Theriogenology 169 (2021) 76–88

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

The ART of bringing extinction to a freeze – History and future of species conservation, exemplified by rhinos

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ARTICLE INFO

Article history:
Received 31 October 2020
Received in revised form
12 April 2021
Accepted 14 April 2021
Available online 18 April 2021

Keywords:
Cryopreservation
Self-sustaining population
Stem cell technologies
In-vitro-gametogenesis
Biobanking

ABSTRACT

The ongoing mass extinction of animal species at an unprecedented rate is largely caused by human activities. Progressive habitat destruction and fragmentation is resulting in accelerated loss of biodiversity on a global scale. Over decades, captive breeding programs of non-domestic species were characterized by efforts to optimize species-specific husbandry, to increase studbook-based animal exchange, and to improve enclosure designs. To counter the ongoing dramatic loss of biodiversity, new approaches are warranted. Recently, new ideas, particularly the application of assisted reproduction technologies (ART), have been incorporated into classical zoo breeding programs. These technologies include semen and oocyte collection, artificial insemination, and in-vitro embryo generation. More futuristic ideas of advanced ART (aART) implement recent advances in biotechnology and stem-cell related approaches such as cloning, inner cell mass transfer (ICM), and the stem-cell-associated techniques (SCAT) for the generation of gametes and ultimately embryos of highly endangered species, such as the northern white rhinoceros (*Ceratotherium simum cottoni*) of which only two female individuals are left. Both, ART and aART greatly depend on and benefit from the rapidly evolving cryopreservation techniques and biobanking not only of genetic, but also of viable cellular materials suitable for the generation of induced pluripotent stem cells (iPSC). The availability of cryopreserved materials bridges gaps in time and space, thereby optimizing the available genetic variability and enhancing the chance to restore viable populations.

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https://doi.org/10.1016/j.theriogenology.2021.04.006
0093-691X/

1. Introduction

Cryopreservation in liquid nitrogen at −196 °C brings most biological processes to a halt, thereby greatly expanding the possibilities of assisted reproduction technologies (ART). The visionary physician and researcher, Kurt Benirschke, in 1975 created the "Frozen Zoo®", the first large-scale systematic cryobank for blood products, DNA samples, tissue, cells and, reproductive material of exotic species at the San Diego Zoo. In anticipation of future technologies, he already intended to extend biological knowledge and to sustain biodiversity, stating that "You must collect things for reasons you don't yet understand." [1]. This collection has expanded since, counting 10,000 cell lines in 2020 [2]. Indeed, biotechnological possibilities have greatly expanded, opening a wealth of new tools and possibilities, many of which are still being implemented or are at the boundaries of our current imagination.

The Anthropocene – the human epoch – is characterized by the dominance of *Homo sapiens* over the planet. Overexploitation of limited natural resources bears the risk of significant negative economical and societal consequences [3] as we fundamentally depend not only on their steady supply but also on the invaluable 'ecosystem services' which intact ecosystems provide [4]. The current unprecedented rate of species extinction is estimated at 100 to 1000 times higher than natural background rates [5,6] (Fig. 1a and b). This earth's sixth great extinction event has already driven 22% of all mammalian species close to being lost forever. Ecological interactions are extremely complex and intertwined so that the loss of a single species may have much larger implications than we can foresee (so-called vortex-effect [7]). In our own interest [5,8], it becomes more and more pressing to contain and ideally reverse this development by protecting ecosystems, species and genetic variability.

Important scientific advances have been achieved in the recent past and will continue to open new pathways for the future of biodiversity conservation. After summarizing the historical, present and future possibilities, limitations, and success stories of species conservation, we will explore the new possibilities arising from advancing cryopreservation methods. Many important examples are connected with rhinoceros species, which grants them an inherent focus in our review; nevertheless – wherever relevant and possible – we attempt to adequately mention examples across all animal taxa.

2. Species conservation strategies

The most fundamental level of species conservation is the protection of natural resources, as habitat loss or degradation for logging and agriculture presents the greatest current threat to biodiversity [9]. Starting in the late 18th century and encouraged by scientists such as Alexander von Humboldt [10], the Yellowstone National Park became the first public protected area (Fig. 2a and b). Nevertheless, large viable habitats are being emptied of wildlife by hunting [11,12], pollution [13], and by invasive species [14]. In many instances now, however, restoring habitat, stopping poaching and removing pollution and invasive species will not solve the problem because remaining clusters of wild animals are too small to be viable [15]. One of the earliest species conservation success stories concerns the southern white rhinoceros (SWR, *Ceratotherium simum*) which recovered from near extinction [16], through hunting bans and habitat protection [17], as well as strategic translocation [18]. Translocations have since become an important tool in wildlife conservation [19–21]. This success renders the SWR – despite lately declining numbers [22,23] – the most abundant rhinoceros species today. In contrast, the sister taxon northern white rhinoceros (NWR, *Ceratotherium simum cottoni*) has dwindled to only two female individuals, as poaching for rhino horn [24,25], leaves the Rhinocerotidae one of the most threatened mammalian families [26,27].

Insurance populations and ex-situ breeding by zoological institutions, crucial to species conservation plans [28,29], have led to at least 13 to 19 well-documented success stories among vertebrates [30–33], comprising the European bison (*Bos bonasus*) [34] (Fig. 2c), the Przewalski horse (*Equus ferus przewalskii*) [35] (Fig. 2c), the Arabian oryx (*Oryx leucoryx*) [36], and the red wolf (*Canis rufus*) [37,38]. Currently ongoing reintroduction efforts include for example the Scimitar-horned oryx (*Oryx dammah*) [39], and the Spix's macaw (*Cyanopsitta spixii*) [40]. Despite considerable challenges and drawbacks, these encouraging examples illustrate the feasibility re-establishing wild populations that had already gone extinct.

To halt mass extinction and irreversible loss of keystone species before suitable solutions are found or habitats restored requires elaborate methods to enhance genetic management. Especially if complicated by small numbers of founder individuals, infertility due to old age, diseases, spatial distance between individuals or suboptimal husbandry, assisted reproduction technology (ART) [41–43] or advanced ART (aART) involving state-of-the-art stem cell and biotechnology (see Fig. 1c) will be necessary. Therein, making use of cryopreserved tissues, cells and reproductive materials is crucial, providing the opportunity to preserve and convey genetic diversity across time and space (Fig. 1d).

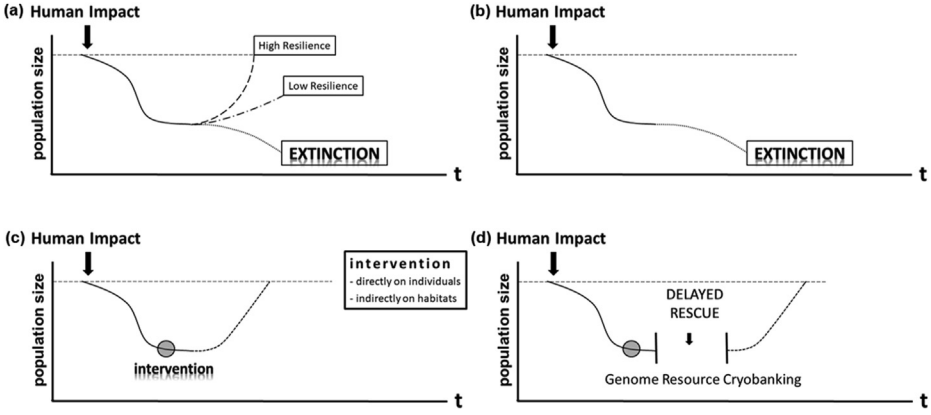

Fig. 1. Different scenarios of population development due to human impact.
Fig. 1. (a) Three different scenarios of the population development after human impact. (b) Reality for a large number of mammals, birds, and reptiles e.g. dodo, Steller's sea cow since 1600s. (c) Interventions as demonstrated in (a)–(d) can help to rescue critically endangered species. (d) The role of cryopreservation if current technologies are not sufficient.

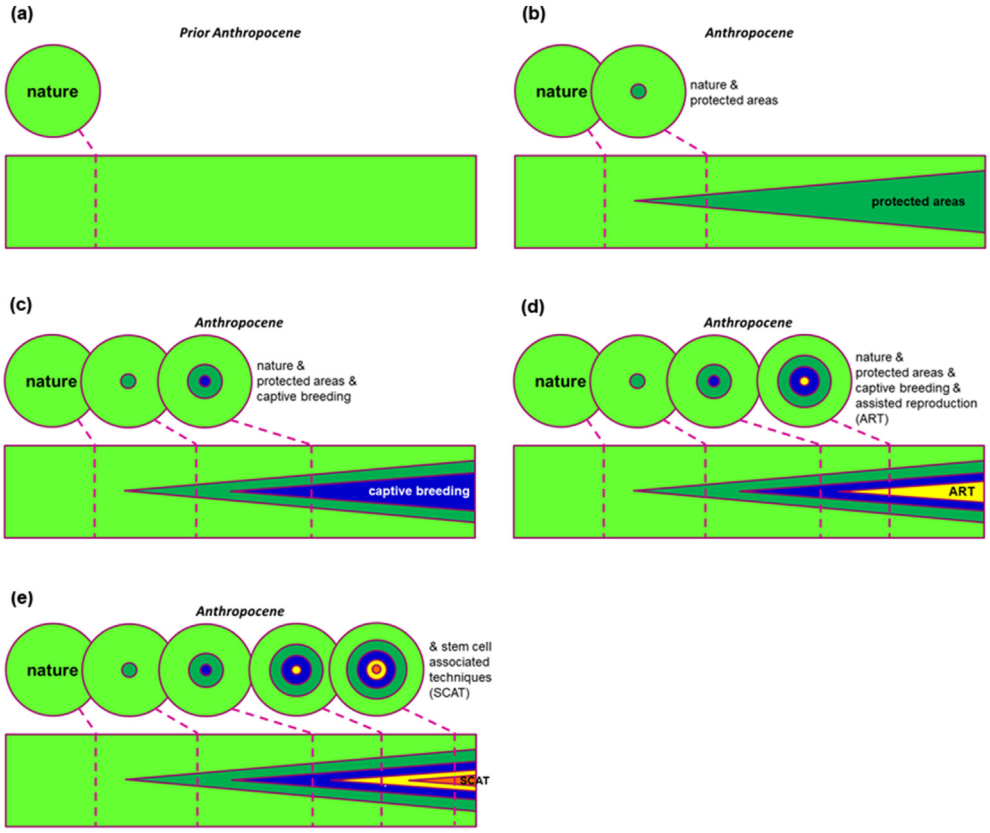

Fig. 2. Different levels of human intervention for species conservation.
Fig. 2. (a) Intact nature prior anthropogenic impact. (b) Situation after foundation of 1st national park (Yellow-Stone National Park) 1872. (c) Captive breeding for reintroduction programs (e.g. European bison, Przewalski horse). (d) Implementation of assisted reproduction (e.g. black footed ferret, Californian condor). (e) *BioRescue* – an international program for saving the northern white rhino from extinction will use SCAT for artificial gamete generation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.1. Assisted reproduction technology (ART)

ART in wildlife species has been extensively reviewed elsewhere (e.g. Refs. [44–46]) and will be only briefly summarized here to illustrate the importance of cryopreservation. Many of the established techniques have been developed and optimized for livestock production. In-depth-knowledge of reproductive anatomy and physiology are an important prerequisite, but only available for approximately 250 species, mainly mammals and birds [47]. Taxonomically closely related domestic or less endangered model species may serve as a blue-print for developing suitable protocols for endangered wildlife species [42]. The first species to profit from the use of ART is the California condor (*Gymnogyps californianus*). Mainly due to lead poisoning it went extinct in the wild in 1987. Artificial incubation, foster parenting, or hand-rearing, state-of-the-art molecular genetics [48,49] and a ban of lead ammunition have restored a largely independent wild population [50] (Fig. 2d).

2.1.1. Hormone monitoring and administration

Endocrine monitoring of steroid metabolites is indispensable for monitoring ovarian cyclicity, infertility, seasonality of testicular activity, pregnancies, and for the determination of optimal timing for reproductive interventions, whereas monitoring corticosteroid levels can help to improve husbandry by assessing stress [51]. Hormonal stimulation is crucial to induce ovulation and manipulate reproductive activities (e.g., superovulation, or estrus synchronization in preparation for artificial insemination (AI), embryo transfer (ET), or contraception) and requires different protocols in different taxa, e.g. Refs. [52–55]. Cryopreservation of the relevant samples allows for long-term collection, systematic evaluation, and safely shipping samples to laboratories.

2.1.2. Semen collection

Semen collection is fundamental to fertility assessment, ART, and gamete cryo-banking [56]. Relevant methods comprise massaging, phantom use, postcoital collection, e.g. Refs. [57,58], electroejaculation (EE) [59] urethral catheterization [56,60], or obtaining epididymal sperm by castration, biopsy, aspiration, or post mortem. Most frequently used methods In wildlife species are EE (from bats [61] to rhinoceros [62]), as well as urethral catheterization (mainly used in carnivores [63]). Semen collection opens up the possibility to move sperm instead of animals between facilities or between the wild and captivity. This permits for the inclusion of individuals into the gene pool that do not naturally mate owing to physical or behavioural handicaps, the use of aliquots for disease screening, or sex-sorting [64,65]. Semen cryopreservation removes the limitations imposed by generations and time, involving even long deceased individuals in reproduction [42,52,66].

2.1.3. Artificial insemination (AI)

AI is the most frequently used ART and has produced viable offspring in more than 50 wildlife species [45], comprising 14 bovid, seven cervid [53,67], three cetacean [68–72], two rhinoceros [73–75], and various wild cat species, including ocelot, Pallas's cat, fishing cat, sand cat, tiger, and clouded leopard [76,77]. Building on

knowledge of the poultry industry, AI supports the recovery of the whooping crane, peregrine falcon [78], houbara bustard [79,80], and Spix' Macaw [81], and was successful in numerous species of raptors, cranes, waterfowl, psittacines, and passerines [82].

Relevant contributions of AI to species recovery programs are limited to (i) the black-footed ferret (*Mustela nigripes*), by increasing genetic diversity while reducing inter-generational time [[83]]. Laparoscopic AI with fresh or frozen semen, even 20 years after their cryopreservation [42], has generated more than 8000 offspring and 4400 releases [83–85] (Fig. 2d). (ii) Giant panda (*Ailuropoda melanoleuca*) populations, decreasing due to habitat loss, poaching, bamboo flowering [86], low reproductive success [87] improved – besides by hand rearing of twin cubs [88] – due to AI with fresh and frozen-thawed semen [89], (Fig. 2d). (iii) AI in captive Asian (*Elephas maximus*) and African elephants (*Loxodonta africana*) [90–93] helps to avoid breeding-related transfers and increase genetic exchange, also with wild populations [94].

2.1.4. Ovum pick up (OPU)

The collection of oocytes (OPU) is more invasive, complex, and costly than that of semen. It is either performed timely post mortem, laparatomically, transcutaneously under ultrasound-guidance, transvaginally, or transrectally. OPU is regularly performed in domestic or laboratory species such as cattle, deer, horses, and macaques. It has been rarely applied in wildlife [45], where it is reported mainly for black [95] and Sumatran rhinoceros [96], and recently for the northern and southern white rhinoceros [52,96].

2.1.5. In vitro fertilization (IVF) and embryo transfer (ET)

In vitro fertilization (IVF) with fresh or frozen-thawed semen has been developed and optimized for humans, laboratory animals, and livestock [45] to increase the female genetic contribution to the gene pool. IVF is followed by in vitro culture of embryos and ET. Obstacles are oocyte or embryo retrieval, the vulnerability of the large oocyte to cryo-damage, more complex handling and culture, and costly equipment. Moreover, synchronization of embryo development and facilitating foeto-maternal recognition is a major obstacle to overcome. Exact knowledge of reproductive cycles is crucial for successful reimplantation. Consequently, as compared to AI, relatively few live offspring have been produced following IVF-ET.

Since the first successful mammalian embryo transfer in the rabbit in 1890 [97], the first non-domestic species to give birth to live offspring after ET was the baboon (*Papio cynocephalus*) [98]. Successful interspecies embryo transfers were achieved from eland (*Tragelaphus oryx*) and gaur (*Bos gaurus*) to domestic cow (*Bos Taurus*) [99,100], and from bongo (*Tragelaphus euryceros*) to eland [101]. Despite standard use in domestic species, and despite further successes in IVF and ET of felid [102–107], bovine [108], deer [109–111], and primate species as models for human ART [112], this technique has not played a major role in the genetic management of mammalian wildlife species so far [113]. Successful hormonally induced gamete harvesting, *in-vitro* fertilization, and embryo development have generated large numbers of viable amphibian offspring, including the endangered Wyoming toad (*Bufo baxteri*) and Mississippi gopher frogs (*Rana sevosa*), which were released to the wild [114,115].

2.2. Advanced assisted reproduction technology (aART)

The term advanced assisted reproduction technology was coined in 2004 [116] and refers to methods that require extensive laboratory equipment and expertise which lie beyond those needed for the more "classical" ART methods. While some of these advanced techniques have been available for decades, recent developments have opened up new pathways which may present the only hope for critically endangered species such as the northern white rhinoceros or the Bornean subspecies of the Sumatran rhinoceros (*Dicerorhinus sumatrensis harrissoni*). In both cases the effective founder population is already too small for established species recovery programs. Therefore, the incorporation of new cellular resources in combination with cryopreservation, such as tissue biopsies, blood samples, or fibroblast cultures can open up a new avenue in conservation. Contrary to cloning, this cellular material may be subjected to stem-cell associated techniques (SCAT), yielding artificial gamete cultures and thereby widen the genetic pool by including – with the help of cryopreservation – samples from deceased or completely infertile individuals [117,118] (Fig. 2e). A strategic roadmap was outlined in "Rewinding the process of mammalian extinction" [66]. Along these lines, high quality hybrid embryos from SWR and NWR gametes [52], as well as pure NWR blastocysts [119] were successfully generated. The blastocysts were also the basis for the establishment of two embryonic stem-cell lines. In addition, we were able to produce integration-free naive and primed iPSC-like cells derived from cryopreserved fibroblast cultures of a deceased northern white rhino. First steps towards the transformation of these primordial germ cells (PGC) were very promising. They expressed typical PGC marker genes Blimp, Stella, Sox17, and Oct4 [120]. These achievements provide the basis for the second phase: The production of artificial oocytes and spermatozoa originally derived from simple fibroblast cultures.

2.2.1. Intracytoplasmatic sperm injection (ICSI)

Intracytoplasmatic sperm injection (ICSI), the injection of sperm into the egg cytoplasm through a micropipette, is extensively used in humans [121], but remains rare in domestic and livestock reproduction owing to low success rates, and the requirement of expensive equipment, and skills [122,123] ICSI nevertheless offers a great advantage when semen characteristics are insufficient for IVF. Attempts of implementing ET and in southern and northern white and Sumatran rhinoceros following ICSI are currently undertaken [52,66].

2.2.2. Somatic cell nuclear transfer (SCNT)

SCNT, alias somatic cloning, has suffered a substantial loss in reputation owing to a range of problems, such as early and late abortions, compromised immune systems, circulatory and respiratory problems, and a high rate of foetal death, probably primarily mediated by atypical epigenetic re-programming [124].

Nevertheless, it has been successfully performed not only in domestic species such as cattle, horse, pig, and sheep [125], but also in non-domestic red deer (*Cervus elaphus*) [126] and cynomolgus monkey (*Macaca fascicularis*) [127]. Interspecies somatic nuclear transfer has been achieved in several wildlife species such as the Gaur (*Bos gaurus* to *Bos taurus*) [128], including the endangered mouflon (*Ovis orientalis musimon* in *Ovis aries*) [129], African wildcat (*Felis silvestris lybica* in *Felis catus*) [130], grey wolf (*Canis lupus* in *Canis familiaris*) [131,132], sand cat (*Felis margarita* in *Felis catus*) [133], the extinct Pyrenean Ibex (*Capra pyrenaica* in *Capra hircus*) [134], and the Russian sturgeon (*Acipenser gueldenstaedtii* in *Acipenser ruthenus*) [135].

Although somatic cloning and genomic approaches in mammals may become a last desperate option for species conservation [136], it is not very efficient in generating live offspring and major technical and ecological challenges remain unsolved [113]. Cloning may, however, be an interesting option for non-mammalian vertebrates such as amphibians (in which the technique was pioneered) or species where breeding is threatened or that have even gone extinct [137].

2.2.3. Inner cell mass (ICM) exchange

The inner cell mass (ICM) of an early stage mammalian embryo contains the cells that will determine the developing organism, whereas the trophoblastic vesicle surrounding it gives rise to the placenta. Transferring the ICM of an endangered mammalian species into a trophoblastic vesicle derived from a surrogate female of a different species facilitates implantation and successful gestation of offspring of the endangered species. The foster mother's species will be selected to be taxonomically closely related, but less endangered. This is a highly experimental approach, which has so far been successfully applied between sheep and goat [138,139], and between sheep embryos [140], but may represent a viable option in the future – especially if combined with artificial gametes derived from induced pluripotent stem cells [46]. ICM exchange has not been attempted so far as a solution to the pressing problem of lacking reproductively healthy recipients in critically endangered species.

2.2.4. Stem cell-associated techniques (SCAT)

aART in combination with techniques for generating gametes from stem cells provide a new conceptual strategy for saving critically endangered or practically extinct species. This ambitious and novel approach is currently being developed for rhinoceros species [52,66]. In the future it may serve as a blue-print for applying the potential of in-vitro-derived gametes [139–145] created from induced pluripotent stem cells to the conservation of further species on the brink of extinction.

While SCNT generates copies of existing genotypes, recent advances in stem cell technology [120] have opened a new promising path, using live cells to establish induced pluripotent stem cell (iPSC)-derived gametes. As this approach comprises meiosis, it is capable of generating an enormous variety of new genotypes. By crossbreeding arbitrary individuals (theoretically, iPSC of male donors can be used to produce oocytes, which requires silencing of Y-chromosome-linked gene(s) [142], a pedigree can be designed in the Petri dish to optimally exploit the available genetic diversity. So far, viable offspring from iPSC-derived gametes has been generated only in the mouse (*Mus musculus*; [141,143–145], but efforts are currently undertaken to extend this approach for saving highly endangered taxa, specifically the northern white rhinoceros (*Ceratotherium simum cottoni*). iPSC have been successfully established for several domestic and laboratory species [146], but also for several wildlife species such as the quail [147], several feline species [148], e.g. the endangered snow leopard [149] and for the critically endangered northern white rhinoceros [150,151] (Fig. 2e).

2.3. Cryopreservation

Cryopreservation crucially enhances the possibilities of ART by rendering the use of biological materials independent of time and space.

2.3.1. Cryopreserved materials and associated methods

Various materials can be preserved using cryopreservation for extended, potentially indefinite, periods of time [152]. Different methods have been developed for the long-term cryopreservation of biological samples, with a strong focus on vertebrate species, whereas many other taxa remain unstudied. The major difficulty to overcome is vulnerability to cryo-damage, which depends on cell membrane composition, its permeability regarding both, water and the cryoprotectant, cryoprotectant toxicity, tolerance to osmotic changes, and resistance to cooling and freezing temperatures [153]. Thus, suitable protocols differ substantially between species and material.

Blood samples are suitable for biochemical analyses, as well as for medical inquiries, and may serve as a source of DNA for molecular biological investigations, and – if adequately preserved – also as a source of viable lymphocytes suitable for iPSC protocols.

Tissue samples e.g. of liver or spleen are useful for the extraction of enzymes, and genomic and mitochondrial DNA, and for establishing primary cell cultures. Viable cells for subsequent cell culture can be preserved by freezing tissues with an adequate cryoprotectant such as glycerol or dimethylsulfoxide (DMSO).

Cell culture-based methods have gained considerable importance in basic research and biomedical applications over the past decades. Besides storing nuclear genetic information, cells further contain viable cell organelles and represent an amplifiable source of biological material. Over the past decades, cell lines from a wide range of different taxa [152] have been successfully established and banked. In the context of ART, owing to the development of advanced methods such as SCNT, ICM and in vitro gameteogenesis, cultured and cryopreserved cells will play a crucial role in maintaining and improving population viability of rare and endangered species.

Cell culture is nevertheless expensive and requires well-equipped laboratory space, sterile working conditions and skilled personnel. The most accessible samples are fibrous tissue such as skin or gingiva, and the less abundant lymphocytes that may be recovered from blood; viable cells have even been successfully isolated also from ejaculate, milk [154], and feces [155]. Skin biopsies can be obtained at zoological institutions, e.g. from ear-notches, or opportunistically during handling for quarantine, veterinary interventions such as castrations, surgery, transport, or ultimately during necropsy. If adequately cooled and stored, such tissue may remain viable for several days before further processing and may be either cryopreserved or cultured immediately. Primary cells can be obtained by centrifugation of blood, by mechanical disaggregation or enzymatic digestion of tissues, or by explant culture [156]. The hence cultured cell lines can be frozen, protected with glycerol or DMSO, and stored in liquid nitrogen containers for many years [1]. Such cryopreserved tissues and cell lines provide a viable and expandable source of genetic material and living cells that offer manifold possibilities for molecular and basic research.

Growing fibroblasts to sufficient numbers requires time, which increases material costs and risk of contamination, and repeated passaging of cell cultures may result in decreased viability. Unless primary cells are derived from neoplastic tissues or immortalized by mutation or specific intended modifications [157], their lifespan is limited due to undergoing senescence and ceasing to proliferate after a certain number of divisions [158]. Another limitation is the accumulation of mutations and chromosomal aberrations (aneuploidy) that may arise under culture conditions [159]. Therefore, for somatic cell-based technologies it is crucial to closely monitor the quality of the original cell lines and ensure genome integrity including chromosomal stability and the absence of relevant epigenetic alterations during prolonged culture and differentiation, which occur randomly and unpredictably [160]. Cell lines differ in quality based on culture conditions, age of the cell material donor, number of passage and many more factors, rendering quality control crucial to improve the observed low rates of reprogramming following SCNT and iPSC. Much work needs to be done for banked cell lines to become a valuable resource for offspring production. Cell lines can be reprogrammed to iPSC [120], and – if exposed to suitable factors – their pluripotent nature permits them to differentiate into various tissues including gametes.

Reproductive material suitable for cryopreservation comprises gametes, embryos, gonadal tissues, as well as embryonal (ES) and induced pluripotent stem cells (iPSC), which are gaining importance in preserving species biodiversity, as the associated technology evolves. However, progress is hampered due to significant

physiological variations among species and lack of fundamental knowledge in germplasm cryobiology.

Spermatozoa of approximately 116 mammalian species have been cryopreserved, accounting for 2% of all mammals, from which in approximately 45 species live births have been achieved following AI [161] since the first successful cryopreservation in 1949 [162,163] and the first calf produced from cryopreserved semen [163]. This renders cryopreserved semen the most successful and practical resource for propagating endangered species. Cryopreserved sperm of genetically valuable individuals can be stored for decades as a backup for possible future losses in genetic diversity. Ram semen has been reported to sire viable offspring after 50 years of storage without any decrease in pregnancy rates compared to recently frozen semen [164]. Even if thawed sperm quality is poor, it may generate viable offspring using ICSI [165]. Two of the most successful recent conservation programs, the giant panda [166] and the black-footed ferret have incorporated AI using fresh and frozen-thawed semen [167]. For the black-footed-ferret, cryopreserved semen of six of the last 18 survivors was employed to produce eight live offspring by AI, some of which up to 20 years after initial cryopreservation. Their relevance is illustrated nicely by their significant contribution to genetic diversity and heterozygosity in the population, lowering measures of inbreeding by 5.8% [83].

In contrast to mammals, bird sperm cells are fragile and knowledge is limited, complicating semen collection and processing [78]. Due to poor outcomes of cryopreservation in cranes [168], AI was not implemented in crane genetic management. Frozen-thawed sperm has generated viable offspring in a few amphibians [169] and fish [170] species, but so far not in reptiles, in which only artificial insemination with chilled semen has been accomplished so far [171].

One obstacle to the broad application of sperm cryopreservation is the variability in sensitivity to cryoprotectants and low temperatures from species to species. Tolerance to glycerol ranges from less than 2% in mice to 6% in chinchillas [153,172]. Sperm cryopreservation protocols are continuously extended and improved 173, e.g. by slow-freezing [174], optimizing freezing of small [175] and large [176] volumes; directional freezing [167,177,178] and double-freezing [179]. Even more challenging are amphibian and fish studies, as their sperm remain immotile as long as they are in seminal plasma. Motility is induced and quickly exhausted when they are released into a lower osmolarity environment, in the case of frogs, in urine [54,180,181]. Thus, amphibian and fish cryopreservation protocols need to keep sperm inactivated during handling and storage by mimicking the testicular environment. Anuran sperm has been successfully cryopreserved following non-invasive collection via hormonal induction [54]. Lately, sperm of a variety of coral species has been cryopreserved for the in vitro production of larvae and restauration of reefs [182].

Oocytes are a scarce resource compared to sperm cells, and less accessible. They can be obtained only invasively or post mortem [183], and are more difficult to handle [153]. Therefore, they are underrepresented in cryobanks and oocytes of only a few species have been preserved so far using vitrification [45,184]. Due to their large size, structure and lipid-rich yolk composition, frozen-thawed oocytes are more vulnerable to cryo-damage and until now, have not yielded any offspring in wild mammalian, fish, or amphibian species [167]. Survival and function of frozen-thawed oocytes has improved with the development of minimum volume vitrification (MVV), exhibiting extremely fast cooling rates that solidifies the sample into a glass-like state, thereby avoiding harmful intra- and extracellular ice crystal formation [185]. Using this method, oocytes of four carnivore species (Mexican grey wolf [186], serval, and Pallas's cat [187], and lion [188]), one antelope (chousingha [189]), and one marsupial (Tasmanian devil [190]) have been cryopreserved, but the developmental competence of the cryopreserved oocytes has only partially been reported.

Embryos of approximately 51 species (1% of mammals) have been cryopreserved, the success of which in terms of post-thaw viability and further development is not always reported [161]. Live births after ET of frozen-thawed embryos have been achieved in merely 25 species [161]. Similar to oocytes, differences in cryoprotection and freezability of fertilized eggs among different taxa will require considerable research. Vitrification [184,191] has been found to preserve the developmental potential of human embryos better than the earlier developed slow-freezing [192,193] or ultra-rapid freezing [194]. As of now, our bank contains few embryos with thus far unproven viability. Additionally, purebred SWR and purebred NWR embryos have been generated using harvested oocytes that have been fertilized with cryopreserved sperm of dead NWR males [52,66,195] and are currently stored in liquid nitrogen [119].

- Gonadal tissues are designed to mature functional gametes, but they also continue to contain gamete precursor cells of various different developmental stages. These spermatogenic and oogenic cells are another viable resource for obtaining functional spermatozoa and oocytes [153]. As they lack a metaphase spindle, are smaller and metabolically less active compared to mature gametes and contain low amounts of lipids [196], they are more cryoresistant. Hormonal treatment of the female to stimulate follicle development for OPU results in just a handful of oocytes for collection under general anaesthesia. Therefore, collecting oocytes by stimulating the ovary to develop them in vivo brings limited opportunities for successful population recovery. One approach is to harness the full potential of the ovary, which contains thousands of immature oocytes in primordial follicles – which can be obtained opportunistically during OPU or by using a needle-derived ovarian cortex biopsy – by developing an in vitro follicle culture method. The exceptional power of this technique is that, even when animals have died, their cryopreserved ovarian tissue can still lead to offspring. Establishing methods to culture and grow these follicles in the lab would vastly increase the chance of successful in vitro embryo production for endangered species. A first step in this process is to xenotransplant ovarian tissue into mice to verify the health and growth potential of collected frozen-thawed ovarian tissue (e.g. for endangered rhinoceros species, Fig. 3). By xenografting or in vitro culture of banked ovarian and testicular tissue, including that derived from neonatal and prepubertal individuals [196–198], mature gametes may be obtained, although this has not yet been achieved in non-domestic species.

Ovaries or ovarian tissue of a variety of mammalian species have been cryopreserved, e.g. of several felines [199,200] and the black-footed ferret (*Mustela nigripes*), amongst others [45].

Cryopreserved ovarian tissues from wombats, elephants, wallabies, and lions have been transplanted into immunodeficient mice, consistently resulting in the formation of morphologically normal secondary or antral follicles [200]. While it is still a long way to obtain viable mature oocytes for successful IVF, nevertheless, this approach represents an important step towards the inclusion of female gametes into ART.

For amphibians, direct cryopreservation of immature ovarian follicles may be a viable option, but would require methods such as xeno-transplantation to obtain mature, ovulated oocytes. For obtaining viable oocytes from cryo-preserved primordial germ cells, the generation of chimeras would be required to produce adults that can yield viable gametes; this may be a feasible future possibility also in fish and birds [55,167].

Comparable to ovarian tissue, in theory germ cells contained in testicular tissue can resume spermatogenesis to produce viable

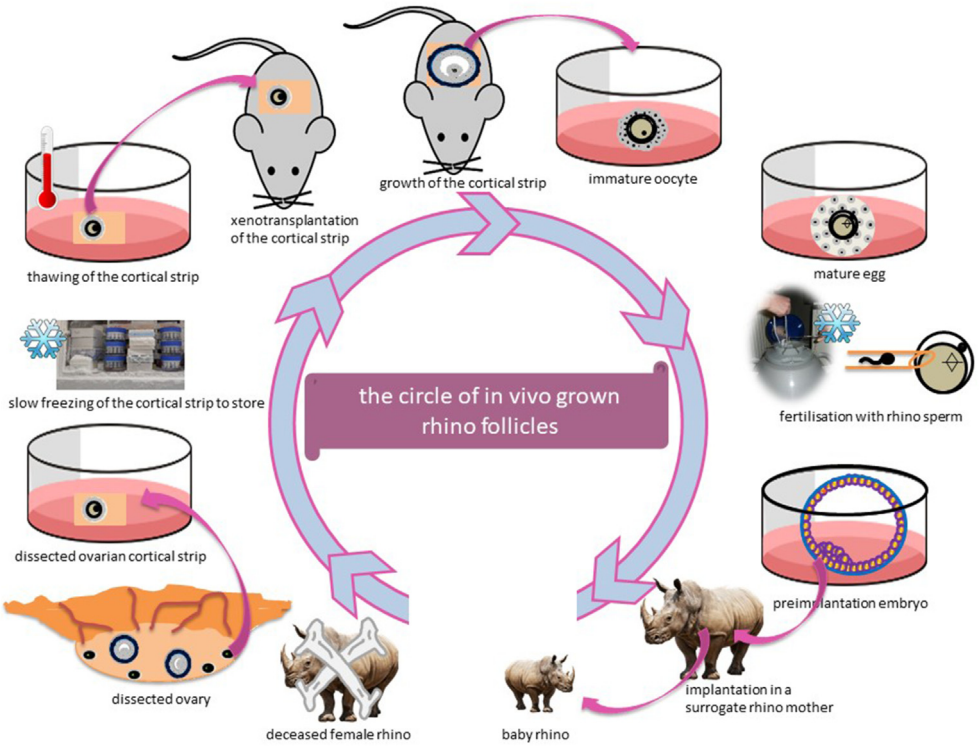

Fig. 3. Xenotransplantation of rhinoceros ovarian tissue into mice to verify the health and growth potential of collected frozen-thawed ovarian tissue.

spermatozoa, even in tissue of neonatal and prepubertal individuals [183]. To achieve this, transplantation or grafting are needed. ICSI of sperm recovered from grafted cryopreserved testicular tissue has generated live offspring in the mouse and rabbit [201], pig [202,203], and rhesus macaque (*Macaca mulatta*) [204], in birds (e.g. quails, *Coturnix japonica*) [205], and in fishes, such as hatched larvae of the critically endangered cyprinid honmoroko (*Gnathopogon caerulescens*) [206]. Testicular tissue of various mammalian wildlife species has been cryopreserved [207] without reported successful generation of live offspring so far.

2.4. Cryobanks

Genome resource banking (GRB) is the systematic collection, storage, and redistribution of biomaterials in an organized, logistical, and secure manner, which together with associated genomic information, are essential for progression of biomedicine, health, and basic research. Especially animal germplasm (sperm, eggs, embryos, ovarian, and testicular tissues) employed in combination with ART offers great potential to decelerate the loss of gene diversity in captive populations.

Unfortunately, currently there are no suitable alternatives to cryobanking samples at preferably −196 °C in liquid nitrogen. The associated disadvantages comprise the risk of cross-contamination with pathogens in liquid nitrogen, danger of accidents, as well as high energetic costs and dependence on a constant energy supply. Alternatives such as dry biobanking by exsiccating samples via freeze- or vacuum-drying (i.e. lyophilization), have yielded some successes, such as fertilizing ovine oocytes with freeze-dried spermatozoa using ICSI [208]. However, reliable protocols are not established as of now to an extent that the return to biological activity after rehydration is ensured, but the technology may offer a much cheaper and more stable solution for biodiversity storage in the future [161].

Biobanks offer countless possibilities. Their main advantage is that they maintain genetic variability across time and space. Cryopreservation of viable cells can keep otherwise extinct species suspended and buy time until the methodology and technology are in place to bring the species back to regular existence [209]. Cryobanks of endangered animal species represent an extremely valuable backup of today's biodiversity. Original genetic material can be maintained without removing genetically valuable individuals from the wild, and decrease the interval between generations [165,167]. Naturally, they are also confronted with constraints of space and resources given that storage tanks, maintenance and regular liquid nitrogen supply are costly. Long-term commitment of a cell bank sponsor is needed to secure the perpetuity of the collection, and mirroring of the bank, preferably in geographically different sites is highly recommended [1]. Optimally, an automated, digitised sample storage system is implemented. Whereas the majority of existent cryobanks established at zoos aim at preserving mainly genetic material or other non-alive items, extending this classical concept to living materials, and using advanced cryopreservation techniques, cellular methods and state-of-the-art stem cell technologies will open up many novel possibilities.

Preservation of genetic material from wildlife is mostly derived from zoos due to the ease of access and include many species that are threatened, extinct in the wild, or completely extinct. The oldest cryobank for wildlife-derived samples is the San Diego Frozen Zoo® with more than 10,000 cell lines across 11 mammalian orders, oocytes, sperm and embryos representing almost 1000 different taxa [1,2,152], established in 1975. In addition, the Frozen Ark Consortium [210,211], was founded. In the 1990s, the biobank at IZW Berlin was established with now more than 150 different living cell lines and tissues and reproductive material of approximately 250 different exotic species, amongst them cryopreserved semen from a total of 45 species [210,212]. Further initiatives include the

National Institute for Environmental Studies in Japan founded in 2002, containing cell lines of many avian and mammalian species, the amphibian ark (www.amphibianark.org), the Israel-German Ark of Life (IGAL), founded in 2016, that preserves tissues and cells of wildlife and exotic species from the largest wildlife clinic in the Middle East at Ramat Gan Safari, as well as the frozen Zoo in australia (http://www.australianfrozenzoo.org.au/). Most recently, CryoArks [213] and and in 2020, the biobank Nature's SAFE (Saving Animals From Extinction) were established in the UK (https://www.natures-safe.com/). Unusually, Nature's SAFE is an independent charity and is dedicated to collecting samples from endangered species with the view to regeneration when required. Several of these banks have been established when the generally accepted paradigm still excluded the possibility of reprogramming differentiated cells, thus before the recently arising possibilities could be foreseen. It therefore seems safe to assume that future applications will be just as much beyond our current imagination.

3. Discussion

At the current rate of species extinction, urgent action is needed to preserve as much biodiversity as possible. However, when recapitulating the initial and recent encouraging success stories of species recovery by measures ranging from simple habitat protection to the incorporation of increasing levels of assisted reproduction technology, and with the recent emergence of state-of the-art stem cell biotechnology, we may gain hope for the future. The incorporation of increasingly sophisticated genetic and reproductive tools has offered us strategies for minimizing the occurrence of genetic diseases, as in the California condor [48], retain high genetic diversity in black-footed ferrets using frozen-thawed semen that had been stored for 20 years [83] and re-build viable species from population sizes that would otherwise doom the species to extinction (Fig. 1b–d).

Threats to nature may be increasing - but so are the opportunities and tools at our disposition.

This is illustrated best with the northern white rhinoceros (*Ceratotherium simum cottoni*), of which only two female individuals are left. Cryopreserved tissue samples, somatic cell lines, iPSCs, and spermatozoa of 12 (5.7) individuals have been stored [66] and have, so far, allowed us to create so far five blastocysts of a species with no male individual alive. Encouraging for the prospect of recovering the NWR from these available resources, the genomes of the preserved specimen show levels of heterozygosity comparable to the SWRs, with higher levels of genome-wide heterozygosity and slightly lower levels of autozygosity in the NWR compared to the SWR [214], sparking hope for this charismatic keystone species. We may still be in time to someday reintroduce this landscape architect back to its now empty home in the central African bushland.

Zoos and zoological research institutions are key players for conserving genetic variability and provide reliable access to valuable material. Sample collection would optimally be implemented into the routine of zoo veterinarian work. A global network of cell culture repositories is missing. Although the number of GRB has been increasing over the past decades, their integration into common strategies, a unified data base, and concerted contribution to the management of captive populations could be further extended. Accessible and interconnected databases are needed to combine the available range of samples of a given species, pooling the existing resources for successful conservation efforts and research, thereby lifting the impact for biodiversity preservation on a higher level. It is further highly desirable to render the collections safe by mirroring across several distant locations. A further important impediment to globally concerted research efforts and international cooperation is grounded in the exchange of samples and specimens. The many national and international legislations and rules intended to prevent importation of exotic diseases and misuse – the Nagoya protocol, CITES, TRACES, national export- and import permits, to name a few – can render sample exchange very costly in terms of time and money, sometimes almost impossible. With the help of governments and legislation, conservation research should be facilitated in this respect.

4. Conclusion

Cryopreservation is an indispensable tool. In combination with assisted reproduction technology, it can become an integral part of successful species conservation management. The entire information of an organism is contained in each of its cells and this information can be preserved in liquid nitrogen for decades or even centuries. Recent advances in stem-cell technology may allow for reprogramming these cells to gametes, ultimately resulting in entirely new individuals, conceived from the material of a small skin biopsy. Many obstacles are still in our way before we can apply these techniques to one, and a hopefully further on to a broad range of animals. As of now, we will not be able prevent the extinction of many populations; but by preserving cells of as many species as possible, we may be having the chance to bring them back in the future. Now, as much as in 1984 [1], we must go on and "collect things for reasons we don't yet understand."

Acknowledgements

This study was funded by the BMBF project "BioRescue" (01LC1902A), by the Nadace ČEZ project PR21/46514, by Richard McLellan and Merck KGaA, Darmstadt, Germany to C.G., and. by Fondation Hoffmann to S. A. Williams. The Authors like to thank the German Society of Cryobanks (GDK) for support of our conservation research. Thanks to Derek Ott and Jan Zwilling for carefully reading the manuscript.

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