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PERSPECTIVE

PROGRESSIVE TECHNIQUES FOR WILD MAMMAL OOCYTE CRYOPRESERVATION AND RECOVERY

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Abstract

The loss of wild biodiversity has encouraged the development of assisted reproduction techniques, such as in vitro fertilization, intracytoplasmic sperm injection and somatic cell nuclear transfer. The recovery and cryopreservation of oocytes derived from antral follicles are important steps to ensure the efficiency of these techniques. The capability of embryonic development depends on the success of these steps, especially for wild mammals, whose availability is limited, and accessibility is low. In general, it is possible to obtain from a few units to dozens of oocytes depending on the species and collection technique used. The cryopreservation protocols for domestic species are used as a starting point for studies on phylogenetically close wild species; however, the results are modest. Studies show viability after thawing ranging from 37-70% and metaphase II does not exceed 42%. Currently, the main goal is to optimize these results by improving or comparing different cryopreservation and recovery methods. The susceptibility of oocytes to injury during cryopreservation shows inter-specific differences. Optimal methods differ even between species that belong to the same phylogenetic group. Moreover, vitrification has been a promising technique for establishing biobanks. Protocols for evaluating the efficiency of these processes have been studied in several species. Therefore, this review discusses the use and development status of oocyte recovery and cryopreservation techniques in wild mammals and evaluates the success and perspectives of conservation of immature or matured oocytes in different species. The factors that impact successful oocyte cryopreservation must be established for each species.

Keywords: gamete rescue; in vitro maturation; vitrification; wildlife conservation.

INTRODUCTION

The reduction of genetic diversity in wild mammals has become an increasingly serious problem over the years. According to the International Union for the Conservation of Nature (IUCN), over 45,300 species are at a high risk of extinction, with 26% being mammalian (1). The continents with the most threatened mammal species are Sub-Saharan Africa, South and Southeast Asia and South America (2). These areas face significant human interference, leading to the destruction and loss of natural habitats and the hunting and illegal trafficking of animals (3). The increased number of endangered species can cause several ecological

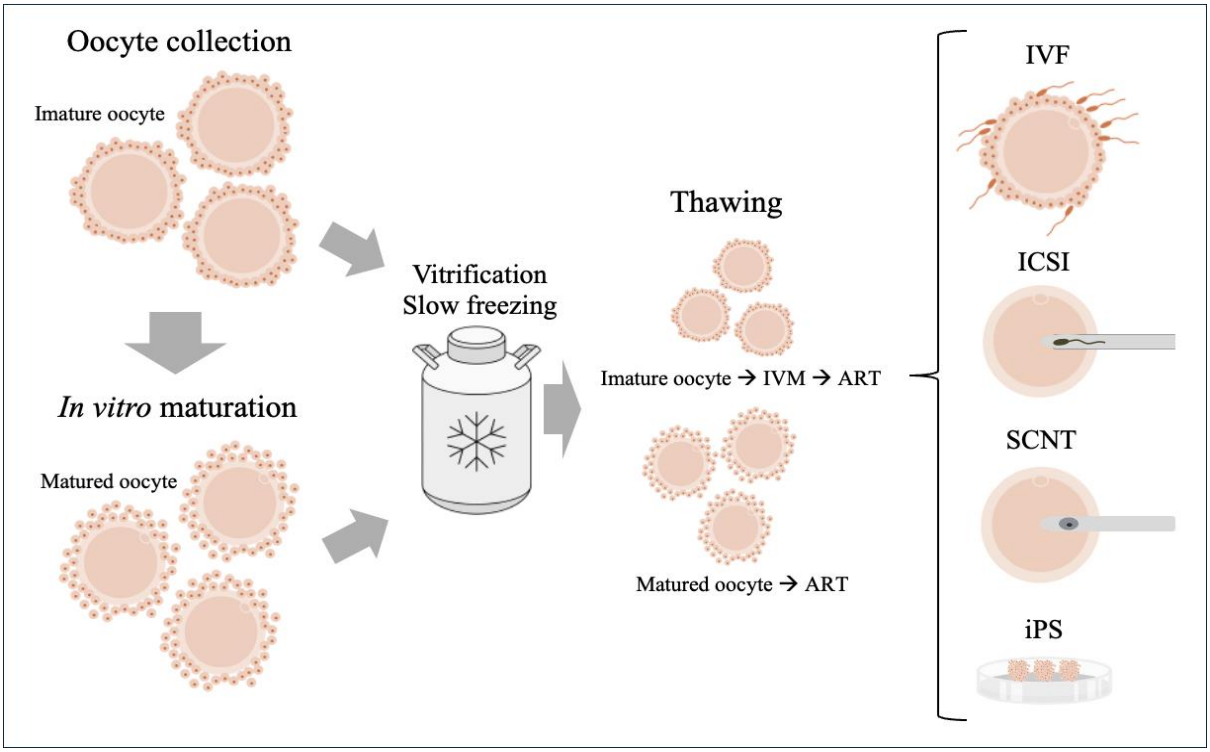

Figure 1. Steps and applications of oocyte cryopreservation techniques in wild mammal species. IVM: in vitro maturation. ART: assisted reproductive techniques. IVF: in vitro fertilization. ICSI: intracytoplasmic sperm injection. SCNT: somatic cell nuclear transfer. iPS: induced pluripotent stem cells.

and economic problems, directly impacting the countries involved (4). To address this issue, studies are exploring ways to preserve the genetics of these wild animals. This can be done through in situ conservation, where animals are preserved in their natural habitat, or ex situ conservation, by preserving them outside their natural habitat. Oocyte cryopreservation stands out among the ex situ conservation techniques (5). This method not only preserves genetic inheritance but also provides study material for the creation of oocyte biobanks and enables the development of assisted reproductive techniques (ARTs) such as in vitro fertilization (IVF), somatic cell nuclear transfer (SCNT), intracytoplasmic sperm injection (ICSI) and induced pluripotent stem (iPS) cells (Fig. 1). Oocyte cryopreservation has been successful in some domestic species. However, in addition to the limited availability of study samples, many protocols established for domestic species do not meet the needs of wild animals, resulting in low oocyte viability % (6).

Despite advances in the development of cryopreservation techniques, oocytes are particularly challenging to preserve due to their high sensitivity to low temperatures (7). As a result, they exhibit low fertilization and percentage blastocyst production (4, 8). This occurs because oocytes, being large cells with limited cytoplasmic dimensions, experience increased plasma membrane and cytoskeleton permeability, leading to damage by excessive ice crystal formation (9, 10). Moreover, low temperatures negatively affect the biochemical conditions of the oocyte, leading to alterations in protein conformation and aggregation, organelle denaturation and pH imbalance, which cause irreversible damage to the gametes (11). Therefore, the most significant challenges in oocyte cryopreservation are developing specific conditions for the cryopreservation process and determining the appropriate freezing method, as each organism has its unique characteristics and cryotolerance properties (12). Thus, it is essential to understand female physiology, especially of the oocyte, to ensure efficient cryopreservation. When it comes to wild mammals, the limited availability of study material hinders the progress of these research efforts (13). Hence, prior knowledge of essential cryobiological factors is crucial to ensure the viability and development of oocytes before and after cryopreservation. Thus, this review aims to

analyze the use and development status of oocyte recovery and cryopreservation techniques in wild mammals and to evaluate the success of cryopreservation of immature or matured oocytes in different wild mammal species.

Table 1. Main results of oocyte cryopreservation in wild mammals.

Species	IUCN status	Oocyte collection	Recovery (oocytes/female)	Cell viability	Ref
European mouflon (*Ovis aries musimon*)	VU	Slicing	4.4	1 - 15 COCs viable by donor	(31)
Red-rumped agouti **(***Dasyprocta leporina*)	LC	Slicing	18.1	315 COCs viable (83.1%)	(30)
Jaguar (*Panthera onca*)	NT	LOPU	14.5	159 COCs viable (99.3%)	(14)
Puma (*Puma concolor)*	LC	LOPU	42.1	416 COCs viable (98.8%)	
White-tailed deer (*Odocoileus virginianus texanus*)	LC	LOPU	9.8	44 COCs viable (72.7%)	(19)
Cheetah (*Acinonyx jubatus)*	VU	LOPU	16	NI	(35)
Collared peccary *(Pecari tajacu*)	LC	Follicular aspiration	6.5	42 COCs viable (71.2%)	(24)
Paca (*Cuniculus pacas*)	LC	LOPU	17.2	NI	(20)
Tiger (*Panthera tigris*)	EN	Slicing	11	16 COCs viable (72.7%)	(33)
Lion (*Panthera leo*)	VU	Slicing	11	26 COCs viable (78.8%)	(33)
Leopard (*Panthera pardus*)	VU	Slicing	21.3	46 COCs viable (71.9%)	(33)
Nilgai (*Boselaphus tragocamelus*)	LC	Slicing	73.8	320 COCs viable (61.8%)	(32)
Black rhinoceros (*Diceros bicornis*)	CR	Follicular aspiration	15.8	83 COCs viable (87.3%)	(25)
Indian Blackbuck (*Antilope cervicapra*)	LC	Slicing	21.8	93 COCs viable (70.9%)	(29)
Mhorr gazelle (*Gazella dama mhorr*)	CR	LOPU	5.8	29 COCs viable (82.8%)	(21)
Rhesus monkey (*Macaca mulatta*)	LC	LOPU	5.5	NI	(16)
Minke Whale *(Balaenoptera bonaerensis**)***	NT	Follicular aspiration	33	2657 COCs viable (91.3%)	(27)
Black bear (*Ursus americanus*)	LC	Follicular aspiration	50	1034 COCs viable (43%)	(26)
Tiger (*Panthera tigris)*	EN	LOPU	28.5	426 COCs viable (93.4%)	(22)

IUCN: International Union for the Conservation of Nature; LOPU: laparoscopic ovum pick-up; NI: not indicated; COCs: oocyte-cumulus complex; VU: vulnerable; LC: least concern; NT: near threatened; EN: endangered; CR: critically endangered.

ADVANCES IN THE RECOVERY OF IMMATURE OOCYTES FROM WILD MAMMALS

The first step in establishing methodologies for long-term conservation is oocyte recovery. Understanding this step improves oocyte retrieval rates as the recovery protocol is adapted according to the species (14). Furthermore, the recovery methodology must maintain the morphology and viability of these cells to improve the success of the technique, which will be subsequently applied (15). Immature oocytes can be collected in vivo using laparoscopic ovum pick-up (LOPU) associated or not with hormonal stimulation (16). In this case, anesthesia, surgery, and post-operative care must be carried out with the utmost rigor to ensure the safety and well-being of the animals (17). On the other hand, in vitro oocyte recovery, such as follicular aspiration or slicing, is performed after ovariectomy or by obtaining ovaries post-mortem (18). The choice between in vivo and in vitro oocyte recovery depends on several factors, such as opportunity, species, purpose and animal condition.

Oocyte recovery by LOPU has been carried out in different wild mammals (Table 1), such as ungulates (19), felines (14), primates (16) and rodents (20). In wild ungulates, Maraboto et al. (19) reported 72.7% viable oocytes from white-tailed deer (*Odocoileus virginianus texanus*). Similar results are also found in the endangered species *Gazella dama mhorr* in a study conducted by Berlinguer et al. (21), where 58.6% oocyte recovery was achieved. This technique was also performed in jaguars (*Panthera onca*) and pumas (*Puma concolor*) after superovulation treatment (14). As a result, the authors obtained a total of 421 oocytes from 10 pumas, with 416 qualified as viable and 160 oocytes from 11 jaguars, with 159 qualified as viable, proving the effectiveness of the oocyte retrieval technique. LOPU also showed positive results in tigers (*Panthera tigris*), with a total of 456 oocytes recovered from 468 aspirated follicles, achieving 97.4% recovery of, with only 30 oocytes classified as degenerated (22). Studies with primates are also being conducted, where LOPU allowed 40.5% recovery of oocytes in rhesus monkeys (*Macaca mulatta*) (16). In pacas (*Cuniculus paca*), representing one of the few available data of wild rodents, oocyte retrieval by LOPU resulted in 32.5% recovery, considered a significant advancement for the conservation of the species (20).

Follicular aspiration using a syringe and needle is well-established for obtaining oocytes (23). In wild ungulates, good results have been observed with collared peccaries (*Pecari tacaju*) using follicular aspiration after euthanasia, allowing 83.1% oocyte recovery, with 42 oocytes considered viable (71.2%) (24). Moreover, high recovery was obtained in rhinoceroses (*Diceros bicornis*), as reported by Stoops et al. (25), who recovered 95 oocytes from five females, with an average of 15.8 oocytes collected per female. In black bears (*Ursus americanus*), follicular aspiration allowed the recovery of 2,403 oocytes from 48 animals, showing promising results for the species (26).

Within the order Cetartiodactyla, the study by Iwayama et al. (27) showed good oocyte quality through follicular aspiration recovery in minke whales (*Balaenoptera bonaerensis*) from both adult and prepubescent animals. A total of 1,568 oocytes were obtained from 112 ovaries of adult whales, while 1,341 oocytes were recovered from 64 ovaries of prepubescent animals, totaling 2,909 oocytes recovered. Moreover, the gametes showed good morphology and were later designated for cryopreservation. Fujihira et al. (28), using the follicular aspiration method in minke whales, obtained a good percentage of oocytes at metaphase II from adult animals (60.9%) and prepubescent animals (53.1%) after the in vitro maturation process. These results show that follicular aspiration can be applied for the conservation of several species of different orders.

Regarding the slicing technique applied for oocyte collection, which involves making cuts on the surface of the ovary, 131 oocytes were obtained from Indian blackbuck (*Antilope cervicapra*), with 93 classified as viable (29). In wild rodents, such as the red-rumped agouti (*Dasyprocta leporina*), 379 oocytes were obtained and 315 (83.1%) were classified as viable (30). Temerário et al. (31), who aimed at short- and long-term storage of oocyte-*cumulus* complexes from European mouflon (*Ovis aries musimon*), recovered 49 high-quality oocytes from 11 females. For the Nilgai (*Boselaphus tragocamelus*), this technique allowed a 36.9% recovery, with a total of 517 oocytes collected from 14 ovaries, of which 320 were considered viable, demonstrating the efficiency of the

methodology and its ability to maintain oocyte quality (32). In wild felines, Rao et al. (33) obtained 22 oocytes from two tigers (*Panthera tigris*), 33 oocytes from three lionesses (*Panthera leo*) and 64 oocytes from three leopards (*Panthera pardus*).

Although oocyte recovery techniques can be applied to various species in different conditions, it is essential to carefully evaluate oocyte viability, especially for wild species at risk of extinction (6). This is due to the need to maximize the use of biological material, as opportunities for manipulation are limited, making it crucial to apply these cells in ARTs or long-term conservation, such as cryopreservation (34).

GENERAL ASPECTS OF OOCYTE CRYOPRESERVATION IN WILD MAMMALS

The preservation of endangered species has driven the development of techniques such as germplasm cryopreservation (35). The cryopreservation of female gametes stands out as a fundamental approach for maintaining genetic diversity (7). This technique can be applied in various phases of the reproductive cycle of females, allowing the preservation of oocytes at different stages of development (37). Oocyte cryopreservation can be performed using vitrification and slow/conventional freezing (38) (Fig. 2). Since the results vary in distinct species, studies have compared slow/conventional freezing with vitrification to determine the optimal cryopreservation method for wild mammalian oocytes (7).

The slow freezing protocol for oocytes involves gradual cooling and osmotic removal and transfer of intracellular water to the extracellular medium. This medium is composed of an osmotic solution rich in cryoprotectants, which help protect cells during freezing (13). Although it is considered effective, this method is relatively expensive due to the need for specialized equipment that allows controlled temperature reduction with gradual cooling (39). This control is critical to prevent the rapid formation of ice crystals, which can damage cells, thus allowing ice crystals to form in a slow and controlled manner (6). On the other hand, vitrification is a process that solidifies liquid, avoiding the formation of crystals and resulting in an amorphous solid (38). In this procedure, the cells are exposed to highly concentrated cryoprotective solutions, promoting fast dehydration, followed by a rapid drop in temperature, which can reach -200 °C/min (40). This method is highly efficient in avoiding cell damage caused by ice crystals during cooling (41).

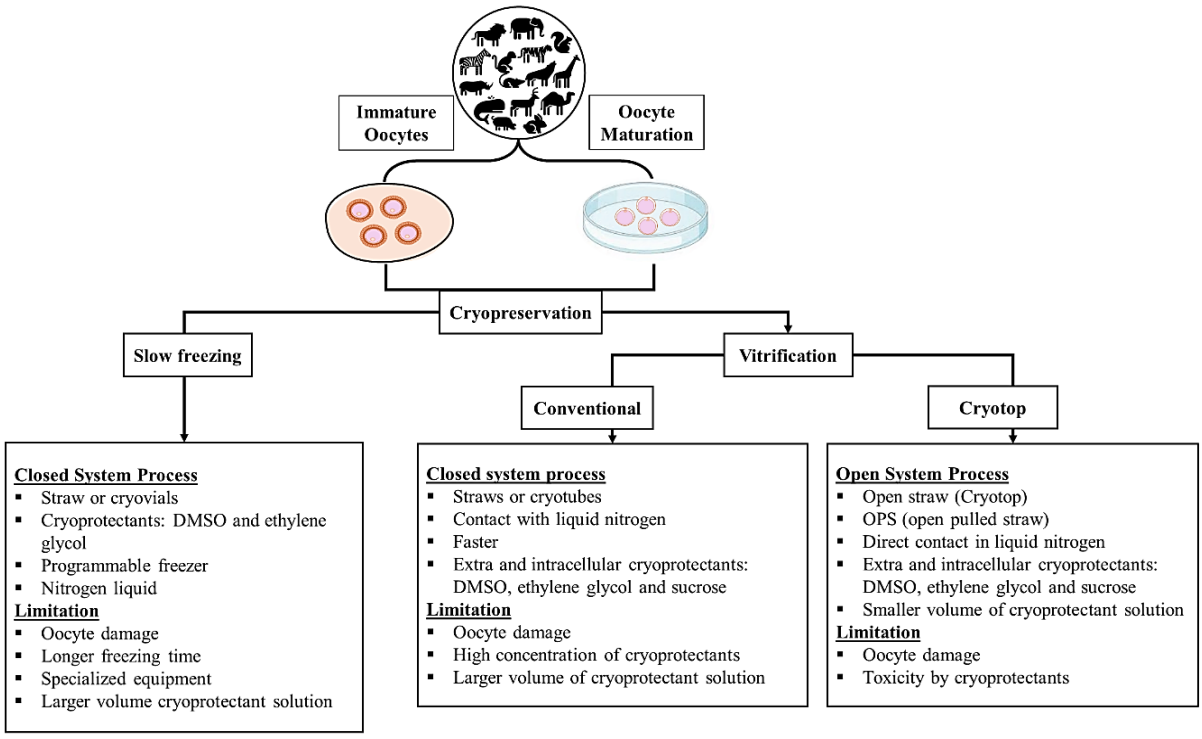

Figure 2. Methods for cryopreservation of wild mammalian oocytes.

Vitrification can be performed through two primary methods: closed and open systems (41). In closed systems, the contact of the cells with liquid nitrogen is avoided by using sealed canes or cryovials, ensuring greater safety against contamination. In open systems, the oocytes are placed in open canes, as in the Cryotop system, which allows faster cooling through direct contact of the medium with liquid nitrogen but may present a risk of contamination (42).

The first immature oocytes were cryopreserved in 1959 from mice by Sherman and Lin (43) and from sheep by Averill and Rowson (44). Cryopreservation of wild oocytes was first successful in 1998, using the slow-freezing method with immature oocytes from squirrel monkeys (*Samini spp*.) and gorillas (*Gorilla gorilla*) (45, 46). Cryopreservation of oocytes still presents difficulties since they are highly sensitive to temperature changes and cryoprotective solutions (40). Another obstacle is maintaining the integrity of the communication between cumulus cells and immature oocytes, which can be interrupted by the formation of ice crystals (31).

The search for higher oocyte quality percentage after thawing led to investigations on their optimal stage of development for cryopreservation. Studies indicated that immature oocytes maintained higher viability due to the greater resistance of the meiotic spindle to temperature variations (9). This has been observed in immature oocytes derived from primary follicles of the Tasmanian devil (*Sarcophilus harrisii*) undergoing vitrification, where these oocytes displayed high survival after cryopreservation (47).

Studies with vitrified immature oocytes obtained from four-horned antelope chousingha (*Tetracerus quadricomis*) showed low maturation percentage (7). Studies in African lion (*Panthera leo*) demonstrated that vitrified immature oocytes could develop to the metaphase II stage but did not develop beyond the 4-cell embryonic stage (48). In addition to allowing simultaneous cryopreservation of large numbers of samples, vitrification of immature oocytes requires less equipment but a higher concentration of cryoprotectants compared to the slow freezing technique (31).

In addition, studies on vitrification of oocytes from Mexican gray wolves (*Canis lupus baileyi*) using the Cryotop method demonstrated that the oocytes maintained viability after thawing (42). Despite many advances in cryopreservation techniques, post-thaw survival of oocytes is still significantly lower compared to other cell types, as they display many morphological and functional defects. The level of these defects vary between protocols and species, which makes it necessary to adapt the protocol to each species studied (7).

CRYOPRESERVATION OF IMMATURE AND MATURED OOCYTES OF WILD MAMMALS

Oocyte morphological and molecular conditions create technical barriers that affect cryopreservation and induce sensitivity to mechanical stress, causing osmotic imbalance (49). Thus, one possibility to ensure the effectiveness of cryopreservation is to use immature gametes soon after collection (31). However, when it comes to wild animals, it is challenging to indicate an appropriate technique for cryopreservation of their genetic material due to the lack of knowledge about sensitivity to cryoprotectants and resistance to low temperatures (7, 11). Thus, it has been possible to test the cryopreservation of immature oocytes from animals, such as mouflon (*Ovis aries musimon*) (31), four-horned antelope (*Tetracerus quadricornis*) (7) Mexican wolf (*Canis lupus baileyi*) (42), Tasmanian devils (*Sarcophilus harrisii*) (47) and silver fox (*Vulpes vulpes*) (50) (Table 2).

Genetic diversity hinders the effectiveness of cryopreservation processes since a single technique may or may not be suitable depending on the physiological condition of the animal (7). An alternative to this is using protocols established for domestic animals in endangered species as a basis for further studies (51), as demonstrated by Boutelle et al. (42), who compared the vitrification using Cryotop applied to domestic dogs and Mexican wolves (*Canis lupus baileyi*). In this study, although the viability of the dogs' oocytes showed a slight reduction upon thawing, wolf's oocytes showed around 30 to 50% viability after thawing, demonstrating that this technique enabled the cryopreservation of the genetic material of these animals. However, it still needs improvement.

Table 2. Cryopreservation technique for oocytes of wild mammals at different stages of development.

Oocyte	Species	IUCN status	Cryopreservation technique	Main results	Ref
Immature	*Ovis aries musimon*	VU	Vitrification - 15% EG, 15% DMSO and 0.5 M sucrose	19.2% oocytes reached MII and 1PB stages	(31)
	Panthera leo	VU	Vitrification - 15% EG, 15% DMSO and 0.5 M sucrose	42.9% oocytes able to mature after vitrification	(48)
	Vulpes vulpes	LC	Vitrification – 15% EG, 7.5% DMSO	41.9% oocytes resumed meiosis	(50)
	Canis lupus baileyi	LC	Vitrification - 15% EG, 15% DMSO and 0.5 M sucrose	41.4% intact oocytes viable	(42)
	Tetracerus quadricornis	VU	Vitrification - 20% EG, 20% DMSO and 0.6 M sucrose	29.4% oocytes achieved maturation	(7)
	Sarcophilus harrisii	EN	Vitrification - 18% EG, 18% DMSO and 1 M sucrose	70% viable oocytes	(47)
	Alopex lagopus	LC	Vitrification - 15% EG, 15% DMSO +21% Ficoll and 0.35 M sucrose	11% oocytes reached MII stage	(66)
	Macaca mulatta	LC	Slow freezing	Oocytes did not reach MII stage	(39)
	Balaenoptera bonaerensis	NT	Vitrification - 30% EG or 15% EG and 15% DMSO and 0.5 M sucrose	46% oocytes retained cleave after parthenogenetic activation and ICSI	(28)
	Saimiri sciureus	LC	Slow freezing	37% oocytes viable after thawing	(63)
Mature	*Okapia johnstoni*	EN	Vitrification - 15% EG, 15% DMSO and 0.5 M sucrose	40% oocytes reached MII stage	(53)
	Leptailurus serval	LC	Vitrification - Cryotech vitrification media	70% viable cells	(54)
	Felis manul	LC	Vitrification- Cryotech vitrification media	60% viable cells	(54)

IUCN: International Union for the Conservation of Nature; DMSO: dimethyl sulfoxide; EG: ethylene glycol; MII: metaphase II; 1PB: first polar body; LC: least concern; NT: near threatened; VU: vulnerable; EN: endangered; CR: critically endangered.

Although vitrification is widely used in oocyte cryopreservation processes, it becomes a risky technique in wild animals because the sensitivity of these gametes to cryoprotectants is unknown (11). Studies that have developed vitrification in wild animals, although allowing germplasm conservation, showed a reduction in oocyte development after thawing. Rao et al. (7) demonstrated that it was possible to cryopreserve immature oocytes of *Tetracerus quadricornis* through the vitrification technique. However, compared to fresh oocytes, there was a reduction in the number of oocytes reaching the mature stage (69.3% *vs.* 29.4%).

Within oocyte cryopreservation, it is still possible to carry out long-term conservation of already matured oocytes (52), which is an important factor when obtaining female gametes from an endangered species. When there is no availability of the male gamete for in vitro embryo production, being able to store the oocyte "prepared" for subsequent IVF is essential. This is evidenced by Simone et al. (53), who cryopreserved matured oocytes of Okapi (*Okapia johnstoni*), a species belonging to

the *Giraffidae* family already declared endangered. In this study, 10 COCs proceeded to in vitro maturation, where four reached the nuclear stage of metaphase II. These were then directed to vitrification, which maintained the viability of the gametes, subsequently fertilized by ICSI. However, there was no success in embryonic development.

Previously, Nowak et al. (54) studied the cryopreservation of matured oocytes of serval (*Leptailurus serval*) and Pallas' cat (*Felis manul*), wild feline species that are suffering from population decrease. Vitrification was performed only with matured oocytes, where 24 serval gametes were cryopreserved, 10 thawed, and seven showed good cell viability (corresponding to 70%). On the other hand, 20 Pallas' cat gametes were cryopreserved, 10 were thawed, and six showed good cell viability, demonstrating the good efficacy of the vitrification technique for this wild feline. Although there is knowledge concerning the cryopreservation of matured oocytes, few studies have been conducted on the cryopreservation in wild animals using gametes after in vitro maturation. This may be related to a higher cryotolerance of immature oocytes since, at the germinal vesicle stage, the gametes have a meiotic spindle that is more resistant to temperature changes (55). In addition, it is worth noting that individual characteristics of the species directly influence whether cellular material of certain species will adapt to different cryopreservation protocols (7) since most methodologies have been previously applied to domestic animals and adapted to wild animals (56). Therefore, more studies are needed to validate these concerns (Table 2).

PERSPECTIVES OF OOCYTE CRYOPRESERVATION IN WILD MAMMALS

Oocytes are the most inaccessible and expensive biological samples that can be used as a source of genetic variability for application in ARTs, and their cryopreservation is technically more challenging compared to sperm, embryos and somatic cells (57). Oocytes are under-represented in cryobanks of wildlife-derived samples, such as the San Diego Frozen Zoo and Frozen Ark Consortium (58).

Although there are reports of birth after oocyte cryopreservation in some domestic species including cattle (59), pigs (60), horses (61) and cats (62), to date, this success has not been achieved in wild species. Cryopreservation has been reported in ungulates, carnivores, marsupials, primates and cetaceans (Table 2), but results on developmental competence after thawing are quite limited. So far, the most advanced stage of development reached was cleavage (28, 63).

There are numerous reasons for the lack of success in the cryopreservation of oocytes from wild species that go beyond the technical elements of the freezing protocol. The lack of knowledge regarding aspects of in vivo reproductive physiology limits the hormonal manipulation of females to optimize oocyte collection without harming the animal's health (64, 65). Furthermore, anesthetic collection procedures put the animal's life at risk, which is a significant factor, especially for species with few specimens (53).

Considering the chance of performing a successful collection in vivo or in vitro, it is necessary to know the appropriate culture conditions for the maintenance and maturation of oocytes (31). Furthermore, after freezing and thawing, it is important to have sufficient knowledge of oocyte physiology for artificial or sperm-induced activation. Therefore, the cryopreservation technique and the preceding and subsequent steps must be established. This can be easily exemplified by the study of Simone et al. (53), who had a unique opportunity to obtain and cryopreserve oocytes from okapi (*Okapia johnstoni*), a species which is classified as endangered. In this study, it was necessary to use oocyte maturation and vitrification protocols from the domestic bovine model due to the lack of sufficient information on the species' reproduction physiology. Therefore, it is difficult to accurately assess oocyte competence, considering that protocols have not been established due to the unique physiological characteristics of okapi.

Despite the difficulties, successful cryopreservation of oocytes is an invaluable source for the genetic representation of wild species and for rescuing populations under threat of extinction (31). Even if there are no established protocols, ensuring the long-term storage of these gametes is essential. This is the primary objective of biobanks that seek to store samples in anticipation of future technologies and knowledge (67). These oocytes can be used in advanced ARTs, becoming a source of genetic

rescue, as has been brilliantly developed for rhinos (58).

FINAL CONSIDERATIONS

Cryopreservation of oocytes from wild animal species has broad applicability for the conservation of genetic diversity and long-term maintenance of samples for the application of advanced ARTs. Thus, it is essential to establish efficient cryopreservation protocols using slow freezing and vitrification techniques and the most efficient cryoprotective solutions according to the species. The vitrification technique has shown greater applicability in wild species and immature oocytes are most used.

The success of oocyte cryopreservation depends on many factors that must be identified and established for each species, especially regarding its reproductive physiology and in vitro culture of oocytes. Therefore, the formation of biobanks containing samples of female gametes should be a priority for the largest possible number of wild species, regardless of whether there is an imminent risk of extinction. This storage should preferably occur before the decline in a species population number, allowing a greater sample number for future studies and for maintaining genetic variability.

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