CryoLetters 42(5), 251 – 260 (2021)

PERSPECTIVE

OOCYTE AND EMBRYO PRESERVATION IN WILD ANIMALS: AN UPDATE

G R Bhat* and K A Sofi

Division of Veterinary Clinical Complex, Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir
*Corresponding author's E-mail: rasool_roshan0127@rediffmail.com

Abstract

The reduction in population genetic diversity due to inbreeding depression and the negative impact of human activity on habitats ultimately generates an extinction debt. Therefore, there is always a dire need to save wild population and to protect biodiversity. Preservation of wildlife female germplasm, i.e., oocytes and embryos, is a promising biotechnological tool to conserve species' biodiversity. Other applied tools of Assisted Reproductive Technology (ART) which assure conservation of endangered species include artificial insemination (AI), embryo transfer technology (ETT), and sperm cryopreservation. Only a few studies show the possibility of adapting the cryopreservation techniques developed for domestic animal female genetic material for use with wild animals. Difficulty is encountered in getting samples, accesses to animals for study, and the standardization of protocols for cryopreservation of such genetic material. Our meta-analysis of the literature (published or in press) and on-going studies found that biobanking for the preservation of vital tissues of wild animals is possible. Somatic tissue sections, ovarian tissues, sperms, oocytes and embryos are potential materials for preservation by vitrification. As vitrification is economical and easily applied, it appears to the best option currently available for the preservation of wildlife female genetics in order to conserve species' biodiversity.

Keywords: cryopreservation; embryo; oocyte; wild animals.

INTRODUCTION

Preservation methods for the world's wild animal genetic resources is urgent, particularly for the leopard, tiger, elephant, gorilla, hangul, markhor, Tibetan wild dog, Tibetan gazelle, chiru, musk deer, double humped camel, etc. Cryopreservation of female gametes dates back to the studies of Chang (1, 2) in the early 1950s when they were able to store rabbit oocytes, zygotes and embryos. Another milestone was Mazur's work (3, 4) on cell specific optimal cooling and warming rates and successful cryopreservation of mouse embryos using 1.5 M dimethyl-sulphoxide (DMSO) as cryoprotectant. This finding formed the basis for successful cryopreservation of mammalian oocytes and embryos. Cryopreservation of oocytes and embryos has been successfully achieved in domestic cat. The cryopreservation of gametes and embryos of wild animals has been reported, with few successful births, by adopting the protocols developed for domestic animals.

Efforts at wildlife conservation has two immediate benefits: emphasing the importance of natural fauna, and protecting biodiversity. The unabated progress of decreasing animal numbers can lead to inbreeding depression, with a loss of genetic diversity and the accumulation dangerous mutations. Inbreeding depression also increases disease susceptibility and weaknesses in offspring. Inbred progenies can have a compromised ability to adapt to changing environments via natural selection. Thus, habitat shrinkage, due to raised human population interventions, can also predispose wild flora species to extinction, as can natural disasters (5). The IUCN Species Survival Commission (https://www.iucn.org/) has stressed the concerning pattern of a rapid decline in wild mammalian species.

For biodiversity conservation, preservation of habitat and captive breeding approaches are recommended strategies. However, the acceptability of the latter is low due to impaired reproductive performance owing to space restriction to animals. In order to compensate for this low performance, assisted reproductive techniques (ARTs), involving gamete and embryo preservation, are promising approaches. Cryopreservation via vitrification is widely used. Compared to the embryo, an oocyte contains a variety of cytoplasmic factors and has good power of differentiation (6), making it a more attractive tissue for preservation. The first successful oocyte vitrification in humans was done by Arav (7), and later by Kuwayama (8). The same principles of cryopreservation are followed in animals. Compared to female gametes and embryos, spermatozoa are convenient for cryopreservation owing to their smaller cell size, lesser cytoplasmic mass and a condensed form of DNA (9). Sperm preservation in wild animals has been carried out by conventional slow freezing methods using programmable bio-freezers. Although the technique is easy to apply, the availability of animals' sperm samples may be difficult.

Spermatozoa have been successfully cryopreserved in a few wild animal species (from live as well as dead animals). Some of the successful wild sperm preservation studies include Asiatic elephant (10), Indian rhinoceros (11) and capuchin monkey (12). However, oocyte and embryo cryopreservation in wild animals have not been standardized due to limitations in the number of dead animals available and the challenges associated with oocyte freezing because of its size and structural properties (13). Both slow freezing and vitrification techniques developed for domestic animals are now being applied to wild felids, to conserve germplasm at a success rate sometimes comparable to that observed with their domestic counterparts.

In situ habitat conservation and ex situ conservation programs including in vivo and in vitro preservation, and also the establishment of germplasm banks, cannot suffice for wildlife biodiversity conservation (5). Nonetheless, preserving the female oocyte and embryos can support a conservation rescue strategy without undermining the natural processes of evolution. In this way, ARTs can connect in situ and ex situ conservation programs (14).

Many factors decrease the adoption of reproductive biotechnology in wild animal conservation. These include, more availability of wild animals for the collection of ovaries, wild life resources being far away from established ART labs and uncertainty in the wild animal death reports. Furthermore, the cryo-injury mechanisms in oocyte are not fully understood, indicating that more insight is needed to understand oocyte structural dynamics so that oocyte cryopreservation methods can be improved (15).

Embryo cryopreservation could enable better future use of genetics. For example, embryo manipulation guarantees the efficient use of genetic material of a valuable female that is physically incapable of reproducing. The genetics from valuable wild females can thus be propagated by transfer to recipients from closely related more abundant (non-domestic or domestic) species. Embryo manipulation procedures have been performed in non-domestic felids but the studies are at a preliminary stage of development so far. To achieve successful pregnancies a routine in zoos, further comprehensive investigations are needed into the cryopreservation, maturation and fertilization of embryos.

Due to limitation in maintaining genetically viable populations of threatened species, the establishment of genetic banks containing semen, oocytes and embryos could protect against the future loss population genetic diversity. In the present review, we discuss in detail the current and potential uses of cryopreservation of female germplasm (oocyte and embryo) to prevent the extinction of threatened wild life species.

PRESERVATION OF FEMALE GERMPLASM TO CONSERVE WILD SPECIES

Preservation of oocytes and embryos has been carried out by slow freezing and vitrification techniques (16) and these remain the current two methods of cryopreservation for mammalian oocytes and embryos (Table 1). However, vitrification has more satisfactory outcomes, due to the avoidance of exceeding a minimal volume. During slow freezing cells or tissues in a balanced low concentration of cryoprotectant solution are slow cooled in a programmable freezer. This results in sufficient cellular dehydration but low enough to avoid cytotoxic

Table 1. Chronology of cryopreservation of wild and laboratory animal female tissues: methods and outcomes.

Species	Method	Material	Outcome	Year (Ref)
Rat (*Rattus norvagicus)*	SF	Ovarian tissue	Takes and the functional activity of the grafts was greater for tissue suspended in serum or saline containing 15% glycerol and cooled either to -79 or to -190°C for 1 h. Such material did not deteriorate during 9 days at -190°C.	1953 (33)
Mouse (*Mus musculus*)	SF	Embryo	Live birth reported following successful storage of blastocysts and eight cell stage mouse embryos at -196℃	1972 (34, 35)
Mice (*Mus musculus*)	SF	Oocytes	Unfertilized oocytes recovered from mice and frozen at -196°C; 35% of oocytes subsequently resulted in live individual births after insemination.	1977 (36)
Rat (*Rattus norvagicus)*	SF	Oocytes	Takes and cultured oocytes. Majority of recovered frozen rat oocytes found normal after culture protocol and penetration procedure.	1979 (37)
Baboon (*Papio* sp.)	SF	In vivo-produced blastocyst	Recovered blastocyst stage embryos successfully frozen and normal offspring after transfer of cryopreserved embryos to foster mother.	1984 (38)
Lowland gorilla (*Gorilla gorilla gorilla*)	SF	Immature oocytes	Immature gorilla oocytes recovered and cryopreserved which were then successfully micromanipulated and fertilized in this comprehensive experiment.	1992 (39)
African elephant (*Loxodonta africana*)	SF	Ovarian tissue	Comprehensive study involving xenografted mice with elephant cells. The recovered elephant antral follicles, cumulus and oocytes were frozen, developed and xenografted.	1998 (30)
Minke whale (*Balaenoptera bonaerensis*)	SF	Oocytes	Takes and cultured cryopreserved minke whale oocytes. 20–30% of the cultured cells resumed meiosis and 3.8% reached the M-II stage.	2000 (40)
Rhesus macaque (*Macaca mulatta*)	SF	Early-stage blastocysts (ICSI)	Takes and quality of oocytes frozen was maintained. Also out of two frozen IVF embryos one pregnancy established.	1989 (41), 2005 (42)

SF, slow freezing; V, vitrification; OPS, open pulled straw.

Table 1 (continued). Chronology of cryopreservation of wild and laboratory animal female tissues: methods and outcomes.

Species	Method	Material	Outcome	Year (Ref)
Cynomolgus monkey (*Macaca fascicularis*)	V	2 to 8-cell stage embryo	Vitrification / slow cooling is more efficient than conventional freezing methods to store early cleavage stage embryos, depicted by 82% post thaw survival.	2002 (43)
Minke whale (*Balaenoptera bonaerensis*)	V, Cryotop and OPS	Oocyte (germinal vesicle-stage)	Vitrification using Cryoptop improved IVM rates up to 30% in Antarctic minke whale immature oocytes in their germinal vesicle stages.	2005 (44)
Rhesus macaque (*Macaca mulatta*)	V	Ovarian tissue	Takes and 70% oocytes survived vitrification and about 90% co-cultured ovarian thawed tissue remained viable.	2005 (44)
Fat-tailed dunnart (*Sminthopsis crassicaudata*)	V, OPS	Granulosa-oocyte complexes (GOC)	Takes and Granulosa cells found more sensitive to vitrification than oocytes. 70% vitrified oocytes found viable. Also, almost no decline in oocyte viability observed.	2009 (26) 2010 (25)
Tasmanian devil (*Sarcophilus harrisii*)				
Mexican gray wolf (*Canis lupus baileyi*)	V, Cryotop	Oocytes	Vitrification using Cryoptop carrier system showed 57.1% intact and viable post-thaw oocytes and the method maintained oocyte viability.	2011 (28)
Serval (*Leptailurus Serval*) and Pallas (*Felis manul*)	V, Rapid-I method	Oocytes	Takes and Cryotech medium and Rapid-I device was found to maintain viability in 70% serval and 60% pallas cat oocytes.	2019 (66)

SF, slow freezing; V, vitrification; OPS, open pulled straw

damage. This conventional freezing method tends to be discouraged unless a costly programmable freezer is available, as precise cooling is needed to reduce the risk of uncontrolled ice crystal formation and growth. In contrast, vitrification, is a cost effective method with wide application. It has been used with variable success in the cryopreservation of oocytes and embryos, and can be used under field conditions when dead wild animals are found and need germplasm preservation. The use of rapid cooling (plunging) results in the formation of a glass-like highly viscous state, avoiding crystallization of water and ice crystal growth which otherwise predisposes cells to various cryo-injuries (17). Vitrification of oocytes and embryos, followed by successful transfer of vitrified embryos, pregnancies and live births have been reported in 1990s in bovines (18, 19).

Gonadal tissue preservation is a promising option for the preservation of wild vertebrates (20). Storage of ovarian tissue directly overcomes oocyte preservation limitations, such as the low number of mature oocytes available in the ovaries, oocyte cryodamage at low temperatures, and challenges of achieving super-ovulation. Ovarian tissue vitrification has been employed for the cryopreservation of wild animal germplasm. For example, germplasm vitrification following gonadal tissue storage has been carried out in kangaroos (*Macropus giganteus*) by Richings (21) and in red deer (*Cervus elaphus hispanicus*) by García-Alvarez (22). In collared peccaries (*Pecari tajacu*), ovarian tissue subjected to vitrification maintained its morphological integrity in >70% of the follicles after rewarming. In the baboon (*Papio anubis*) and cynomolgus monkeys (*Macaca fascicularis*), ovarian tissue

vitrification followed by autografting resulted in good follicle survival, growth, and ovulation (23, 24). Vitrification has been applied to isolated viable immature oocytes of wolves and follicle viability has been maintained for 48 h in carnivorous marsupials (*Sminthopsis crassicaudata*, *Sminthopsis harrisii*, *Dasyurus viverrinus*, and *Dasyurus hallucatus*) using a phosphate-buffered saline (PBS) as the base medium during chilling of ovarian tissue. Also in case of whales, vitrification of ovarian fragments maintained oocyte integrity (25, 26). Weilderman (27) and Boutella (28) reported successful slow freezing and in vitro ovarian tissue culture 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*). Conventional freezing of ovarian fragments, maintained oocyte integrity in agoutis (*Dasyprocta aguti*) (29), African elephants (*Loxodonta africana*) (30), wombats (*Lasiorhinus krefftii*) (31) and whales (32).

Collection and evaluation of wildlife oocytes and embryos

Wild or endangered dead or live animals can be a source of oocytes. Recovery and cryopreservation of oocytes and embryos from dead wild/endangered animals can be achieved by adapting conventional methods of vitrification. As for domestic animal species, ovaries can be separated from the dead animal's genitalia and used to obtain wild animal female gametes i.e., oocytes through follicle puncture, aspiration or slicing techniques. Oocytes in live animals may also be extracted from ovarian tissue biopsies and unilateral or bilateral ovariectomy. Mature or immature oocytes thus obtained can be vitrified and cryopreserved. Recovering oocytes from postmortem ovaries holds tremendous potential for the conservation and establishment of species-specific protocols for assisted reproductive technologies. Direct laparoscopic aspirations of oocyte from 2 mm sized pre-ovulatory follicles of ovaries stimulated with exogenous gonadotropins provides an efficient and thorough method to maximize the oocyte harvest from the female. The method allows for better in vitro evaluation and sorting of oocytes for fertilization and transfer. Easy assessment of ovaries and ease in aspiration of follicles makes the domestic cat an acceptable model for the fine-tuning of oocyte retrieval procedures. In this way, cryopreservation of oocytes and embryos can ensure the long-term storage and protection of existing genetic diversity and an insurance against the loss of wild populations due to epidemics and natural disasters.

A general procedure for oocyte retrieval from felids has been described (45). Ovaries are collected from animals under general anesthesia using isoflurane inhalant drug. The ovaries are evaluated and follicles observed using endoscope. Mature follicles larger than 2 mm are aspirated using a 22G needle attached to polypropylene tubing connected to a sterile collection tube (15 mL). The tube contains TCM 199 culture medium and heparin, and is attached to a vacuum aspiration pump. Following collection, oocytes placed in Petri dishes with culture medium can be observed by stereomicroscopy at 400 X magnification for evaluation and separation of culture grades. The cytoplasm of felid oocytes is often very dark and opaque making it difficult to assess the nuclear structures. Excellent quality oocytes are characterized by a uniformly dark cytoplasm, distinct corona radiata and cumulus cell expansion. Oocytes having cytoplasm with a nonuniform appearance, indistinct corona radiata and cumulus cell mass not expanded are classified as non-regular quality. Oocytes are said to be degenerated when they bear abnormal cytoplasm, no corona radiata or cumulus cell mass. Preliminary evaluation of the oocytes is necessary to separate degenerated and immature oocytes from the mature ones. The oocytes are removed from the debris using a micropipette with suction provided by an attached mouth pipette. They are placed in a new culture dish with fresh cell culture medium. Many such studies of collection, grading and maturation of oocytes have been carried out in sheep (46, 47). According to Umapathy (48) the ovaries of dead Barasingha, Thamin, Swamp and Spotted deer have been a source of immature oocytes which were then successfully matured in vitro (first polar body extrusion) and subjected to parthenogenetic activation. Interestingly the effect of the duration between the death of animal and recovery of ovaries was not significant on recovery of culture grade oocytes, in vitro maturation and development. The

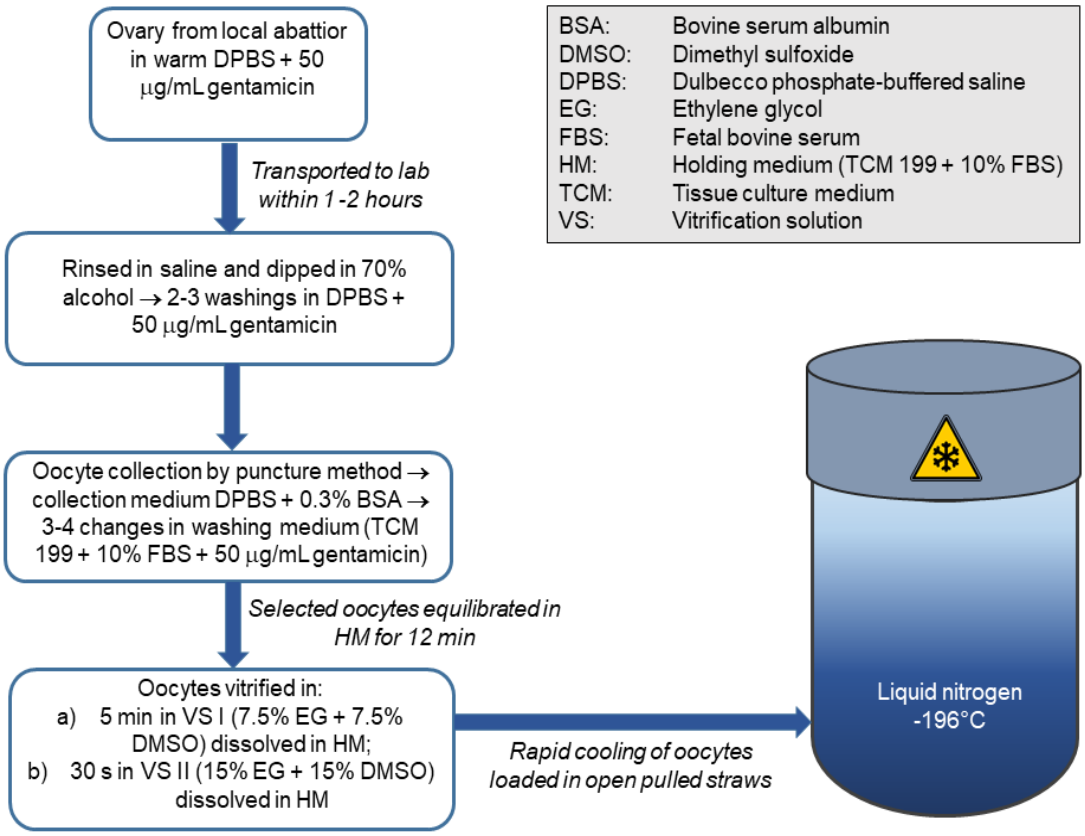

Figure 1. Vitrification protocol routinely applied for domestic animal tissue cryopreservation.

oocytes were categorized as mature when the corona radiata and the cumulus oophorus cells are loosened in appearance and expanded and immature when the corona radiata cells were found to be tightly compacted around the oocytes, and degenerated when the oocyte appeared abnormally pale or lacked any corona radiata cells around it. The mature and immature oocytes were placed in separate culture dishes. All oocytes were washed three times with fresh medium while under light weight paraffin oil. The overlying paraffin oil maintained the oocytes within cell culture medium droplets that form when the oil is layered atop. The immature oocytes then needed to be cultured in cell culture medium for another 24 h and then reassessed. All oocytes were incubated in 5% CO_2 at 38°C. The oocytes could tolerate lower temperatures for short periods of time, however, they were very susceptible to higher temperatures. Mature oocytes were prepared for in vitro fertilization (IVF). After 24 h, the cultured immature oocytes were reevaluated and matured ones were inseminated by exposure to prepared spermatozoa (IVF) or intracytoplasmic sperm injection (ICSI). The immature oocytes collected in cats became mature in 24-32 h in culture media (49). The first IVF embryos were reported in Brazil jaguar (*Panthera onca*) embryos. Also, cat embryos are often cultured in media developed for embryos of other species or other cell types (50). However, systematically refined media and culture systems can make transfer of IVF-derived embryos more successful (51). A well- defined and optimized culture medium for wild animal embryos should be a priority to achieve more births.

Embryo manipulation techniques show promise towards greatly advancing conservation biology. To generate embryos using oocytes and spermatozoa collected from post mortem ovaries and testicles. Zoo researchers employ several techniques for successful embryo manipulation including: laparoscopic transabdominal oocyte aspiration, oocyte in vitro maturation, oocyte in vitro fertilization (IVF), embryo assessment, and embryo transfer (ET). In bovine (52), in vitro produced embryos, after rinsing with PBS and

20% FCS, have been incubated in holding medium containing 1.5 M ethylene glycol (EG) and 1 M dimethyl sulfoxide (DMSO) and vitrified in vitrification solutions 1 and 2 (PBS supplemented with 20% FCS) for 3 min and 30 s, respectively. Embryos straws were then immediately shifted to liquid nitrogen. For retrieval, embryos examined under the stereoscope, are assessed as good or excellent based on symmetry, shape and evenness. Good embryos appeared symmetrical, spherical, and uniformly dark. While fair and poor-quality embryo appeared rather degenerated, pale and misshaped having fragmented cells within it. The good to excellent embryos were collected in cell culture medium at the two to four cell stage for transfer into the oviducts of the recipient which preferably was the same individual or another female of the same species.

A schematic diagram of a routinely followed oocyte vitrification protocol in domestic animals, as reviewed in the literature, is briefly described in Figure 1.

Cryopreservation of oocytes and embryos

Oocytes collected by in vivo pickup or from dead wild animals can be frozen for extended periods of time for the establishment of germplasm banks and subsequent IVF to produce embryos. A consistent oocyte and embryo cryopreservation method has not been established in any species yet. However successful births have been reported from frozen-thawed oocytes in cattle, sheep, and horses (53, 54, 55). Compared to spermatozoa and embryos, oocytes are extremely sensitive to chilling as oocytes typically have a low permeability to cryoprotectants and there is seasonal variation in sensitivity of its membranes towards low temperatures (56). Further mature metaphase II oocytes are more susceptible to cryoinjuries compared to immature ones. Immature oocytes, particularly those at the germinal vesicle stage, often lack cortical granule and have higher membrane permeability for water and cryoprotectants. These features render the cells more resistant to chilling injury than mature oocytes. Resumption of meiosis of isolated immature oocytes of Asian antelopes (*Tetracerus quadricornis*) after vitrification followed by in vitro maturation has been reported. Some major differences in plasma membrane composition [i.e., less polyunsaturated fatty acids (PUFA) in the oocytes compared to embryos (57)], presence of cortical granules, and spindle formation at metaphase II (MII) stage of meiosis (58) makes oocyte cryopreservation challenging compared to embryos.

In a study in rusty spotted cat (*Prionailurus rubiginosus*), immature cumulus oocyte complexes collected from postmortem ovaries and culture grades matured in modified TCM-199 when maintained in an incubator with 5% CO_2 under humidified air at 38.5°C for 36 h (59). The TCM-199 medium was supplemented with 0.3% BSA (fatty acid-free) (w/v), 10 μg/mL FSH, 6 IU/ml LH, 1 μg/mL 17β-estradiol, 0.36 mM pyruvate, 2.2 mM calcium lactate, 2.0 mM L-glutamine, 100 IU/mL penicillin and 0.1 mg/mL streptomycin (59). The oocytes were then successfully loaded and cryopreserved in liquid nitrogen using Cryotop method (60). Loading of oocytes in open pulled straws followed by emersion in liquid nitrogen has also been reported (61).

Vitrification of oocytes works on the principle of effectively suspending biological activity of cells or gonadal tissues whilst preserving their functional status (3), by exposure to liquid nitrogen at -196°C (62). However, at such a low temperature, morphological and functional cryodamage has been reported in immature, in vitro matured or ovulated oocytes. Moreover, many critical ultrastructural elements for maintenance and development of oocytes can be damaged (63). Also post thaw (37°C or below) oocyte zona pellucida or cytoplasmic membrane fractures and cytoskeletal or chromosomal alterations have been reported in animals (64). These damages are attributed to ice crystal formation, osmotic injury and toxic effects of cryoprotectants during cryopreservation.

In vitro embryo production (IVP) in domestic animal models might be reliable for the mass production of embryos and be adapted for use in the cryopreservation in wild animals (65, 66, 67). Embryo cryopreservation allows widespread transfer of embryos 'from rare, severely endangered animals to more abundant recipients so as to regenerate or maintain population. More investigations into basic embryology and embryo preservation is necessary to make embryo transfer a successful and valuable tool in species preservation. The first successful vitrification of mouse embryos using a relatively large volume sample (0.25 mL straw) was achieved by applying a mixture of DMSO, acetamide, and polyethylene glycol in a

straw that was plunged into liquid nitrogen (65). Successful Minimal Drop Size (MDS) method was reported by Arav (7, 66). The authors applied the minimal size and volume used for vitrification of 0.07 mL. This approach maintained embryos without damage by controlling desiccation by using a vitrification solution about 50% lower in concentration than that generally used for large-volume vitrification.

CONCLUSION

Female germplasm cryopreservation can be used to counteract the risks of genetic loss within in situ settings as a result of habitat contraction, reduction in population sizes and increasing inbreeding depression. It is also a valuable technique that can complement captive rearing. Vitrification and cryopreservation of oocytes and embryos are promising tools in efforts to conserve wild animal genetic resources. However, the documented literature in this regard is sparce. The propagation of domestic animals using assisted reproductive techniques, more likely oocyte and embryo preservation, has become more routine and the lessons learned could help to preserve wild animals. Some of the most endangered wild animal species worldwide are in the Himalayas. These species need comprehensive strategies for their conservation and preservation. For example, Hangul (Kashmir stag), Ladakhi Yak and Double Humped Camel populations have declined to critical levels. Future investigations need to focus on adapting available vitrification and cryopreservation protocols, or modifications thereof, for wild species. Special emphasis should be given to safe and effective collection and vitrification procedures. In addition, there are challenges to be solved around embryo transfer as that requires anaesthesia, surgery and hormonal priming of the recipient females. Nonetheless, we believe that future research can better ensure the preservation of wild species genetics.

Acknowledgements: The authors are highly thankful to DST-SERB, India for funding of our research proposal “Oocyte vitrification for long term preservation of important animal genetic resources of temperate Himalaya”. This review was prepared in relation to the proposal.

REFERENCES

1. Chang MC (1948) *J Gen Physiol* **31**, 385–410.
2. Chang MC 1952 *J Exp Zoo* **121**, 351–381.
3. Mazur P (1970) *Science* **168**(**3934**), 939–949.
4. Mazur P (1963) *J Gen Physiol* **47**, 347–369.
5. Corson WH & Gomes Camaru A (1996) *Manual Global de Ecologia*, 2nd edition, Augustus, São Paulo, Brasil.
6. Tachibana M, Kuno T & Yaegashi N (2018) *Reprod Med Biol* **17**, 421–433.
7. Arav A (1992) in *New Trends in Embryo Transfer*, (eds) Lauria A & Gandolfi F, Portland Press, Cambridge, UK, pp 255-264.
8. Kuwayama MG Kato O & Leibo SP (2005) *Reprod Biomed Online* **11**(**3**), 300-330.
9. Saragusty J 2012 in *Current Frontiers in Cryobiology*, (ed) Katkov I, BoD – Books on Demand, pp 293-368.
10. Saragusty J, Hildebrandt TB, Behr B, Knieriem A, Kruse J & Hermes R (2009) *Anim Reprod Sci* **115**, 255–266.
11. Stoops MA, Atkinson MW, Blumer ES, Campbell MK & Roth TL (2010) *Theriogenology* **73**, 1104–1115.
12. Oliveira KG, Miranda SA, Leão DL, Brito AB, Santos RR & Domingues SFS (2011) *Anim Reprod Sci* **123**, 75–80.
13. Comizzoli P (2017) *Anim Reprod* **14**, 30-33.
14. Andrabi SM & Maxwell WM (2007) *Anim Reprod Sci* **99**, 223–243.
15. Otoi TK, Yamamoto N, Koyama S, Tachikawa & Suzuki T (1996) *J of Vet Med Science* **58**(**8**), 811–813.
16. Joseph S & Amir Arav (2011) *Reproduction* **141**, 1–19.
17. Amir A (2020) *Theriogenology* **150**, 263-267.
18. Lopez-Gatius F & Camon-Urgel J (1989) *Zuchthygiene* **24**, 255–258.
19. Tachikawa S, Otoi T, Kondo S, Machida T & Kasai M (1993) *Mol Reprod Dev* **34**, 266–271.
20. Comizzoli P, Songsasen N, Hagedorn M & Wildt DE (2012) *Theriogenology* **78**, 1666-1681.
21. Richings NM, Shaw G, Temple-Smith PD & Renfree MB (2006) *Reprod Fertil Dev* **18**(**6**), 677–688.
22. García-Alvarez O, Maroto-Morales A & Berlinguer F (2011) *Theriogenology* **75**, 65–72

23. Lima GL, Costa LL & Cavalcanti DM (2012) *Reprod Domest Anim* **47**, 1–612.
24. Lima GL, Luz VB & Alves AMCV (2012) *Anim Reprod* **29(4)**, 261.
25. Czarny NA& Rodger JC (2010) *Cryobiology* **60**, 322–325.
26. Czarny NA, Harris MS & Rodger JC (2009) *Reprod Fertil Dev* **21**, 640–648.
27. Wiedemann C, Zahmel J & Jewgenow K (2013) *BMC Vet Res* **22**, 9–37.
28. Boutelle S, Lenahan K, Krisher R, Bauman KL, Asa CS & Silber S (2011) *Theriogenology* **75**, 647–654.
29. Wanderley LS, Luz HK & Faustino LR (2012) *Theriogenology* **77** (**2**), 260–267.
30. Gunasena KT, Lakey JRT, Villines PM, Bush M, Raath C & Critser ES (1998) *Anim Reprod Sc*. **53**, 265–275.
31. Cleary M, Shaw JM, Jenkin G & Trounson AO (2004) *Reprod Fertil Dev* **16**, 699–707.
32. Zheng YL (2013) *Zygote* **21**(**3**), 246–249.
33. Parkes AS & Smith AU (1953) *Biol Sci*, **140**, 455–470.
34. Whittingham, DG, Leibo, SP & Mazur, P (1972) *Science* **178**, 411-414.
35. Wilmut, I (1972) *Life Sciences* **11** (22), 1071-1079.
36. Whittingham, DG (1977) *Journal of Reproduction and Fertility* **49** (**1**), 89-94.
37. Kasai M, Iritani A & Chang MC (1979) *Biology of Reproduction* **21** (4), 839-844
38. Pope CE, Pope VZ & Beck, LR. (1984) *Fertility and Sterility* **42**, 1, 143-145.
39. Lanzendorf SE, Holmgren WJ, Schaffer N, Hatasaka H, Wentz AC & Jeyendran RS (1992) *Journal of Assisted Reproduction and Genetics* **9** (**4**), 358-364.
40. Asada M, Horii M, Mogoe T, Fukui Y, Ishikawa H & Ohsumi, S (2000) *Biology of Reproduction* **62** (**2**), 253-259
41. Wolf DP, Vandevoort CA, Meyer-Haas GR, Zelinski-Wooten MB, Hess DL, Baughman WL & Stouffer RL (1989) *Biology of Reproduction* **41(2**), 335-346.
42. Yeoman RR, Wolf DP & Lee DM (2005) *Fertility and Sterility* **83**(**4**), 1248-1254.
43. Curnow EC, Kuleshova LL, Shaw JM & Hayes, ES (2002) *American Journal of Primatology* **58**(**4**), 169-174.
44. Iwayama H, Hochi S, Kato M, Hirabayashi M, Kuwayama M, Ishikawa H, Ohsumi S & Fukui, Y (2005) *Zygote* **12**(**4**), 333-338.
45. Rodrigues da Paz RC (2012) in *Current Frontiers in Cryobiology*, (ed) Katkov I, BoD – Books on Demand, pp 369-388.
46. Sofi KA, Khan MZ, Islam R & Lone FA (2011) *Small Ruminant Research* **96**, 191-194.
47. Sofi KA, Khan MZ, Islam R & Lone FA (2012) *Ind J of Anim Sciences* **82**(**10**), 1190-1192.
48. Umapathy G (2017) *Conservation of Endangered Animals Based on Genetic Polymorphism Studies and Assisted Reproduction*. A report submitted to Central Zoo Authority of India Ministry of Environment and Forests (Govt. of India), New Delhi.
49. Miller AM, Roelke ME, Goodrowe KL, Howard JG & Wildt DE (1990) *J Reprod Fertil* **88**, 249-258.
50. Pope CE (2000) *Theriogenology* **53**, 163–174.
51. Gardner DK, Pool TB & Lane M (2000) *Semin Reprod Med* **18**, 205–218.
52. Sandal AI & Ozdas O B (2015) *Turk J Vet Anim Sci* **39,** 688-692.
53. Maclellan LJ, Carnevale EM, Coutinho da Silva MA, Scoggin CF, Bruemmer JE & Squires EL (2002) *Theriogenology* **58**(5), 911–919.
54. Otoi TK, Yamamoto N, Koyama S, Tachikawa & Suzuki T (1996) *J of Vet Med Science* **58**(**8**), 811–813.
55. Pereira RM & Marques CC (2008) *Cell and Tissue Banking* **9**(**4**), 267–277.
56. Zeron Y, Ocheretny A, Kedar O, Borochov A, Sklan D & Arav A (2001) *Reproduction* **121**, 447-454.
57. Saragusty J & Arav A (2011) *Reproduction* **141**(**1**), 1-19.
58. Chen SU, Lien YR, Chao KH, Ho HN, Yang YS & Lee TY (2003) *Molecular and Cellular Endocrinology* **202**(**1-2**), 101–107.
59. Rao BS, Mahesh YU, Suman K, Charan KV, Nath R & Rao KR (2015) *In Vitro Cell Dev Biol Anim* **51**(**1**), 19-25.
60. Rao, BS Umamahesh Y, Venu Charan K, Suman K, Sekhar N & ShivajiS. (2012) *Cryobiology* **64**(**3**), 176–184.
61. El-Raey M & Nagai T (2014) *J of Reprod and Infertility* **5** (1), 01-13
62. Woods EJ, Benson JD, Agca Y & Critser JK (2004) *Cryobiology* **48**(**2**), 146–156.
63. Tharasanit T, Colleoni S, Galli C, Colenbrandera B & Stout TAE (2009) *Reproduction*, **137(3),** 391–401.
64. Vajta G & KuwayamaM (2006) *Theriogenology* **65**(**1**), 236–244.

65. Rall WF & Fahy GM (1985) *Nature* **313** (**6003**), 573-575.
66. Arav A, Yavin S, Zeron Y, Natan Y, Dekel I & Gacitua H (2002) *Mol Cell Endocrinol* **187**, 77-81.
67. Nowak A, Kochan J, Prochowska S, Partyka A, Mlodawska W, Witarski W, Skotnicki J, Grega T, Palys M & Nizanski W (2019) *CryoLetters* **40** (4), 226-230