ovarian fragments maintained oocyte integrity in agoutis (*Dasyprocta aguti*[29]), African elephants (*Loxodonta africana*[30]), and wombats (*Lasiorhinus krefftii*[31–33]), while vitrification of ovarian fragments maintained oocyte integrity in whales.[34]

In addition, the resumption of meiosis of isolated immature oocytes of Asian antelopes (*Tetracerus quadricornis*) after vitrification followed by in vitro maturation has been reported.[35]

## The preservation of male gametes

Systematic cryopreservation and storage of male gametes from endangered species circumvents the problem of homozygosis in isolated populations by introducing new genetic material across populations and facilitates genetic exchanges between captive areas/zoos/research centers or countries.[36] It is possible to cryopreserve semen, epididymal spermatozoa, or spermatogonial stem cells that are either isolated or in testicular tissue.[37] Each cell type has specific requirements during cryopreservation. Differences in the physical size and shape of spermatozoa may influence their susceptibility to thermal

shock and resistance to osmotic stress during the freeze–thaw process when the stability of the membrane is challenged.[38] Furthermore, epididymal spermatozoa are not exposed to the complex secretions of the accessory sex glands, and this could alter both the sensitivity to chilling and the resistance to freezing of ejaculated spermatozoa.[39]

Electroejaculation is the most frequently used method for semen collection in wild species because it prevents manipulation risks during the procedure and requires chemical restraint of the animal. Other methods for semen collection include the use of internal artificial vaginas or vaginal condoms.[40] In addition, post-coital sperm recovery has proved to be successful method of semen collection in marmoset monkeys[41] and rhinoceros.[42]

The protocols used for cryomethods in the preservation of sperms of domestic animals have been adapted for use in wild species of interest.[43] Protocols such as chilling allow sperm cell preservation for short periods of time, while freezing is used for longer periods. Semen cryopreservation is an established technique and differences in efficacy may be attributed to particularities of each species.[4] In general, spermatozoa from most mammalian species have displayed good results after SF (–0.5°C/min), recovering at least some motility after thawing.[44] Tris and TES are among the most employed diluents used in semen preservation,[45] while coconut water has been used in the capuchin monkey,[46] the agouti,[47] and the collared peccary.[4] In addition, milk-based cryoextenders have also been used to successfully freeze the sperm from species such as the Asian elephant (*Elephas maximus indicus*)[48] and the Indian rhinoceros (*Rhinoceros unicornis*).[49] In searching for an ideal diluent for semen cryopreservation, many additives have been tested with the aim of fostering increased sperm viability after thawing. For example, adding detergent-active-ingredient substances (such as sodium dodecyl sulfate [SDS], or Equex) to the freezing medium improves the quality, longevity, and fertility of the sperm after thawing.[50] The use of Equex for semen cryopreservation in gray wolves (*Canis lupus*),[51] and alpacas (*Vicugna pacos*)[52] improved sperm longevity. The addition of antioxidant substances reduces the effects of cold osmotic shock and stress due to an imbalance between the reactive oxygen species (ROS) and the antioxidants.[53] In this regard, Thuwanut et al[54] evaluated the impact of adding vitamin E, Trolox (a vitamin E analog), and glutathione peroxidase (GPx) to the thawed spermatozoa of the flat-headed cat (*Prionailurus planiceps*) and concluded that the addition of GPx reduced oxidative stress and increased the success of cryopreservation.

In relation to CPAs, the use of egg yolk and glycerol has been studied in wild species.[4,55–57] Glycerol (4%–6%) in the CPA allows the recovery of thawed spermatozoa in a diverse range of species including felids,[55,58] marine mammals,[59,60] Asian elephants (*Elephas maximus*),[61,62] rhinos (*Ceratotherium simum*, *C.s. cottoni*),[63] leopards (*Neofelis nebulosa*),[64] bears (*Ursus arctos*),[65] monkeys (rhesus monkeys),[66] collared peccaries (*Pecari tajacu*),[57] and many other species. Although glycerol is used for sperm cryopreservation of a wide range of species, some studies point out that there are some restrictions in its use due to its cytotoxic effects.[67] Glycerol toxicity may cause protein denaturation, actin interaction and alterations, and direct disruption of the plasma membrane.[68] Because of this, substances such as dimethylsulfoxide (DMSO) and dimethylacetamide have been proposed as important alternatives to glycerol for semen cryopreservation in saltwater crocodiles (*Crocodylus porosus*)[69] and kangaroos (*Macropus giganteus*).[70]

Collection of the epididymal spermatozoa directly from the cauda of the epididymis and vas deferens is also an option for use in wild and captive animals that have high genetic value, and for animals killed accidentally.[71] This method provides morphologically viable cells that retain the ability to undergo capacitation, bind to the zona pellucida, and fertilize the oocyte.[72,73] Factors such as the size of the epididymis and the diameter of the vas deferens influence the choice of technique and its success for the recovery of sperm.[74] The flotation method is performed with the epididymis immersed in a buffered medium. Numerous sections in the epididymis cauda are made with a blade, followed by recumbency for some minutes. Then the sperm will migrate to the medium and can be recovered after removing the tissue pieces.[75] This is the procedure of choice for small animals such as deer and squirrels, given the small size of the epididymis,[76–78] but it has also been used for large animals (camels, antelopes, European bisons, and Cantabrian brown bears).[75,79–81] Retrograde flushing is another method of sperm collection that involves the injection of a buffered medium inside the vas deferens using a syringe. The pressure pushes the sperm toward an incision in the cauda epididymis, generally near the junction with the corpus, where the gametes are recovered.[82] It allows samples to be obtained with a lower level of contamination by blood cells.[83] Sperm cells derived from this technique have been efficiently cryopreserved in red deer,[84] African buffalos,[85] and agoutis.[47]

Cryopreservation of testicular tissues is a potential means of preserving male genetic material in endangered animals that die unexpectedly.[86] This technique enables the preservation of cell integrity and the endocrine functions of

the testes,[87] allowing the preservation and posterior use of spermatozoa[88] and/or spermatogonial stem cells.[64] Recent developments in autografting and xenografting testes demonstrate the potential value of cryopreserving gonadal tissues.[89,90] Nevertheless, a major obstacle limiting the application of this technology is the low efficiency of sperm production in many species.[91]

A number of CPAs have been used to protect testicular tissues against cryoinjury in order to improve the quality of spermatozoa recovered after testicle cryopreservation. Such CPAs include both penetrating and nonpenetrating CPAs such as sucrose, trehalose, glycerol, ethylene glycol (EG), 1,2-propanediol (PrOH), and DMSO. Nevertheless, the efficacy of these CPAs depends on several factors such as CPA concentration, cooling rate, and the species being studied.[92]

SF is the conventional method used to cryopreserve testicular biopsies. Advantage is taken of the regulatory properties of extracellular ice formation to dehydrate cells during cooling and avoids toxicity to cells by exposing them to lower concentrations of CPAs while slowly decreasing the temperature. However, this method is time inefficient and requires expensive programmable freezers.[93] Vitrification is another cryopreservation strategy adopted to preserve testicular biopsies. Vitrification is a fast and convenient method that involves a solidification process without crystallization that avoids the biologically damaging effects associated with ice crystal formation by using higher concentrations of CPAs and ultrafast cooling rates.[93,94]

Regardless of the method used, testicular tissue cryopreservation is a promising way to preserve male germplasm and has been successfully used in domestic species (porcine,[95] mouse,[93] cat,[92] bovine[96]), wild species (*Antilope cervicapra*,[97] monkeys,[98] *Felis chaus*, *Panthera leo*, *Panthera pardus*, *Rusa timorensis*, *Muntiacus feae*, *Sumatran serow*, *Capricornis sumatraensis*[54]), and human.[99] Recently, Borges et al[100] performed cryopreservation of testicular tissue of the collared peccary (*Peccary tajacu*) using a solid surface vitrification method with CPAs (EG, dimethylformamide [DMF] or DMSO, at 3 M or 6 M concentration). It was observed that EG preserved the nuclei and epithelium of the testicular cells.

## Embryos and somatic cells

Advances in cell biotechnology have increased the interest in the creation of cell banks as sources of different cell types, but this requires knowledge of cell-type specific cryopreservation. Cryobanking of cells and tissues is an important and useful approach both for human applications and for the conservation of endangered mammalian species.[101] Additionally, the cryopreservation of embryos and somatic cells offers several important logistic and economic advantages, including the preservation of embryos in excess of the number of available recipients, and national and international movement of embryos.[102]

Some valuable embryo biotechnologies have been developed for mustelids. Amstislavsky et al[103] used SF to obtain important results in the intraspecies and interspecies embryo transfer of endangered animals. Taylor et al[104] published results demonstrating the successful use of a novel microinjection system in the cryopreservation of llama embryos and confirmed pregnancy by ultrasound.

Embryos of the African lion (*Panthera leo*) resulting from intracytoplasmic sperm injection using frozen–thawed spermatozoa obtained by percutaneous epididymal sperm aspiration and mature oocytes were cryopreserved and subsequently transferred.[105] Cryopreservation by conventional SF and vitrification in South American camelids (alpacas and llamas) yielded promising results using conventional methods.[102,106] The authors found no differences in trophoblastic vesicle survival after 24 hours among control groups and those exposed to EG or propylene glycol.

Lattanzi et al[107] tested the viability of llama hatched blastocysts using vitrification and SF and observed re-expansion of vitrified and slow-frozen embryos of 54% and 57%, respectively. Similarly, Von Baer and Del Campo[108] tested the effect of vitrification by open pull straw (OPS) on the morphology and survival of llama hatched blastocysts and found that re-expansion of embryos after thawing was acceptable, but no pregnancies were obtained. Transmission electron microscopy (TEM) revealed a high lipid content in the cytoplasm of llama embryos and may contribute to low survival after vitrification.

Recent advances in gamete and embryo biotechnologies are demonstrating the utility of using somatic cells to obtain induced pluripotency. Induced pluripotent stem (iPS) cells derived from fibroblasts is a new approach to potentially obtain gametes from somatic cells, since iPS cells could be later differentiated into the required cell type.[109] Production of iPS cells from fetal fibroblasts was first reported in the mouse.[110] Concerning highly endangered species, pluripotency has also been induced in somatic cells from a primate, the drill (*Mandrillus leucophaeus*), from the nearly extinct white rhinoceros (*Ceratotherium simum cottoni*),[111] as well as from the snow leopard (*Panthera uncia*).[112]

A pioneering study by Caamano et al[113] reported that a single basic protocol can provide a method for obtaining, culturing, and cryopreserving skin fibroblasts from a wide range of wild animals. In that study, skin biopsies were taken of the brown bear (*Ursus arctos*), and from carcasses of accidentally killed individuals from the following species: gray wolf (*Canis lupus*), red fox (*Vulpes vulpes*), Eurasian badger (*Meles meles*), fallow deer (*Dama dama*), Pyrenean chamois (*Rupicapra pyrenaica*), Western roe deer (*Capreolus capreolus*), and wild boar (*Sus scrofa*). It was possible to obtain, culture, freeze, and thaw skin fibroblasts successfully from all the species studied.

Recently, León-Quinto et al[109] evaluated the cryosensitivity of fetal skin cells in comparison with adult cells from the critically endangered Iberian lynx. Responses to cryoinjury were analyzed in both thawed cell types by means of cell viability and functionality, using freezing media including the permeating CPA (DMSO), either alone or along with the non-permeating CPA sucrose. Data showed a difference between fetal and adult skin cells concerning their cryopreservation sensitivity and requirements, as well as their recovery time after thawing, with survival rates of 54%±4% in thawed fetal cells (vs 89%±6% for thawed adult cells).

Cryopreservation of adult cartilage cells, fetal cartilage cells, and lung tissue has produced promising results and could be very important in biodiversity conservation by biobanking. However, studies on wild animals are scarce and our knowledge is currently limited to domestic animals and marine mammals.[114–116]

## Final considerations

Both in situ and ex situ conservation strategies can benefit from reproductive biotechniques such as artificial insemination, gamete micromanipulation, cell and tissue cryopreservation, in vitro culture, and grafting. In addition, these techniques can also be used to obtain data on the reproductive physiology of wild species. For example, in vitro culture or grafting of gonadal or somatic tissues of threatened species is a great option for their conservation. Germplasm banks can be complemented with the preservation of cell types such as embryos and blood and skin that can be used for the application of other biotechniques in order to preserve species.

There is still very little information on the physiology of wild species, and this is a problem because these data are needed for the improvement and applicability of reproductive techniques for these species. Nevertheless, the techniques reported here are very encouraging and hold great promise for the development of new methods to aid in biodiversity conservation in the future. A practical example for the use of cryopreservation to safeguard animal genetic resource is the Frozen Ark, which is supported by the Natural History Museum, the Zoological Society of London, and the Institute of Genetics at the University of Nottingham (UK); and the Global Genome Initiative (Smithsonian Institution, Washington, DC, USA). These institutions established a consortium of major zoos, aquaria, museums, and research institutions in many countries around the world, aiming to ensure genetic sources preservation.

## Disclosure

The authors report no conflicts of interest in this work.

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