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Review Article

Advancements in assisted reproductive technologies for the conservation of endangered mammalian species - A review

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Abstract

The protection of endangered mammal species has emerged as an urgent issue because of habitat destruction, climate change, and threats caused by human activities. Advanced assisted reproductive technologies (ARTs) offer innovative solutions to counter declining population trends. Methods like in vitro fertilization, embryo transfer, artificial insemination, and gamete cryopreservation have demonstrated their potential in overcoming reproductive challenges in these species. Additionally, emerging approaches like cloning and stem cell technology provide avenues for preserving genetic diversity and even reviving species that are facing extinction. These technologies enable the rescue of genetic material from deceased or infertile individuals, facilitate cross-institutional breeding programs, and minimize the risks of inbreeding in small populations. However, their application faces ethical, logistical, and species-specific challenges, requiring interdisciplinary collaboration among conservationists, veterinarians, and reproductive biologists. Despite these challenges, ARTs represent a promising tool in the global effort to safeguard biodiversity and restore balance to fragile ecosystems. This abstract underscores the importance of integrating scientific advancements with conservation strategies to secure the future of endangered mammalian species.

Keywords: Animals, Conservation, Mammals, Reproductive Technologies.

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Introduction

Nowadays, extinction is happening at a much faster rate than speciation because of destructive human activities like poaching, overfishing, and hunting. The goal of animal conservation is to comprehend and protect biodiversity because the extinction

of a single species can have a significant effect on how well entire ecosystems function [1 - 3]. Over the last two centuries, 46 mammalian species have vanished, and numerous others are in danger of extinction, with populations of less than 1000 individuals [4]. The International Union for Conservation of Nature [4] reports that 30% of invertebrates, 60% of plant species, 25% of mammals, 12% of birds, 20% of reptiles, 30% of amphibians, and 20% of fish are in danger of going extinct [5]. Numerous populations of these mammalian species are small, dispersed, and lack genetic exchange in their habitat, which makes inbreeding and homozygosity more common and ultimately results in poor fertility and an inability to adapt to changes in the environment [6]. According to recent studies, more than 25% of mammalian species are under threat of extinction, with certain flagship species, such as the northern white rhinoceros, reduced to only two non-reproductive females globally. Similarly, many huge carnivores and mega herbivores live in fragmented groups of fewer than 500 mating individuals, where genetic drift and inbreeding depression severely limit fertility and survival. In such small communities, natural mating is frequently insufficient to sustain a population increase. These realities highlight the critical necessity for conservation projects to use assisted reproductive technologies to preserve genetic variety and avert irrevocable biodiversity loss [5].

In addition to safeguarding organisms in their natural environments (in situ conservation), it is essential to sustain robust populations in captivity (ex situ) to support potential reintroduction efforts in the future. However, infertility, inadequate space, health and husbandry issues, unadopted diets, or altered sexual behavior can all compromise reproduction fitness in captivity [6, 7].

Thus, assisted reproductive technologies (ART) can be used to maximize conservation breeding in order to get around the problems mentioned above. Over the past several decades, these tactics have been strongly advocated in an effort to maintain small populations of rare species and enhance breeding management [8]. The importance of employing assisted reproductive technologies (ARTs) to enhance reproductive success and ensure the long-term conservation of valuable genetic resources, given the increasing threat of extinction of vertebrate species [9]. Along with artificial insemination (AI), embryo transfer (ET), and in vitro fertilization (IVF), several other tools and techniques have been developed [10, 6]. Germplasm cryobiology is one of these valuable tools that has been essential in creating biorepositories that capture current genomic diversity [11]. These methods encompass (i) well-established conventional assisted reproductive technologies (ART), including semen collection, artificial insemination (AI), transvaginal ovum pick-up (OPU), and in vitro fertilization (IVF), hormone monitoring and administration, and trans cervical embryo transfer (ET), and (ii) advanced ART (aART) techniques, which necessitate specialized laboratory equipment and knowledge beyond what is required for the more "classical" ART techniques. The latter include inner cell mass (ICM) exchange, somatic cell nuclear transfer (SCNT), IVF with intracytoplasmic sperm injection (ICSI), and stem-cell associated techniques (SCAT) [12]. The combination of living cell biobanking and advanced assisted reproductive technologies (aART) is essential for reestablishing both genetic diversity and population size [13, 14]. The successful application of assisted reproductive technologies to severely endangered species demonstrates its practical relevance. For example, artificial insemination with cryopreserved sperm has helped to restore genetic variety in the black-footed ferret (Mustela nigripes)

population. Similarly, artificial insemination and semen cryobanking have been critical in the conservation breeding of giant pandas, while advanced ARTs are presently the sole viable method for recovering the functionally extinct northern white rhinoceros. These stories demonstrate how ARTs have progressed beyond theory to become critical instruments for species survival [15, 16].

The main objective of this article is to review reproductive biotechnologies that are currently in use to maintain the fertility of mammalian species populations as well as new technologies related to resolving conservation issues.

Research methods

This narrative review is based on a comprehensive assessment of peer-reviewed literature obtained from Web of Science, Scopus, PubMed, and Google Scholar. Relevant articles were found using keyword combinations such as assisted reproductive technologies, wildlife conservation, endangered mammals, cryopreservation, in vitro fertilization, cloning, and stem cells. Priority was given to original research publications and authoritative reviews with a focus on studies demonstrating the practical use of ARTs in mammalian conservation. Reference lists for relevant papers were also reviewed to guarantee thorough coverage.

Cryopreservation or biobanking of living cells

Cryopreservation or biobanking of living cells is the method of keeping organisms, tissues, or cells at extremely low temperatures (usually in liquid nitrogen at -196°C) to maintain their viability for future use. Cryobanking or biobanking is the only component of biodiversity conservation that can significantly aid in species recovery and population management [15 - 17]. The rate of extinction makes it difficult to create specialized conservation plans for every endangered species of plant or animal. Biobanking is an effective backup strategy to buy as much time as possible for as many species to coexist with humans on Earth. Living cells contain all the organelles and machinery needed to support and reproduce life in addition to genetic information, even though many biobanks collect dead materials, for example, tissue kept for pathophysiological reasons in alcohol, formalin, , or frozen without cryoprotectants or genetic examinations or blood serum for hormone or biochemical analyses [12]. In the black footed ferret, artificial insemination with sperm cryopreserved for almost 20 years effectively produced offspring and restored lost alleles to the population, contributing to a measurable increase in genetic diversity [15, 17]. In the giant panda, artificial insemination accounts for more than 60% of captive births worldwide, significantly increasing reproductive output in managed populations [16].

Living cell preservation offers additional opportunities but also necessitates specialized knowledge, work, skills, and technical prerequisites like, for instance, sterile working conditions, cryoprotectants, cell culture media or sperm extenders, and specialized cryopreservation protocols. One source of biological material that can grow is somatic cells, but living gametes can also be used to create embryos. With the development of stem cell technology, this strategy has a great deal of promise to protect biodiversity on Earth [18]. For many species, zoological establishments like traditional zoos, safari parks, or animal shelters serve as an ark, giving them comparatively easy access to appropriate biomaterials. A variety of biomaterials can produce appropriate cell material for culture, such as hair follicles, blood feathers, gingival swabs, the cellular content in urine, the isolated buffy coat

from whole blood, or even the mucous layer on fecal matter. These biomaterials can be collected minimally or non-invasively, either during veterinary procedures or post-mortem. Following a successful culture, these living cells can be kept for hundreds or even thousands of years in central bio archives at -196°C in liquid nitrogen (lN2) [19 - 21]. Biopsy material, cultured fibroblasts, and embryonic stem cells only need simple, reliable cryopreservation methods with 10% DMSO, such as slow freezing over liquid nitrogen vapor. The freezability of these delicate biomaterials varies greatly, though, and is frequently species- and even individual-specific in the case of oocytes, spermatozoa, and preimplantation embryos [22 - 25].

The development of the vitrification method, which was primarily used for embryos and Metaphase II (MII) oocytes, revolutionized cryobiology and gave the human IVF industry a new cryopreservation option with better cryosurvival and clinical results [26 - 28]. It is possible to use either open or closed systems for vitrification, meaning that direct contact with lN2 is not necessary. Cryosurvival rates are lower in closed systems, but biosafety is increased by preventing cross-contamination with liquid nitrogen. Vitrification is still not widely used in wildlife reproduction, though [29, 30]. Table 1 given below gives the overview of biobanking methods and their applications.

Intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF)

Intracytoplasmic Sperm Injection (ICSI) is a procedure that facilitates fertilization by directly introducing a single sperm into an egg, commonly used in cases of male infertility where as In Vitro Fertilization (IVF) is a technique in which eggs and sperm are fertilized outside the body in a laboratory, resulting in embryos that are later implanted into the uterus to initiate pregnancy. During the IVF procedure, a maximum of 10^4 capacitated spermatozoa and a mature, haploid ovum (M-II) are temporarily cultured for a maximum of 12 hours. The zona pellucida and vitelline membrane, or oolemma, will be penetrated by a single competent sperm cell. If karyogamy is successful, the resulting zygote begins cleavage 24 to 36 hours after fertilization [12]. More recently, IVF programs in the white rhinoceros have produced numerous viable blastocysts from cryopreserved gametes, marking a significant step toward preventing species extinction [13 - 14].

ICSI, which uses a micromanipulator to inject a single sperm cell into a MII oocyte, may be helpful for male subfertility or endangered species because it requires a significantly lower sperm count and semen quality. The morphological and motility characteristics of the available sperm cells are used by the embryologist to choose the single sperm.

Table 1: Bio-banking methods and applications.

Biomaterial Type	Preservation Method	Conservation Value	References
Spermatozoa	Slow freezing or vitrification	Male genetic diversity preservation	[22, 24]
Oocytes	Vitrification	Female genetic material conservation	[29, 30]
Somatic Cells	Slow freezing with DMSO	Cloning, iPSC generation	[21]
Embryos	Vitrification or slow freezing	Complete genotype preservation	[13, 14]
Primordial Germ Cells	Vitrification	Future gamete production	[52]

The sperm cell is aspirated into the injection capillary after being rendered immobile by causing a tail injury. It is comprised of two hydraulic actuator arms, one with an injection capillary and one with a holding capillary, and a high-resolution inverted microscope. While human IVF labs usually use glass tubes that resemble needles and have an inner diameter of 4 to 5 μM for traditional ICSI capillaries, with the piezo drill method, a blunt glass capillary is used with an inner diameter of typically 5 μM that uses micro-vibrations to pass through the vitelline membrane and zona pellucida. Murine ICSI and equine IVF labs are the primary uses for the latter [31].

In vitro embryo production

The process of producing embryos is known as in vitro embryo production (IVP) outside the body by collecting eggs and fertilizing it using sperm in a lab. After that, the resultant embryos can be placed inside a uterus for development. It is common practice to cultivate embryos to the blastocyst stage in specialized media. This process can be one-step or two-step, meaning that the media can be renewed either way as the embryo progresses from the zygote to the blastocyst stage. Beneficial compounds are re-supplied through media exchange, which also eliminates metabolic products that are beneficial, like growth factors. Depending on species-specific factors, the time it takes for embryos to develop into early blastocysts can vary from 4 to 11 days. Typically, a traditional 5%-CO2 incubator with reduced oxygen is used for culture. However, the embryologist can alter the conditions of each culture according to the embryo's performance during the incubation period by using time-lapse incubators like the Embryoscope TM (VitroLife) or GERY (Merck). This choice is particularly helpful in cases where the cultured species is unfamiliar. Usually, embryos early in the blastocyst stage (unhatched) are either used fresh in an embryo transfer program or cryopreserved in lN2 [13, 14]. Optimizing the species-specific culture conditions can help prevent negative epigenetic effects, like the large calf syndrome that had been reported as a result of in vitro embryo production [32].

Cloning or nuclear transfer of somatic cells (SCNT)

Reproductive cloning creates an animal with a nucleus that shares the same genome sequence as the somatic cell's donor by introducing genetic material from a somatic cell into an oocyte that has had its nucleus removed [33]. Nuclear transfer from somatic cells is a cloning technique which is used in order to create genetic duplicates of an individual by transferring the nucleus of a somatic cell into an oocyte from which the nucleus has been removed and then using an electric shock to trigger mitosis. For instance, it supported the Przewalski's horse *(Equus ferus przewalskii)* and the black-footed ferret *(Mustela nigripes)* as part of various species rescue initiatives [34, 35]. In order to improve particular traits, bring back extinct species, or manage invasive species, SCNT may be used in conjunction with gene editing [36]. Oocytes and somatic cells from various species are used in interspecies SCNT (iSCNT). These can be used for species conservation or to create ESC-like cell lines from the inner cell mass (ICM) of blastocysts [37]. On the other hand, mitochondrial heteroplasmy is primarily responsible for the low success rates in producing live animals. Only 11 species have produced live offspring thus far: *Camelus bactrianus*, *Canis lupus*, *Canis latrans*), *Ovis aries musimon*, *Felis lybica*, *Felis margarita*, *Capra pyrenaica*, *Bos gaurus*, *Bubalus bubalis*), and *Caracal caracal* [38].

Inner cell mass exchange (ICM)

Inner Cell Mass (ICM) exchange is a technique used in embryology and

developmental biology where the ICM of a blastocyst is removed and replaced with the ICM from another blastocyst. The use of closely related species as surrogate mothers is possible for species with a small number of individuals. Using micromanipulation, it is possible to transplanting the donor inner cell mass into the surrogate's empty trophoblastic vesicle will produce an interspecies trophoblast-ICM chimera for interspecies embryo transfer. For sheep and goats, for instance, this has been accomplished. Despite the potential for critically endangered species, the combination of in vitro gametogenesis and ICM has not yet been successfully implemented [12].

Embryo transfer (ET)

Embryo Transfer (ET) is the process of placing an embryo into the uterus of a female to achieve pregnancy, commonly used in assisted reproduction and animal breeding. In endangered mammals, a several of embryo transfer methods have been described. The transcervical ET is the procedure that is used the most. In larger mammals, the transfer catheter can be manually inserted through the rectum to observe the ipsilateral uterine horn; in smaller species, transrectal or transabdominal ultrasound can be used to observe the procedure. Transferring the blastocyst embryo at an early, unhatched stage is preferable [4, 39, 40].

The embryo can be inserted non-invasively into the oviduct through the infundibulum pathway (2 to 4 cell embryo) or directly into the upper ipsilateral uterine horn (blastocyst) using the second ET technique, which is carried out via laparoscopy. These methods have been used on big cat species [41].

The size and anatomical peculiarities of megavertebrates, like rhinoceroses and Asian and African elephants (*Elephas maximus* and *Loxodonta africana*), necessitate a different approach when performing embryo transfer. The most successful and minimally invasive ET technique for megavertebrates has been demonstrated to be the incorporation of the Corpus luteum graviditatis into the ipsilateral uterine horn near the ovary under ultrasound guidance [12].

In vitro gametogenesis (IVG) using stem cells to produce gametes

In vitro gametogenesis (IVG) is a process where stem cells are used to create sperm or eggs in a laboratory, allowing the production of gametes outside the body for reproductive purposes. The fundamentals of reprogramming living cells to produce induced pluripotent stem cells (iPSC) were established [42]. In order to create future in vitro gametogenesis systems, [43] created the first wildlife iPSC lines and then successfully differentiated white rhinoceros (*Ceratotherium simum*) iPSC to primordial germ cells (PGC) [44, 20].

IVG is a complicated process that starts with the primordial germ (PGCs) cells' development and replicates the stages of the embryo from gastrulation to puberty. PGCs move to the gonadal ridge and mature into gametes there, after developing early in the embryo, around gastrulation. Because mouse (*Mus musculus*) embryos are accessible at all developmental stages, we have the most comprehensive understanding of PGC specification in this species. Since the initial demonstration of in vitro-derived mouse PGC generation, gametes for both males and females have matured in culture [45 - 47].

Live offspring can be produced by combining these IVG-derived gametes with natural gametes. The cynomolgus monkey (*Macaca fascicularis*), marmoset (*Callithrix jacchus*), and northern white rhino are among the few wild species in which PGCs have been successfully produced [48, 49, 20]. However, only the

mouse model has produced fully developed oocytes or sperm so far. Aggregation of PGCs with gonadal matrix cells (ovaries and testes) is necessary for the full IVG. Since embryonic tissue is unavailable for endangered species, these supporting cells must be produced from iPSCs. iPSCs have already been shown to differentiate into ovarian somatic-like cells in mice [50]. Additionally, the production of female gametes from a male cell line is an exciting possibility [50]. But implementing it will take a lot of time and resources to implement IVG in critically endangered mammal species. Table 2 summarizes information about the present-day advanced assisted technologies.

Restoring genetic diversity through gene editing

Restoring genetic diversity through gene editing is the process of using advanced genetic tools, like CRISPR, to introduce or modify genes in a population to increase genetic variation and improve traits such as health, resilience, or fertility. It involves using advanced molecular techniques, such as CRISPR-Cas9, TALENs, or base editing, to introduce genetic variations into a population that has low genetic diversity. Using material preserved in museums to ascertain the genetic composition of the species before the crisis of extinction is one possible future solution for Mammal species that are critically endangered and have a very small gene pool. The genetic profiles of extinct populations could serve as a blueprint for reconstructing genetic diversity in current populations. By using gene editing techniques like CRISPR-Cas9, single nucleotide polymorphisms (SNPs) or even whole genes that have been lost of haplotype patterns can be incorporated into developing fibroblast cultures' cells [51]. The genetically altered cells have the potential to develop into iPSC, which will serve as the foundation for IVG procedures in the future. Lastly, gametes derived from cultures may contain lost genetic patterns that have been previously found in museum specimens. To the best of our knowledge, wildlife species have not yet been subjected to this. Overview of the established ARTs in wildlife conservation is provided in Table 3.

Table 2: Advanced assisted reproductive technologies (aARTs).

Technology	Principle	Conservation Applications	References
Somatic Cell Nuclear Transfer (SCNT)	Nuclear transfer from somatic to enucleated oocyte	Cloning of endangered species	[33, 34]
Intracytoplasmic Sperm Injection (ICSI)	Direct sperm injection into oocyte	Male infertility, limited sperm samples	[31]
Inner Cell Mass (ICM) Exchange	Replacement of ICM between blastocysts	Interspecies surrogacy	[12]
Vitrification	Ultra-rapid freezing using high cryoprotectant concentrations	Gamete and embryo preservation	[26, 28]
Stem Cell Associated Techniques (SCAT)	Use of pluripotent stem cells for reproduction	Gamete production, genetic rescue	[44, 50]

Table 3. Established assisted reproductive technologies (ARTs) in wildlife conservation.

Technology	Description	Key Applications	References
Embryo Transfer (ET)	Transfer of embryos to recipient females	Genetic rescue, population expansion	[5, 22]
In Vitro Fertilization (IVF)	External fertilization of eggs and sperm	Overcoming natural mating barriers	[12]
Cryopreservation	Long-term storage at -196°C in liquid nitrogen	Genetic resource banking	[11, 24]
Sperm Cryopreservation	Freezing of spermatozoa for future use	Male genetic material preservation	[22, 25]

Ethical, genetic, and ecosystem considerations in assisted reproductive technologies

Animal welfare considerations

The use of ARTs on endangered species creates serious animal welfare problems. Procedures like as oocyte retrieval, sperm collection, embryo transfer, and laparoscopy can induce stress, pain, and physiological disruption if not handled properly. Maintaining correct anesthesia protocols, post-operative care, and minimal handling is critical for reducing morbidity and death in donor and recipient animals [34, 41]. Furthermore, therapies in wild or semi-wild animals must include species specific behavioral and social demands to minimize long-term stress that could jeopardize reproductive success. Ethical oversight by institutional animal care committees is strongly advised to verify that all methods meet welfare requirements [34].

Genetic integrity and population viability

Cloning, interspecies somatic cell nuclear transfer (iSCNT), and in vitro gametogenesis are all examples of ARTs that may provide genetic concerns. Cloning can result in epigenetic anomalies, decreased fitness, or unexpected health concerns in offspring, whereas recurrent usage of a small gene pool might increase inbreeding depression [33, 38]. To mitigate these dangers, comprehensive genetic screening of donor and recipient animals, allelic variety monitoring, and long-term progeny observation are required. Biobanking procedures, together with proper pedigree management, help to preserve natural genetic variation and limit the likelihood of maladaptive traits being amplified in small or fragmented populations [2, 15, 52].

Ecosystem and conservation safety

The reintroduction of ART produced individuals into wild populations' demands a thorough ecological risk assessment. Disease transmission, disturbance of existing social systems, resource rivalry, and the introduction of maladaptive features that may have an impact on ecosystem stability are all potential problems [2, 4]. Health monitoring and quarantine methods are crucial prior to release, as is careful matching of individuals to habitats that promote survival and reproductive success. To minimize unforeseen ecological implications, conservation methods should consider the potential effects on trophic relationships, predator prey dynamics, and interspecific competition [2, 41].

Ethical and societal implications

Beyond welfare and ecological implications, ethical issues include consent to intervention (primarily in captive breeding operations), priority of species conservation goals, and equitable resource allocation [34]. The long-term socioeconomic and environmental consequences of utilizing high cost ARTs should be assessed to ensure that interventions do not benefit a small number of species at the price of overall ecosystem stability. Open communication with conservation stakeholders, local communities, and government promotes transparency and public trust in ART based projects. [53].

Challenges and limitations in wildlife ART application

The use of assisted reproductive technologies (ARTs) in wildlife conservation faces numerous challenges. One major limitation is that most endangered mammals lack basic reproductive knowledge, such as estrous cycles, seasonal breeding patterns, hormone profiles, gamete biology, and reproductive anatomy, making it difficult to

develop effective species-specific ART protocols [54]. Even when ARTs are used, success rates are frequently poor; procedures such as artificial insemination, in vitro fertilization, and embryo transfer have only produced limited results in non-domestic species due to species-specific reproductive responses [55]. Logistical and technical constraints further limit implementation: collecting, transporting, and preserving viable gametes and embryos necessitates specialized equipment, controlled surroundings, and immediate access to animals, all of which are rarely practicable in field situations [56]. Additional obstacles include regulatory hurdles, international collaboration challenges, and disparities in national wildlife regulations, all of which limit genetic material sharing and coordinated conservation efforts. These biological, technical, and regulatory constraints underline the need for ongoing study, infrastructure development, and international collaboration to effectively use ARTs for wildlife conservation.

Conclusion

Advanced assisted reproductive technologies (ARTs) play a pivotal role in the conservation of endangered mammalian species by addressing critical challenges in biodiversity preservation. Techniques such as artificial insemination, in vitro fertilization, embryo transfer, cloning and other techniques described in this review offer invaluable tools to overcome reproductive barriers, enhance genetic diversity, and expand population sizes in captive and wild settings. Moreover, the integration of cryopreservation and genomic advancements ensures the long-term storage and utilization of genetic material, safeguarding species against extinction. While these technologies hold immense promise, their success depends on continued research, ethical considerations, and collaboration among conservationists, scientists, and policymakers. Together, these efforts can help secure a sustainable future for threatened mammalian species and the ecosystems they inhabit.

Author's contribution

All authors equally contributed

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